UNIVERSITAT DE BARCELONA

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Characterization of structurally-related second-generation synthetic cathinones

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2020

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University of Barcelona

Faculty of Pharmacy and Food Science

Department of Pharmacology, Toxicology and Therapeutic Chemistry

DOCTORAL THESIS

PhD program in Biotechnology

Dissertation presented by Leticia Duart Castells to apply for the doctorate degree by the University of Barcelona

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2020

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"The more I learn,

the more I realize how much I don't know"

Albert Einstein

Illustrated by Enrique Sánchez Cabanas

This doctoral thesis has been funded by:

Generalitat de Catalunya

Fundació Universitària Agustí Pedro i Pons
Universitat de Barcelona

Ministerio de Economía y Competitividad
Gobierno de España

Obra Social. Fundación "La Caixa"

Plan Nacional sobre Drogas

And it has been done in collaboration with the following institutions:

Medical University of Vienna (Austria)

Universitat de València (Spain)

Universitat Pompeu Fabra (Spain)

ACKNOWLEDGEMENTS

La presente tesis no hubiera sido posible sin la inestimable ayuda y el apoyo incondicional de muchas personas que, de forma directa o indirecta, la han hecho realidad. Por este motivo, me gustaría dedicarles unas páginas y mostrarles mi más profundo agradecimiento.

En primer lloc, agrair als meus directors de tesi, la Dra. Elena Escubedo i el Dr. David Pubill, així com al meu tutor, el Dr. Jordi Camarasa, l'haverme brindat l'oportunitat de realitzar la meva tesi en el seu grup d'investigació. Gràcies pel vostre temps i dedicació, pel vostre suport, ajuda, per tot el que m'heu ensenyat i per haver-me guiat sempre.

Además, agradecer también al equipo de estabulario por su exquisito trabajo, en especial a Marta, David y Tere.

In the same way, I would also like to thank Dr. Harald Sitte, from the Medical University of Vienna (Austria) for offering me the opportunity to do a short-term stay in your lab, it made me grow both personally and professionally. And with that, also to thank all those who were part of the experience: Marco, Tea, Marion, Kathrin, Laurin, Dino, Marija, Eray, Shreyas. Thank you for everything you taught me, for your help and for making my stay such a pleasant experience.

Quiero dar las gracias también a Raul, fuiste mi primer mentor cuando entré a hacer el "treball dirigit". Y al final, seis años más tarde, acabamos mano a mano formando un equipo. Gracias por ayudarme y guiarme siempre, por tus consejos, por intentar animarme en mis momentos de incertidumbre y bajón, por tus conocimientos y por haber sido más que un compañero de trabajo.

Gracias a los que tuvieron la mala o buena suerte de ser mis alumnos por ayudarme a crecer profesionalmente y por vuestra ayuda en el laboratorio. Y con especial cariño, gracias a Sergi Vizcaíno y a Danis, sois unos verdaderos cracks.

Agradecer a todos los compañeros del lab que me han acompañado durante estos 4 años: Gemma, Emma, Miren, Christian, Fotini, Julia, Oriol, Miguel, Nuri, Ana, Amanda, Mar, Quim y Silvia. Gracias por todo lo que me habéis enseñado, por vuestra ayuda, por facilitarme las cosas siempre, por las risas, por las conversaciones, por crear este buen ambiente de trabajo que se respira. Habéis hecho que siempre fuera a gusto a trabajar.

Y en especial, agradecer a aquellos con los que, además, he compartido momentos únicos y muy especiales fuera del laboratorio: Vanessa, Javier, Lucia, Dolors, David, Gaia, Patty y Brigitte. Gracias por las sobremesas, por las celebraciones, por las cenas, por los viajes, por los miércoles de cerveza, por las noches de fiesta... Por las mil y una historias que endulzaron esta etapa. Sin duda, me llevo conmigo a personas realmente extraordinarias.

Gràcies Andres pel teu lab-training, per guiar-me, per ajudar-me amb l'anglès, per fer-me sentir sempre tan valuosa, per les tardes de debat i reflexió i per exigir-me tant mentalment.

Gràcies Ignasi per caminar junts ja desde la carrera i en la nostra tesi, i fer-ho sempre tenint cura de l'altre. Gràcies per tots els dinars, per avisar-me sempre de totes les oportunitats laborals i per transmetre'm seguretat i confiança.

Gracias Vero por ser una de las personas más importantes para mi aun en la distancia. Gracias por escucharme, por tus consejos, por tus masajes,

por tu cariño, por tu apoyo, por creer y confiar en mí siempre, por los viajes juntas... por estar aquí.

Gràcies Anna i Monica per haver estar al meu costat desde que vam començar la carrera. Per absolutament tot, per ensenyar-me el veritable significat de l'amistat.

Quiero dar las gracias a mi nueva familia leona: gracias Marta, Miguel y Pilar. Gracias por todo vuestro cariño, por estar siempre ahí, en lo bueno y en lo malo, por vuestra implicación, por escribirme prácticamente cada día. Os habéis convertido en un pilar fundamental para mi este último año. Sin duda, la vida se vive mejor bailando con vosotros.

Muchas gracias Mario, gracias por ser el mejor amigo del mundo. Y aún más, serlo estando a más de 9000 km de distancia. Gracias por aguantarme, así, tal cual. Gracias por tu apoyo incondicional, por tu sabiduría y por tu optimismo. Te admiro mucho.

Quiero agradecer también a mi familia, especialmente a mi madre, gracias por habérmelo dado absolutamente todo, y a mi hermano Borja, gracias por seguir ahí.

Y por último, y de forma muy importante y especial, agradecerte Kike todo lo que has hecho por mí, tu papel en este último año de mi tesis ha sido crucial y no tengo palabras que puedan realmente expresar lo agradecida que estoy. Gracias por tu apoyo incondicional, día a día, tu paciencia, tu cariño, por ser tan comprensivo conmigo, por darme exactamente lo que necesitaba en cada momento, por tu ayuda, por maquetar esta tesis, por hacer las ilustraciones a mi gusto, por haberme hecho la vida más fácil y más agradable siempre. Gracias por quererme tanto y tan bien. Te quiero.

PREFACE

Over the last years the recreational drug market has changed considerably due to the emergence of New Psychoactive Substances (NPS) such as synthetic cathinones. Hundreds of unknown NPS have appeared marketed as "legal highs", "bath salts" and many other distractive names that have largely allowed their open sale circumventing the law. Hence, the "legal high" phenomenon has become a matter of concern about public safety by the authorities worldwide.

NPS are often traded as replacements to already controlled drugs. In this context, the presence of 3,4-methylenedioxypyrovalerone (MDPV) has spread across the globe as a cheaper and more accessible alternative to cocaine, especially amongst youths. Although MDPV has already fallen under legislative control in most countries, compelling reports indicate that MDPV is among the most commonly detected ingredients in "bath salts" and thus, it is considered one of the most consumed synthetic cathinones. Nonetheless, detailed preclinical and clinical data are still scarce, thereby the pharmacology, toxicology and potential social harms of this new designer drug are barely known. Only one thing is clear: its consumption is often linked to health problems, including severe acute intoxications, drug dependence and even deaths.

In this context, the present doctoral dissertation arises from the need to provide and extend scientific knowledge regarding NPS, especially MDPV, but also about the second-generation synthetic cathinones structurally related to it.

ABSTRACT

Drugs of abuse represent a global problem not only at a health level, but also at a social and economic extent. Recently, a tidal wave of new emerging psychoactive substances has completely modified the illicit drug market, thereby hundreds of new drugs are being consumed by millions of people worldwide regardless most of these substances are hardly known. Among them, a new family of amphetamine derivatives named synthetic cathinones has surfaced, being 3,4-methylenedioxypyrovalerone (MDPV) one of the most popular members of the first-generation of these drugs. MDPV shares mechanism of action with cocaine and its consumption is often linked to severe acute intoxications, drug dependence and even deaths. In this context, this doctoral dissertation aims to contribute to scientific knowledge by providing new insights about MDPV, more concretely, about the neuroadaptive changes underlying its abuse, its addictive properties, its behavioural effects, as well as about its relationship with cocaine, the most consumed psychostimulant worldwide.

In summary, repeated exposure to MDPV induces behavioural abnormalities such as anxiety-related and risk-taking behaviours, aggressiveness and locomotor sensitization. Moreover, it exerts powerful rewarding effects. Importantly, a cross-sensitization and cross-reinstatement between MDPV and cocaine has been evidenced. Nonetheless, despite the great similarities between these two psychostimulants, the intracellular responses that they trigger in reward-related brain areas notably differ.

Whereas the first part of the present doctoral dissertation has been focused on studying MDPV, the second one has been devoted to characterizing the *in vitro* pharmacology as well as the psychostimulant and

rewarding properties of structurally related new second-generation synthetic cathinones.

To sum up, all the compounds tested proved to be potent dopamine and noradrenaline uptake inhibitors, and their potency at inhibiting such transporters varies according to their amino terminal group. Furthermore, all of them exerted psychostimulant and rewarding effects in mice, a fact that evidences their abuse liability.

RESUMEN

Las drogas de abuso representan un problema global no solo a nivel de salud, sino también a nivel social y económico. Recientemente, una ola de nuevas sustancias psicoactivas ha modificado por completo el mercado de las drogas ilegales, por lo que millones de personas en todo el mundo están consumiendo cientos de nuevas sustancias, la mayoría de las cuales apenas se conocen. Entre ellas, ha surgido una nueva familia de derivados anfetamínicos, denominados catinonas sintéticas, siendo la 3,4-mentilendioxipirovalerona (MDPV) una de las catinonas sintéticas de primera generación más populares. La MDPV comparte mecanismo de acción con la cocaína y su consumo está a menudo relacionado con intoxicaciones agudas graves, drogodependencia e incluso muertes. En este contexto, esta tesis doctoral tiene como objetivo contribuir al conocimiento científico proporcionando nueva información sobre la MDPV, más concretamente, sobre los cambios neuroadaptativos subyacentes al abuso de ésta, sus propiedades adictivas, sus efectos conductuales, así como sobre su relación con la cocaína, el psicoestimulante más consumido en todo el mundo.

En resumen, la exposición repetida a MDPV induce anormalidades conductuales tales como comportamientos relacionados con ansiedad y una mayor toma de riesgos, agresividad y sensibilización locomotora. Además, ejerce potentes efectos gratificantes. Cabe destacar que se ha evidenciado una sensibilización locomotora y una recaída cruzada entre cocaína y MDPV. No obstante, a pesar de las grandes similitudes entre ambos psicoestimulantes, las respuestas intracelulares que desencadenan en las áreas cerebrales que forman parte del circuito de recompensa difieren notablemente.

Mientras que la primera parte de la presente tesis doctoral se ha centrado en el estudio de la MDPV, la segunda parte se ha dedicado a caracterizar

la farmacología *in vitro,* así como las propiedades psicoestimulantes y gratificantes de nuevas catinonas sintéticas de segunda generación estructuralmente relacionadas con la MDPV.

En resumen, todos los compuestos testados demostraron ser potentes inhibidores de la recaptación de dopamina y noradrenalina, y su potencia para inhibir dichos transportadores varía según su grupo amino-terminal. Además, todas ellas provocaron potentes efectos psicoestimulantes y gratificantes en el ratón, hechos que demuestran su potencial de abuso.

RESUM

Les drogues d'abús representen un problema global no només a nivell de salut, sinó també a nivell social i econòmic. Recentment, una onada de noves substàncies psicoactives ha modificat completament el mercat de les drogues il·legals, així doncs, milions de persones en tot el món estan consumint centenars de noves substàncies, la majoria de les quals gairebé no es coneixen. Entre elles, ha sorgit una nova família de derivats amfetamínics, anomenats catinones sintètiques, essent la 3,4-metilendioxipirovalerona (MDPV) una de les catinones sintètiques de primera generació més populars. La MDPV comparteix mecanisme d'acció amb la cocaïna i el seu consum està sovint relacionat amb intoxicacions agudes greus, drogodependència i fins i tot, morts. En aquest context, aquesta tesi doctoral té com a objectiu contribuir al coneixement científic proporcionant nova informació sobre la MDPV, més concretament, sobre els canvis neuroadaptatius subjacents a l'abús d'aquesta, les seves propietats addictives, els seus efectes conductuals, així com sobre la seva relació amb la cocaïna, el psicoestimulant més consumit arreu del món.

En resum, l'exposició repetida a MDPV indueix anormalitats conductuals com ara comportaments relacionats amb ansietat i una major presa de riscs, agressivitat i sensibilització locomotora. A més a més, exerceix potents efectes gratificants. Cal destacar que s'ha evidenciat una sensibilització locomotora i una recaiguda creuada entre cocaïna i MDPV. No obstant això, malgrat les grans similituds entre ambdós psicoestimulants, les respostes intracel·lulars que desencadenen en àrees cerebrals que formen part del circuit de recompensa difereixen notablement.

Mentre que la primera part de la present tesi doctoral s'ha centrat en l'estudi de la MDPV, la segona part s'ha dedicat a caracteritzar la farmacologia

in vitro, així com les propietats psicoestimulants i gratificants de noves catinones sintètiques de segona generació estructuralment relacionades amb la MDPV.

En resum, tots els compostos assajats van demostrar ser potents inhibidors de la recaptació de dopamina i noradrenalina, i la seva potència per inhibir aquests transportadors varia en funció del seu grup amino-terminal. A més, totes elles van provocar potents efectes psicoestimulants i gratificants en el ratolí, fets que posen de manifest el seu potencial d'abús.

THESIS STRUCTURE

The present doctoral dissertation is presented in several sections as follows: firstly, a general **Introduction** places the reader in the context of the thesis, providing a comprehensive overview of the topic in which it is framed, in this case, drugs of abuse and drug addiction, and more specifically, the current drug market situation with the emergence of new psychoactive substances like synthetic cathinones. Afterwards, the section **Objectives** collects the principal issues that were aimed to be addressed at the beginning of each investigation. **Methods and Results** section is divided in two well differentiated parts. **Part I** principally studies 3,4-methylenedioxypyrovalerone (MDPV), a first-generation synthetic cathinone. More concretely, Chapter I, II and III define the neurochemical changes underlying MDPV abuse, the behavioural abnormalities induced by the drug and ultimately, its addictive potential. In parallel, as MDPV is considered a cocaine-like psychostimulant, a comparative between cocaine and MDPV effects is always performed. **Part II,** however, extends the study to the new second-generation of synthetic cathinones. Chapter 4 determines the pharmacological profile of five different synthetic cathinones structurally related to α-pyrrolidinovalerophenone (α-PVP) and pentedrone, providing interesting knowledge about the role of the amino-terminal group in their interaction with monoaminergic transporters. Additionally, their rewarding and psychostimulant effects are also evaluated. Thereafter, a general **Discussion** compares and contextualizes the results achieved with the evidence already available in the literature, always trying to clarify the likely existing discrepancies. Finally, the thesis ends with the **Conclusions** derived from the whole study and the **Bibliography** collects all the scientific literature consulted and cited during the thesis development, which, in addition, helped to further discuss the results. The **Annex** contains five

works in which the author substantially participated during the thesis preparation period, but which are not considered part of the main content itself.

Four scientific papers are the result of the present dissertation, two of them already published, another one, accepted for publication, and the last one pending to submit:

Part I

Chapter 1

Duart-Castells, L., López-Arnau, R., Buenrostro-Jáuregui, M., Muñoz-Villegas, P., Valverde, O., Camarasa, J., Pubill, D., Escubedo, E. (2019). Neuroadaptative changes and behavioral effects after a sensitization regime of MDPV. *Neuropharmacology*. *144*:271-281.

Chapter 2

Duart-Castells, L., López-Arnau, R., Vizcaíno, S., Camarasa, J., Pubill, D., Escubedo, E. (2019). 7,8-Dihydroxyflavone blocks the development of behavioral sensitization to MDPV, but not to cocaine. Differential role of the BDNF – TrkB pathway. *Biochemical Pharmacology. 163*:84-93.

Chapter 3

Duart-Castells, L., Blanco-Gandía, M.C., Ferrer-Pérez, C., Puster, B., Pubill, D., Miñarro, J., Escubedo, E., Rodríguez-Arias, M. (2020). Cross-reinstatement between 3,4-methylenedioxypyrovalerone

(MDPV) and cocaine using Conditioning Place Preference. *Progress in Neuro-Psychopharmacology & Biological Psychiatry [accepted for publication].*

Part II

Chapter 4

Duart-Castells, L., Muralter, M., Nadal-Gratacós, N., Puster, B., Berzosa, X., Estrada-Tejedor, R., Niello, M., Bhat, S., Sitte, H., Pubill, D., Camarasa, J., Escubedo, E., López-Arnau, R. Role of the amino terminal group in the pharmacological profile of novel synthetic cathinones structurally related to α-PVP and pentedrone. Comparative of their rewarding and psychostimulant effects *[pending to submit].*

Importantly, the latter arises from the author's short-term stay at the Medical University of Vienna (Vienna, Austria) during the last year of the thesis preparation period.

Additionally, four extra scientific papers, as well as one book chapter, have been published during the time period of the thesis as a result of the author's participation in other projects carried out by members of the same research group. Nevertheless, the content of these publications has not been included in the core thesis since they are beyond its scope and/or the author contributed as a collaborator instead of leading the investigation.

- Ciudad-Roberts, A., **Duart-Castells, L.**, Camarasa, J., Pubill, D., Escubedo, E. (2016). The combination of ethanol with mephedrone increases the signs of neurotoxicity and impairs the neurogenesis and learning in adolescent CD-1 mice. *Toxicology and Applied Pharmacology 293*:10-20.

- López-Arnau, R., Buenrostro-Jáuregui, M., Muñoz-Villegas, P., Rodríguez-Morató, J., Ciudad-Roberts, A., **Duart, L.**, Camarasa, J., De la Torre, R., Pubill, D., Escubedo, E. (2017). The combination of MDPV and ethanol results in decreased cathinone and increased alcohol levels. Study of such pharmacological interaction. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*. *76*; 19-28.

- López-Arnau, R., Luján, M.A., **Duart-Castells, L.**, Pubill, D., Camarasa, J., Valverde, O., Escubedo, E. (2017). Exposure of adolescent mice to 3,4-methylenedioxypyrovalerone increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood. *British Journal of Pharmacology. 174*:1161-1173.

- López-Arnau, R., **Duart-Castells, L.**, Aster, B., Camarasa, J., Escubedo, E., Pubill, D. (2019). Effects of MDPV on dopamine transporter regulation in male rats. Comparison with cocaine. *Psychopharmacology (BerI). 236*:925-938.

- **Duart-Castells, L.**, Cantacorps, L., López-Arnau, R., Montagud-Romero, S., Puster, B., Mera, P., Serra, D., Camarasa, J., Pubill, D., Valverde, O., Escubedo, E. (2020). Behavioural and molecular effects of a high fat diet and maternal binge-like alcohol consumption on female mice offspring. *Psychoneuroendocrinology [submitted].*

Book chapter:

- Buenrostro-Jáuregui, M.H. and **Duart-Castells, L.** (2017). Neurobiology of attachment and loving behavior. *The trace of pleasure. From the regulation to the addiction*. Juárez J. Manual Moderno. ISBN 9786074487428.

The scientific outputs of the thesis and the extra contributions were shared with the scientific community in different national and international congresses as poster presentations, as well as in different research meetings as oral presentations:

Poster presentations:

- Ciudad, A.M., **Duart-Castells, L.,** Camarasa, J., Escubedo, E., Pubill, D. Alcohol enhances mephedrone-induced signs of neurotoxicity and impaired neurogenesis in adolescent CD1 mice. *Society for Neuroscience*. 17-21 October 2015. Chicago (EEUU)
- Buenrostro-Jáuregui, M., López-Arnau, R., Muñoz-Villegas, P.C., Rodríguez-Morató, J., Ciudad-Roberts, A., **Duart, L.,** Pubill, D., Camarasa, J., de la Torre, R., Escubedo, E. Modulating properties of Ethanol on dopamine levels, rewarding and psychostimulant effects of 3,4-methylenedioxypyrovalerone (MDPV). *Society for Neuroscience*. 12-16 November 2016. San Diego (EEUU)
- Luján, M.A., López-Arnau, R., **Duart-Castells, L.**, Pubill, D., Camarasa, J., Valverde, O., Escubedo, E. Early exposure of mice to MDPV increases the vulnerability to cocaine in adulthood. *37th SEF National Meeting with guest society, the British Pharmacological Society*. 18-21 June 2016. Barcelona (Spain)
- **Duart-Castells, L.**, Buenrostro-Jáuregui, M., Muñoz-Villegas, P., López-Arnau, R., Camarasa, J., Pubill, D., Escubedo, E. Repeated exposure of adolescent mice to 3,4-methylenedioxypyrovalerone activates transcriptional mechanisms that persist until adulthood and are similar to those activated by cocaine. *Society for Neuroscience*. 11-15 November 2017. Washington DC (EEUU)

- Escubedo, E., **Duart-Castells, L.,** López-Arnau, R., Pubill, D., Camarasa, J. The BDNF – TrkB signaling pathway is involved differently in the development of locomotor sensitization and place conditioning by MDPV and cocaine. *World Congress of basic and clinical pharmacology*. 1-6 July 2018. Kyoto (Japan).
- Blanco-Gandía, M.C., **Duart-Castells, L.,** Rodríguez-Arias, M. Cross-reinstatement between 3,4-methylenedioxypyrovalerone (MDPV) and cocaine using the conditioning place preference paradigm in adult male mice. *1st International Congress. XLVI National Journeys of Socialdrugalcohol*. 7-9 March 2019. Málaga (Spain). Awarded as the best poster of the Congress.
- Fernández, D., **Duart-Castells, L**., Borrell, J.I., Camarasa, J., Pubill, D., Escubedo, E. Effect of CXCR4 antagonists on psychostimulants-induced hyperlocomotion in mice. *EBPS Biennal Meeting*. 28-31 August 2019. Braga (Portugal)
- **Duart-Castells, L**., Blanco-Gandía, M., Buenrostro-Jáuregui, M., Pubill, D., Rodríguez-Arias, M., Escubedo, E. Cross-reinstatement by 3,4-methylenedioxypyrovalerone (MDPV) and cocaine of mice conditioned to both drugs. *Society for Neuroscience*. 19-23 October 2019. Chicago (EEUU).

Oral presentations:

- **Duart-Castells, L**. Study of how the consumption of new synthetic drugs increases vulnerability to cocaine. Doctoral Journey in Pharmacology of the Universitat Autònoma de Barcelona (UAB) and Universitat de Barcelona (UB). Organized by the Academy of Medical and Health

Sciences of Catalonia and the Balearic Islands, Catalan Society of Pharmacology. Barcelona (Spain). June 2017.

- **Duart-Castells, L.** Study of the effects of maternal alcohol consumption during pregnancy and lactation, and subsequent vulnerability to cocaine, in mice. Institute of Neuropsychiatry and Addictions, Emili Mira Centre. Research Institute in Nutrition and Food Safety (INSA). Barcelona (Spain). May 2018.

- **Duart-Castells, L.** The exposure to the "cannibal drug" during adolescence increases the vulnerability to cocaine abuse. "*XI* Research Day" of the Faculty of Pharmacy and Food Science, University of Barcelona (UB), in collaboration with Federació Farmacèutica (Fedefarma). Barcelona (Spain). October 2018. Awarded with the second prize.

- **Duart-Castells, L.** Neurochemical and psychopharmacological study of the abuse of 3,4-methylenedioxypyrovalerone (MDPV) in adolescence and subsequent vulnerability to cocaine in adulthood. Centre for Addiction Research and Science (AddRess colloquia). Centre for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna. Vienna (Austria). June 2019.

ABBREVIATIONS

α-PpVP	α-piperidinevalerophenone
α-PVP	α-pyrrolidinovalerophenone
4-HNE	4-hydroxy-2-nonenal
5-HT	Serotonin
7,8-DHF	7,8-Dihydroxyflavone
AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
ANA-12	N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide
AP-1	Active activator protein- 1
APA	American Psychiatric Association
Arc	Activity-regulated cytoskeleton-associated protein
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
CDK5	Cyclin-dependent kinase-5
CNS	Central nervous system
CPP	Conditioned place preference
CRE	cAMP response element
CREB	cAMP response element binding protein
DA	Dopamine

DARPP-32	Dopamine- and cAMP- regulated phosphoprotein, Mr 32 kDA
DAT	Dopamine transporter
DEA	Drug Enforcement Administration
DR	Dopamine receptor
DS	Dorsal striatum
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EPM	Elevated plus maze
EU	European Union
GluA2/GluR2	AMPA glutamate receptor subunit 2
HEK 293 cells	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
ICSS	Intracranial self-stimulation
i.p.	Intraperitoneally
mBDNF	Mature brain-derived neurotrophic factor
MDMA	3,4-methylenedioxymethamphetamine
MDPV	3,4-methylenedioxypyrovalerone
mPFC	Medial prefrontal cortex
NA	Noradrenaline
NAcc	Nucleus accumbens
NET	Noradrenaline transporter

NFκB	Nuclear factor-κB
NPS	New psychoactive substance
OCT	Organic cation transporter
OF	Open field
OFC	Orbitofrontal cortex
PFC	Prefrontal cortex
PKA	Protein kinase A
PP-1	Protein phosphatase-1
proBDNF	Precursor BDNF
(Q)SAR	(Quantitative) structure-activity relationship
RIT	Resident intruder test
SA	Self-administration
s.c.	Subcutaneously
SERT	Serotonin transporter
TH	Tyrosine hydroxylase
TrkB	Tropomyosin receptor kinase B
UNODC	United Nations Office on Drugs and Crime
VMAT	Vesicular monoamine transporter
VS	Ventral striatum
VTA	Ventral tegmental area
W	Withdrawal
WHO	World Health Organization

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1 INTRODUCTION

1.1. Drugs of abuse and drug addiction

A *drug* is, from a broad point of view, a chemical substance that has known biological effects on humans or other animals. However, and more specifically, the term *drug* can be used with different meanings. Generally, in pharmacology, a *drug* refers to any natural or synthetic chemical that has a physiological effect and so it is used in medicine to treat, cure, prevent or diagnose a disease, or to enhance physical or mental well-being. Alternatively, in common usage, the term *drug* can also refer to a substance, often a psychoactive substance, that people take because of its pleasant effects without medical purposes, the recreational use of which is usually illegal. Since the beginning of humanity, the human being has been looking for drugs for both purposes: either to palliate or prevent pain, and/or to experience pleasant sensations and relieve distress. Nevertheless, in the present thesis the term *drug* (or *drug of abuse* to distinguish from the medicinal drugs) will be used with the latter meaning.

The World Health Organization (WHO) defines a psychoactive drug as a chemical substance that, when taken in or administered into one's system, affects mental processes (i.e. cognition or affect) (WHO, 1994). In other words, psychoactive substances act primarily upon the central nervous

system (CNS) where they alter the brain function modifying temporarily perception, mood, consciousness, cognition and/or behaviour.

Psychoactive substances encompass a great deal of substances that can be classified according to their chemical structure, mechanism of action and thus, their physiological effects. The main drug categories are nicotine, alcohol, cannabinoids, stimulants, opioids, depressants (sedatives, hypnotics), hallucinogens and dissociative drugs.

Repeated use of a psychoactive substance can lead to substance dependence or drug addiction, a cluster of behavioural, cognitive and physiological phenomena. Drug addiction can be defined as a chronic and relapsing disorder in which the user (referred to as an addict) shows a compulsion to seek and take the drug despite serious harmful consequences, losing the control in limiting intake. Thus, a higher priority is given to the drug rather than other activities and responsibilities. Typically, tolerance (a decreased response to a drug that occurs with continued use) is prominent and a withdrawal syndrome frequently occurs when substance use is interrupted, increasing risk for relapse (APA, 2013; WHO, 1994; Camí & Farré, 2003). The WHO and the American Psychiatric Association (APA) encourage the use of "substance dependence" rather than "drug addiction". However, in the present thesis, both terms will be used interchangeably.

1.1.1. Neuroanatomical substrates of drug addiction

Even though the diverse groups of drugs are chemically divergent molecules with very different mechanisms of action, all of them act on the same neuronal system at different levels, and thus, the resultant addictions

share many important features. Drugs of abuse activate the same brain areas that are activated by natural rewards (food, sex, water...). These brain areas conform the so-called brain reward system, which is the responsible of eliciting positive emotions like pleasure, producing associative learning (i.e. classical conditioning and operant reinforcement) and affecting decision-making in such a way that induces approach behaviour towards the stimulus (Berridge & Kringelbach, 2015; Schultz, 2015). Accordingly, drugs of abuse, as well as food, water or sex, are stimulus that can act as rewards and reinforcers.

Research on drug addiction has in large part defined the neurocircuitry of reward, called the mesocorticolimbic dopaminergic system, which originates in neurons in the ventral tegmental area (VTA) (Kalivas, 2002). This system includes (Figure 1):

- The mesolimbic circuit, with dopaminergic projections from cell bodies of the VTA to limbic structures such as the nucleus accumbens (NAcc, region of the ventral striatum), amygdala and hippocampus. It is involved in the acute rewarding effects, memory, and conditioned responses linked to craving and the emotional and motivational changes of the withdrawal syndrome.

- The mesocortical circuit, involving dopaminergic projections from cell bodies of the VTA to the prefrontal cortex (PFC), orbitofrontal cortex (OFC), and anterior cingulate cortex (ACC). It is implicated in the conscious experience of taking drugs, drug craving and the compulsive behaviour (Camí & Farré, 2003).

The two circuits, mesolimbic and mesocortical, operate in parallel and interact with other brain areas but also with each other through projections

from the glutamatergic neurons of the PFC to the NAcc and VTA, as well as through GABA neurons of the NAcc which project to the PFC and VTA (Goldstein & Volkow, 2002; Hyman & Malenka, 2001; Kalivas, 2002).

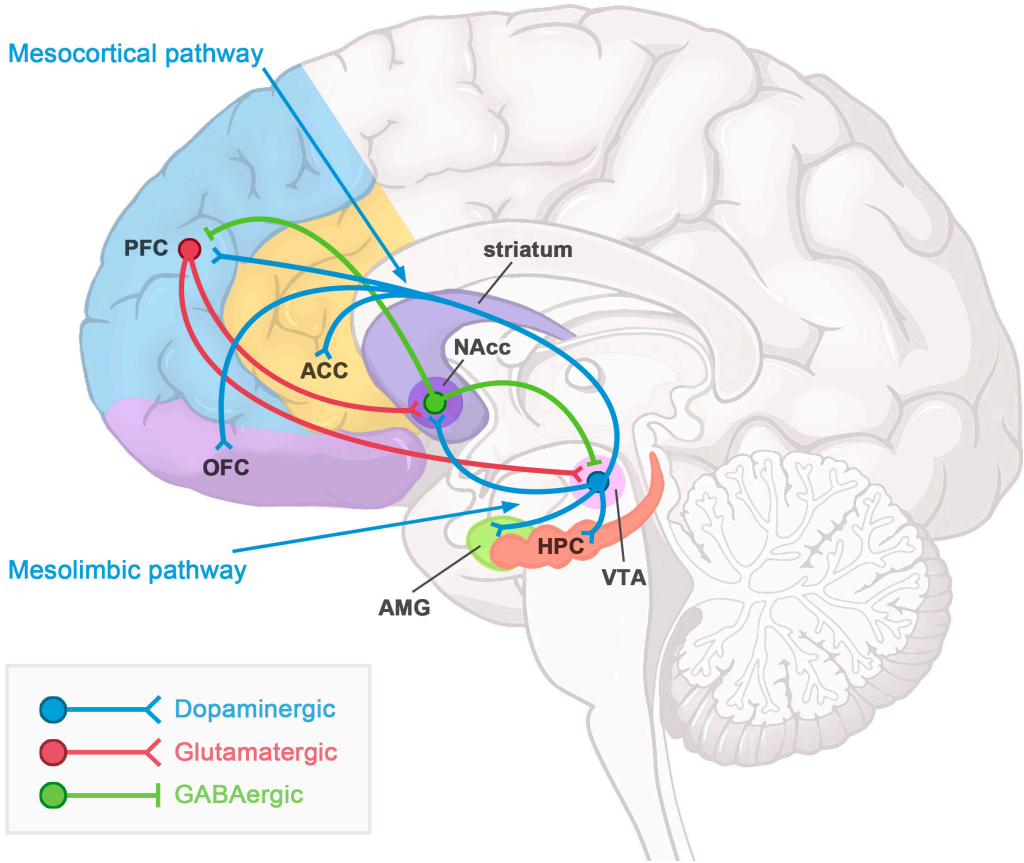

Figure 1. Principal neurocircuitries of the brain reward system: the mesolimbic and mesocortical dopaminergic pathways and their interactions. *The image shows the neuroanatomical location of the main brain areas that comprise the reward system as well as a simplified schematic of the major dopaminergic, glutamatergic and GABAergic connections within the circuitries, in the human brain. VTA, ventral tegmental area; NAcc, nucleus accumbens; PFC, prefrontal cortex; OFC, orbitofrontal cortex; ACC, anterior cingulate cortex.*

In brief, drugs of abuse directly and powerfully activate the reward system by increasing firing of dopamine (DA) neurons in the VTA, leading to subsequent increase of DA released in the NAcc, which causes positive emotions, reinforcement of the behaviour, promotes the associative learning about the stimulus and anticipates its rewarding effects (Camí & Farré, 2003). Importantly, after few stimulations by natural rewards there is a rapid habituation, something that does not happen with drugs of abuse, thus every administration stimulates the release of DA (Di Chiara, 1999). Such DA surges resemble, and in some instances greatly surpass, the physiological increases triggered by natural rewards (Volkow et al., 2011). Taken together, repeated drug administration can lead to a profound perturbation of the brain's reward mechanisms in such a way that drugs gradually, progressively and insidiously replace natural rewards as the major shaper of behaviour (Nestler, 2013). Furthermore, when the drug is withdrawn, there is a substantial decrease in DA levels in the NAcc (Nestler, 2001a). The resulting hypodopaminergic state would explain an addicted individual's decreased sensitivity to natural rewards and the perpetuation of drug use as a means to temporarily compensate for this deficit (Volkow et al., 2011).

Drugs of abuse act initially at the synapse and activate the reward system through diverse mechanisms like mimicking (opiates) or activating (nicotine, alcohol) endogenous opioid pathways that innervate the VTA and NAcc. Others as cannabinoids or stimulants, act directly in the NAcc through other mechanisms (Nestler, 2001a) (Figure 2). There is compelling evidence that these diverse mechanisms are central in mediating the acute rewarding and reinforcing properties that are shared by all drugs of abuse (Koob & Nestler, 1997; Koob et al., 1998; Robbins & Everitt, 1996; Robinson & Berridge, 2000; Wise, 1998).

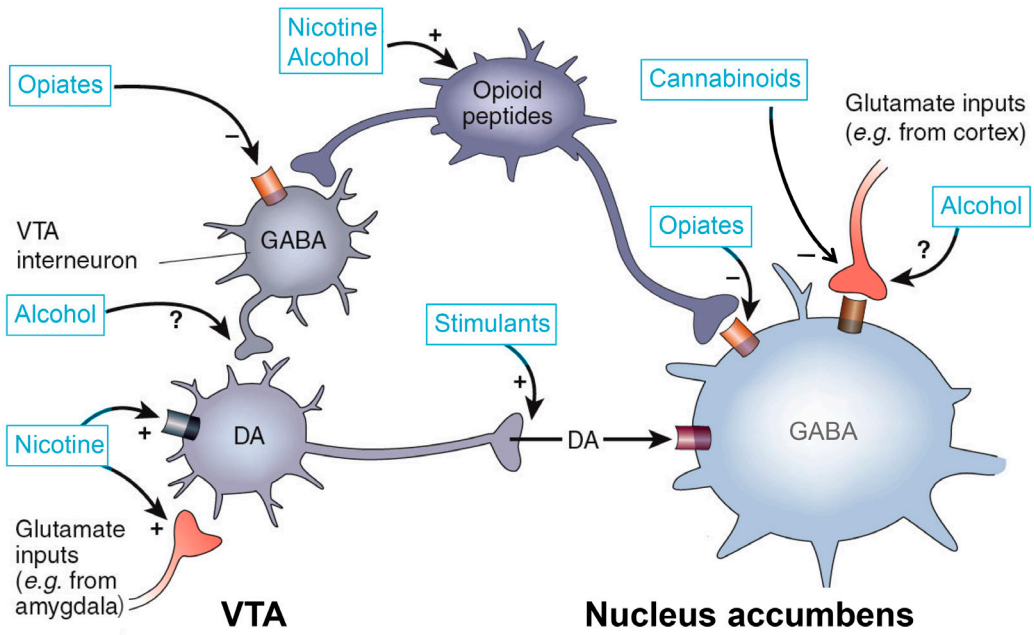

Figure 2. Convergence of drugs of abuse on the ventral tegmental area – nucleus accumbens (VTA-NAcc) reward circuitry. *Drugs of abuse activate the reward system of the brain through different mechanisms that all converge in mediating the same effects: their rewarding and reinforcing properties. Adapted from Nestler, 2013.*

1.1.2. Molecular neurobiology of drug addiction

Upon initial exposure, drugs of abuse enter the brain and bind initially to protein targets located at the synaptic clefts (i.e. receptors, transporters or ion channels) (Nestler, 2001a). This extracellular binding perturbs the synaptic transmission at specific synapses since it alters the postsynaptic

intracellular messenger pathways, causing the acute behavioural effects of the drug (i.e. euphoria, sedation...). However, these acute initial actions of the drugs do not really explain drug addiction *per se*, but repeated drug administrations are required to develop a state of addiction (Nestler, 2001b). Repeated exposure to a drug causes repeated perturbation of the intracellular pathways, which first initiates and then maintains the long-lasting molecular and cellular adaptations that underlie addiction. In this sense, the process of addiction can be considered as a form of drug-induced neural plasticity (Nestler, 2001a; Nestler et al., 1993). The proposed mechanisms by which chronic exposure to a drug of abuse causes stable changes in the brain include translational and posttranslational protein modifications and alterations in protein trafficking, as well as changes occurring at the level of gene expression (Nestler, 2012). Thereby, drugs of abuse, via their initial actions at the synapse, produce changes in intracellular messenger pathways that signal to the nucleus and regulate the activity of numerous transcription factors and many other types of transcriptional regulatory proteins (Nestler, 2001b). Alteration in transcription factors, would, in turn, alter the expression of their target genes. These nuclear changes gradually and progressively built by repeated drug exposure lead to stable changes in gene expression, which, in turn, change neuronal function and, ultimately, behaviour (Nestler, 2001a; Nestler et al., 1993; Robison & Nestler, 2011).

Chronic exposure to drugs has been reported to cause adaptations in many intracellular messenger pathways. Nonetheless, the present thesis will be focused especially on one: the dopamine receptor 1 (DR1) and subsequent upregulation of the cyclic adenosine monophosphate (cAMP) intracellular signalling pathway in the NAcc, which involves the drug-regulated transcription factor that has been shown to play important roles in addiction within the brain's reward circuitry, especially in the NAcc: the transcription

factor ΔFosB. A simplified schematic depiction of this signalling pathway is provided on page 23, Figure 3, immediately after the detailed description of each molecular factor.

1.1.2.1. DR1 – cAMP intracellular signalling pathway

The striatum itself can be divided into two main regions: the ventral (contains the NAcc) and the dorsal striatum. The ventral striatum, particularly the NAcc, plays a key role in motivational salience, reward, reinforcement and cognition, whereas the dorsal striatum primarily mediates cognition involving motor function, certain executive functions (i.e. impulsivity, inhibitory control) and stimulus-response learning (Kim & Im, 2019; Taylor et al., 2013; Yager et al., 2015). However, there is a small degree of overlap since the dorsal striatum also takes part in the reward system so, along with the NAcc, it mediates the encoding of new motor programs associated with future reward acquisition (i.e. conditioned motor response to a reward cue) (Taylor et al., 2013).

The whole striatum is comprised of multiple neuronal phenotypes. However, the majority of striatal neurons are GABAergic inhibitory medium spiny neurons, which in rodents constitute the 90-95% of all striatal neurons (Yager et al, 2015). In these neurons, DA can bind to five different subtypes of postsynaptic receptors: dopamine receptor 1, 2, 3, 4 or 5 (DR1, DR2, DR3, DR4 and DR5, respectively), all of them G-protein-coupled receptors. Depending on the intracellular response they trigger, they are divided in two main families: the DR1-like receptors: DR1 and DR5 and the DR2-like receptors: DR2, DR3 and DR4. The DR1-like receptors are coupled to the G_s protein, and so activate adenylyl cyclase (AC) and increase

the intracellular concentration of the second messenger cAMP. The DR2-like receptors, by contrast, are coupled to the G_i protein and so inhibit the enzyme and decrease cAMP intracellular levels (Nicola et al., 2000). In this sense, striatal medium spiny neurons have two principal phenotypes: those that predominantly express DR1 (*DR1-type*, dynorphin(+), direct pathway) and those that express DR2 (*DR2-type*, enkephalin(+), indirect pathway), although a small subpopulation express both receptors (Nishi et al., 2011; Yager et al., 2015). Classically, these two populations are thought to have opposing effects on their ultimate output structures (Yager et al., 2015).

Dopaminergic modulation by drugs of abuse has extensively studied DR1 and DR2 implication, and in less extent, the rest of receptors. In the NAcc, DR1 activation increases the neuronal excitability, leading to the increase of GABAergic inhibitory outputs that, ultimately, mediate reward-based learning and appetitive incentive salience, which is assigned to rewarding stimuli. Accordingly, such roles of DR1 are implicated in reward, cognition and drug addiction. Conversely, DR2 activation mediates aversion-based learning and aversive motivational salience, which is assigned to aversive stimuli (Baliki et al., 2013).

In the direct pathway, DR1 receptors are coupled to G_s/AC/cAMP which leads to the activation of cAMP dependent protein kinase A (PKA). PKA phosphorylates its substrates such as DARPP-32 (a dopamine- and cAMP- regulated phosphoprotein) and the transcription factor CREB (cAMP-response-element-binding protein) (Nishi et al., 2011).

Dopamine- and cAMP-regulated phosphoprotein, Mr 32 KDa (DARPP-32)

Phosphorylation at Thr 34 by PKA converts DARPP-32 into a potent inhibitor of protein phosphatase-1 (PP-1). The inhibition of PP-1

thereby controls the phosphorylation and activity of many down-stream physiological effectors, including neurotransmitter receptors, ion channels, pumps and transcription factors (CREB, ΔFosB and c-Fos) (Nishi et al., 2011).

cAMP response element-binding protein (CREB)

CREB is a cellular transcription factor that regulates the transcription of genes that contain a certain DNA sequence called cAMP response element (CRE) within their regulatory regions (i.e. dynorphin, the activity-regulated cytoskeleton-associated protein (Arc), c-Fos, the brain-derived neurotrophic factor (BDNF) and ΔFosB) (Manning et al., 2017), but only when phosphorylated at Ser 133 by kinases such as PKA (Shaywitz & Greenberg, 1999). The best-known effect of CREB is that it induces dynorphin expression which, in turn, decreases DA release in NAcc and thereby, causes dysphoria (anhedonia). CREB activation, however, is relatively short-lived and reverts to normal within a few days after drug exposure. Therefore, CREB seems to contribute to the generation of negative emotional states during early phases of withdrawal, but it is not likely to mediate directly the more stable behavioural abnormalities associated with addiction (Nestler, 2001a, 2001b), while ΔFosB does.

ΔFosB

ΔFosB is a member of the Fos family of transcription factors encoded by the *fosB* gene (Morgan & Curran, 1995). To become active, this family proteins need to heterodimerize with Jun family proteins, forming the active activator protein-1 (AP-1) transcription factor that binds to AP-1 sites present in the promotor regions of certain genes (target genes),

regulating their transcription. The expression of Fos family proteins is induced by many drugs of abuse and is especially prominent in the ventral (NAcc) and dorsal striatum (Perrotti et al., 2008) However, their profile of expression differs according to the drug administration pattern. When the drug (i.e. cocaine) is administered acutely, the expression of several Fos family proteins (c-Fos, Fra-1, Fra-2) is rapidly induced but, because they are highly unstable forms, their expression is transient and return to basal levels within 4-12 hours of administration (Nestler, 2008). By contrast, after chronic administration of drugs of abuse, the same Fos family members show tolerance, that is, reduced induction compared with initial drug exposures (Chen et al., 1997; Hiroi et al., 1997; Renthal et al., 2008). By contrast, biochemically-modified isoforms of ΔFosB are barely induced by an acute drug administration while they accumulate within the same brain regions after repeated drug exposure due to their high stability and thus, long half-lives (Alibhai et al., 2007; Chen et al., 1997; Hiroi et al., 1997, 1998; Jorissen et al., 2007; Renthal et al., 2008; Winstanley et al., 2009b). In this sense, the ΔFosB protein can persist in neurons for at least several weeks after drug cessation, providing a molecular mechanism by which drug-induced changes in gene expression can persist for relatively long periods of time. Hence, ΔFosB is considered as a sustained "molecular switch" that helps to initiate and then maintain an addicted state (Nestler, 2001a, 2008). Such accumulation of ΔFosB has been observed for virtually all drugs of abuse, even though the degree of induction observed in NAcc (core vs shell), dorsal striatum and other brain areas differ among drugs (Nestler, 2008; Perrotti et al., 2008). Moreover, that induction seems to be selective for the DR1 subset of medium spiny neurons (Chen et al., 1997; Hiroi et al., 1998), and this cellular pattern of induction is specific for addictive drugs (Kelz & Nestler, 2000; Moratalla et al., 1996).

Drug induction of ΔFosB in NAcc is thought to be a response to the pharmacological properties of the drug itself (Perrotti et al., 2008) and its role in addiction has been largely studied. ΔFosB mediates sensitized behavioural responses to drugs of abuse (Kelz et al., 1999). More specifically, ΔFosB expression is involved in the increased locomotor and rewarding responses to cocaine and causes increased cocaine-seeking behaviour and self-administration (Whisler et al., 1999). Altogether, it may represent a mechanism for relatively prolonged sensitization to cocaine and contributes to relapse after lengthened periods of abstinence (Kelz & Nestler, 2000).

Interestingly, CREB and ΔFosB are two transcription factors that seem to exert opposite effects: while CREB inhibits drug reward, ΔFosB enhances it. Moreover, activation of CREB generally resolves within a few days to a week after drug withdrawal, whereas induction of ΔFosB lasts considerably longer. That would explain the initial period of dysphoria known to predominate during early withdrawal, followed by a more sustained period of sensitized responses to the drugs (Nestler, 2001b).

ΔFosB, as a transcription factor, exerts its effects on behaviour by regulating the transcription of its target genes. It mainly acts as a transcriptional activator but also represses a smaller subset of genes. In fact, ΔFosB is implicated in close to one-quarter of all genes influenced in NAcc by chronic cocaine exposure (McClung & Nestler, 2003). To date, several target genes of ΔFosB have been identified, including c-Fos, the AMPA glutamate receptor subunit 2 (GluA2), cyclin-dependent kinase-5 (CDK5) and the nuclear factor-κB (NFκB).

c-Fos

One candidate gene is ***c-Fos***, a member of the same family of transcription factors as ΔFosB. c-Fos is an immediate early gene whose

expression is repressed by ΔFosB, which helps to create the molecular switch: from the induction of several short-lived Fos family proteins after acute drug exposure to the predominant accumulation of ΔFosB after chronic drug exposure (Renthal et al., 2008). Importantly, the determination of c-Fos expression is used as an indirect measure of neuronal activity since it is usually expressed transiently and rapidly within some neurons following depolarization (Dragunow & Faull, 1989; VanElzakker et al., 2008). Accordingly, an increase in mRNA encoding c-Fos is a transsynaptic marker of recent neuronal activation (Day et al., 2008).

AMPA glutamate receptor subunit 2 (GluA2)

Another of its target genes is the ***GluA2 subunit*** (so-called GluR2) (Kelz et al., 1999). In addition to DA, glutamate projections from the PFC to the NAcc, VTA and amygdala are also involved in the long-term neuroadaptations underlying addiction (Kalivas, 2004). There are both ionotropic and metabotropic glutamate receptors. The subunits of the ionotropic receptors are classified according to their pharmacological properties and sequence similarity into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate or N-methyl-D-aspartate (NMDA) receptor families (Hollmann & Heinemann, 1994). AMPA receptors, in turn, are composed of four types of subunits: GluA1, GluA2, GluA3 and GluA4, which combine to form tetramers. In the NAcc, GluA1 and GluA2 subunits are expressed in virtually all medium spiny neurons, whereas GluA3 and GluA4 expression is relatively low (Schmidt & Pierce, 2010). Most AMPA receptors contain the GluA2 subunit, which reduces permeability to Ca^{2+} and thus, decreases its overall current. Accordingly, GluA2 is critical not only for many aspects of AMPA receptors function, but also for normal brain function. However, the

subunit composition of AMPA receptors varies with cell type, development and brain region (Song & Huganir, 2002).

As mentioned before, ΔFosB expression increases the responsiveness of an animal to the rewarding and locomotor-activating effects of cocaine, and these effects appear to be mediated partly by the induction of the GluA2 subunit expression in the NAcc (Kelz et al., 1999). Growing evidence indicates that cocaine indirectly influences glutamate transmission producing persistent maladaptive forms of neuroplasticity that lead to cocaine-seeking behaviour, and alters subsequent physiological responses to cocaine, including increased trafficking and surface expression of AMPA receptors during extended withdrawal (Boudreau & Wolf, 2005). Moreover, this increase co-occurs with a decrease of the extracellular glutamate levels in the same brain area (Baker et al., 2003). Altogether, these adaptations are hypothesized to facilitate glutamate release in response to a new cocaine injection and thus, lead to the reinstatement of drug-seeking behaviour. More specifically, such increased glutamate transmission through AMPA receptors is mediated by different trafficking of AMPA receptor subunits, including increased surface expression of GluA2-lacking AMPA receptors (Conrad et al., 2008), a finding that, in turn, is consistent with the decrease in whole-cell calcium current observed in the NAcc of rats during cocaine withdrawal (Zhang et al, 2002). Collectively, some discrepancies must be noted at this point since increased GluA2 expression levels by ΔFosB seems to result in an increased surface expression of GluA2-lacking AMPA receptors.

Cyclin-dependent kinase-5 (CDK5)

CDK5 is another target gene induced by ΔFosB. CDK5 is a kinase linked to multiple functions within the CNS. Namely, CDK5 participates in

neural development (migration, differentiation and neuronal connections), regulates synaptic transmission (DA signalling, release and modulation of synaptic vesicles recycling), has an important role in neurodegeneration, learning, memory and, importantly, in synaptic plasticity by regulating neurite outgrowth and synapse development (Angelo et al., 2006; Cheung & Ip, 2007; Hawasli & Bibb, 2007). Among all the substrates that CDK5 phosphorylates to mediate its functions, it is relevant to highlight the phosphorylation and thus, stabilization and increase of the activity of **tyrosine hydroxylase (TH)**, the key enzyme of the synthesis of DA (Angelo et al., 2006). Additionally, CDK5 also phosphorylates DARPP-32 at Thr 75, which results in a decreased DR1/cAMP signalling, considered to be an adaptive change to drug addiction, concretely, an homeostatic negative feedback against the behavioural effects of drugs (Bibb et al., 2001; Nishi et al., 2011).

CDK5 is induced in the NAcc by chronic but not acute cocaine exposure (Bibb et al., 2001; McClung & Nestler, 2003; Peakman et al., 2003) and its expression has been directly linked to increases in dendritic spine density of NAcc medium spiny neurons (Norrholm et al., 2003; Pulipparacharuvil et al., 2008) that are associated with chronic cocaine administration (Robinson & Kolb, 2004; Russo et al., 2010). Indeed, recently, ΔFosB has been considered necessary and sufficient for cocaine-induced dendritic spine growth, probably and, in part, through CDK5 (Maze et al., 2010).

Nuclear factor-κB (NFκB)

Finally, a fourth well-known induced target gene by ΔFosB is the ***NFκB***. The NFκB is a protein complex that acts as a transcription factor which is rapidly activated by diverse stimuli. It has been better studied

for its role in immunity (Smith et al., 2006), inflammation (Monaco et al., 2004) and cancer (Escárcega et al., 2007; Vlahopoulos, 2017). However, in the CNS it has been recently shown to mediate cell survival and to have an important participation in synaptic plasticity, learning and memory (Albensi & Mattson, 2000; Meffert & Baltimore, 2005; Meffert et al., 2003). NFκB is induced in NAcc by chronic cocaine exposure (Ang et al., 2001; Russo et al., 2009b), and so, along with CDK5 (Bibb et al., 2001; Norrholm et al., 2003; Pulipparacharuvil et al., 2008; Russo et al., 2009b), is required for cocaine's induction of dendritic spines of NAcc medium spiny neurons. In the caudate-putamen (dorsal striatum), NFκB induction is associated with increases in locomotion, whereas in the NAcc, NFκB contributes to sensitization to the rewarding effects of the drug (Ruffle, 2014; Russo et al., 2009b).

Recently, ongoing research using more sophisticated techniques like genome-wide gene and chromatin assays are providing a rich list of further putative genes that may be targeted by the transcription factor ΔFosB. In that list we can find additional neurotransmitter receptors, proteins involved in pre- and postsynaptic functions, ion channels, intracellular signalling proteins, proteins that regulate the neuronal cytoskeleton and cell growth, as well as proteins that regulate chromatin structure (McClung & Nestler, 2003; Renthal et al., 2008, 2009). There is increasing knowledge regarding to the latter, proteins that regulate the chromatin structure, since many genes are under control of not only transcriptional factors but also epigenetic mechanisms including chromatin remodelling. It is known that repeated exposure to drugs induces long-lasting epigenetic changes in the brain, modifying the chromatin structure of DNA via

histone acetylation, phosphorylation and/or methylation (Biliński et al., 2012). This field is particularly interesting since chromatin remodelling is considered a critical mechanism that leads to aberrant transcriptional changes in NAcc, contributing to drug-induced plasticity that may underlie aspects of drug addiction (Kumar et al., 2005; Renthal et al., 2008, 2009). In this sense, it has already been reported the essential role of the histone methyltransferase G9a in cocaine-induced plasticity (Maze et al., 2010).

Histone Methyltransferase G9a

G9a specifically catalyses the mono- and di-methylated states of histone H3 at lysine 9 (H3K9me2), which acts as a repressive mark that controls epigenetic regulation within the NAcc (Covington et al., 2011; Tachibana et al., 2001, 2002). Concretely, reduced G9a expression plays an essential role in mediating the development of addiction through a ΔFosB-dependent mechanism. Indeed, ΔFosB regulates G9a expression in the NAcc and both, ΔFosB and G9a, regulate some of the same target genes (i.e. *fos*) (Robison & Nestler, 2011). The interactions between G9a and ΔFosB have already been described in such a way that, after acute cocaine, G9a expression increases and methylates the *fosB* gene, inhibiting its transcription and thus, initially limiting the induction of ΔFosB. However, after repeated cocaine exposure, as ΔFosB accumulates, it represses G9a expression and thus, the methylation of the *fosB* gene, thereby potentiating its own further induction. Moreover, such G9a repression after chronic cocaine exposure is not observed for c-Fos, where *fos* methylation by G9a is increased, consistent with the fact that c-Fos, unlike ΔFosB, is repressed and not induced after repeated drug exposure (Maze et al., 2010).

As noted above, ΔFosB and G9a share many target genes, for example *fos*. However, G9a also targets other genes that have also a central role in the development of drug addiction: *arc* and *bdnf* (Walker et al., 2013; Zhang et al., 2014), amongst others (Maze et al., 2010).

Activity-regulated cytoskeleton-associated protein (Arc)

Arc, an immediate early gene, is localized at dendritic processes, a feature that allows the local synthesis of the protein by demand. In fact, Arc is highly regulated by changes in neuronal activity and, thus, its expression is considered a reliable index of activity-dependent synaptic modifications, that is, a marker for plastic changes in the brain (Fumagalli et al., 2006; Salery et al., 2017). Because of its high sensitivity to changes in neuronal activity and its peculiar localization, Arc represents a preferential target for drugs of abuse. In fact, the effects of different drugs of abuse on Arc expression have been studied after both acute and chronic expositions (Fumagalli et al., 2006; Kodama et al., 1998; Schiltz et al.,2005). Convincing evidence suggest that Arc contributes to the long-term effects of drugs such as cocaine, probably by alteration of the morphology of dendrites and spines observed in different brain regions after both acute and chronic drug exposure (Norrholm et al., 2003; Robinson & Kolb, 1999, 2004).

Brain-derived neurotrophic factor (BDNF)

BDNF is a member of the neurotrophin family of growth factors that acts on certain neurons of the central and peripheral nervous system regulating cell growth, survival and differentiation during the development of the nervous system (Acheson et al., 1995; Huang & Reichardt, 2001). In

the adult nervous system, it has also an important role in activity-dependent remodelling of neural function (Bolaños & Nestler, 2004).

BDNF is initially synthesized as a precursor (proBDNF) and subsequently cleaved into mature BDNF (mBDNF), which can be anterogradely transported contained in vesicles from the cell soma either to dendrites or axon terminals of BDNF synthesizing neurons (Lessmann et al., 2003). Alternatively, proBDNF can also be released by neurons, thereby extracellular proteases such as metalloproteinases and plasmin, can subsequently cleave the pro-region to yield mBDNF (Manti et al., 2017). mBDNF exerts its biological effects mainly through binding to its specific receptor tropomyosin receptor kinase B (TrkB), which can be localized either in the axon terminal or the postsynaptic dendrite. Under basal conditions, BDNF is highly expressed in VTA, amygdala, hippocampus and frontal cortex but, in less extent, in dorsal striatum and NAcc (Altar et al., 1997). Although there is some local production of BDNF in these regions, BDNF is chiefly supplied by anterograde axonal transport from cortical pyramidal neurons in frontal cortex (Li & Wolf, 2015).

There is convincing evidence that BDNF, along with its specific receptor, TrkB, have a key role in the behavioural abnormalities observed in rodents after psychostimulant administration (Li & Wolf, 2015; Pickens et al., 2011; Russo et al., 2009a). More specifically, research done over the last decades points BDNF to be involved in the long-term neuronal adaptations leading to functional modifications in the synapses associated with cocaine abuse and related behaviours, such as behavioural sensitization and drug-conditioning (Ghitza et al., 2010; Horger et al., 1999; Le Foll et al., 2005; McGinty et al., 2010; Russo et al., 2009a). Furthermore, BDNF synthesized in either VTA or neurons originating from the cortex, controls

DR3 expression (Guillin et al., 2001). Interestingly, hyperresponsiveness to drug-associated cues and context-dependent behavioural sensitization might be related to hypersensitive postsynaptic dopaminergic receptors. Among them, DR3 seems to be a key target since it is highly expressed in the NAcc (Di Chiara & Imperato, 1988). In this sense, BDNF along with DR3 play a key role in behavioural sensitization (Bordet et al., 1997; Guillin et al., 2001) and in place preference acquisition (Ciudad-Roberts et al., 2015).

To sum up, initial effects of drugs of abuse on their synaptic targets lead to molecular and cellular adaptations after chronic exposure, some of which must undergo a process of consolidation to form the stable adaptations that underlie the very long-lived behavioural abnormalities observed in many addicts. Altogether, although induction of ΔFosB constitutes the longest-lived molecular adaptation known to occur as a response to a drug of abuse, it is not permanent because, as all proteins, it finally undergoes proteolysis. Thus, it cannot underlie *per se* the near-permanent brain changes. Therefore, these adaptations need consolidation. The mechanisms of such consolidation are poorly known but could involve changes in chromatin or synaptic structure. Additionally, since drugs of abuse produce morphological changes in certain neuronal cell types after chronic exposure, these consolidations could also be mediated by such structural changes in neurons (Nestler, 2001b).

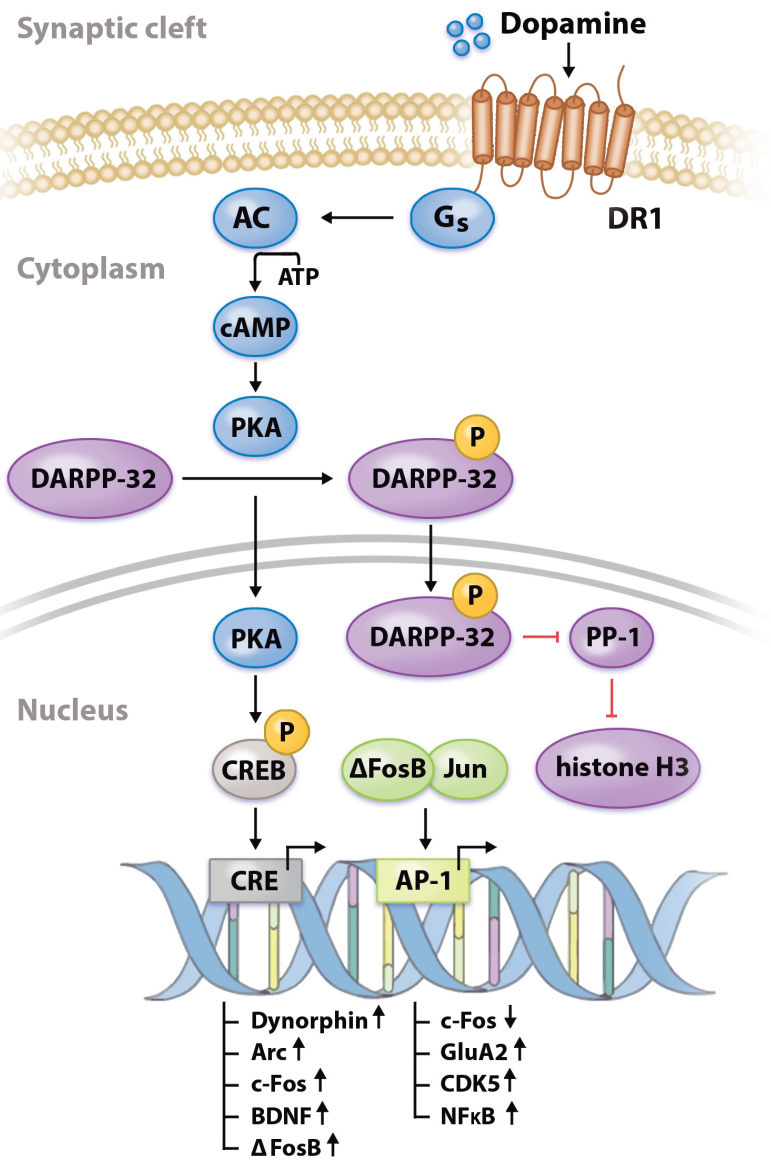

Figure 3. The DR1 – cAMP intracellular signalling cascade triggered within ***DR1-type medium spiny neurons in the NAcc.*** *Dopamine binds to DR1, which is coupled to G_s, thereby activates the cascade AC/cAMP/PKA. PKA phosphorylates DARPP-32 at Thr 34, which accumulates in the nucleus and inhibits PP-1, leading to the increase of histone H3*

phosphorylation, and thus, the expression of certain genes. In parallel, PKA also phosphorylates the transcription factor CREB at Ser 133, which binds to CRE sites in the DNA modulating gene expression (i.e. dynorphin, Arc, c-Fos, BDNF and ΔFosB). After repeated drug exposure, ΔFos accumulates and dimerizes with a member of the Jun family to form the active AP-1 transcription factor which binds to AP-1 sites in the DNA and regulates the expression of its target genes (i.e. c-Fos, GluA2, CDK5 and NFκB).

1.1.3. Animal models for studying drug addiction

Over the last decades, the behavioural abnormalities that define drug addiction have been studied accurately in animal models, especially in rodents (Koob et al., 1998; Robbins & Everitt, 1996; Robinson & Berridge, 2000; Wise, 1998). In this sense, diverse preclinical behavioural paradigms have been modelled to study the addictive properties (rewarding and reinforcing) of drugs, the development of sensitization and tolerance, the withdrawal syndrome, as well as the neuronal substrates and molecular/cellular mechanisms involved in such features.

1.1.3.1. Rewarding properties

Any stimulus that the brain interprets as intrinsically positive or as something to approach is considered a reward (Camí & Farré, 2003). In this sense, drugs of abuse are rewards. The rewarding properties of a drug can be assessed by the conditioned place preference (CPP) paradigm, the procedure of which is represented in Figure 4. In fact, for some drugs, the risk of abuse in humans can be predicted with this type of paradigms.

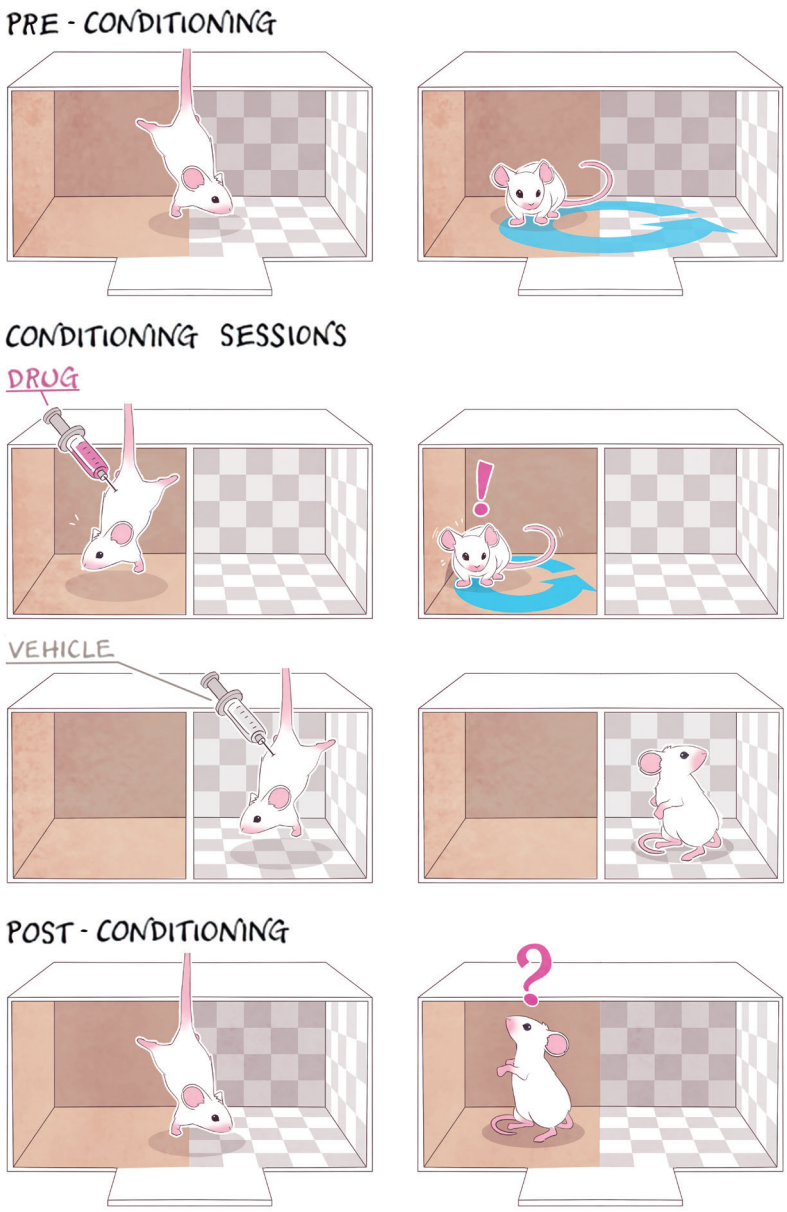

Figure 4. Conditioned place preference paradigm. *The apparatus consists of two main compartments connected by a corridor. The two compartments are disposed with differences in visual and tactile cues. The test is performed in three phases: 1) Pre-conditioning: mice have free*

access and roam among the apparatus. Animals with an initial preference are discarded from the experiment. 2) Conditioning: mice receive the drug and are placed into one compartment (drug-paired compartment). On alternate sessions, they are given the vehicle and placed into the other compartment (vehicle-paired compartment). Treatments are counterbalanced between compartments. 3). Post-conditioning test: performed as the pre-conditioning day, animals have free access among the apparatus and the time spent in each compartment is recorded. The rewarding properties of drugs make the animals prefer the drug-paired compartment, where they stay longer.

The CPP evaluates the conditioned rewarding effects of drugs related to environmental cues. That is, whether certain environmental stimulus have the property of eliciting approach responses such as drug-seeking. Indeed, persistent drug craving and relapse to drug use can be triggered by exposure to contextual cues associated with past drug use and by stress (Self & Nestler, 1998; Shaham et al., 2000). In this sense, the CPP is a model of relapse in drug addiction, in which animals are first trained to acquire a conditioned place preference in a drug-paired compartment. In other words, the rewarding properties of a drug are associated with the features of a given environment (compartment), therefore, after conditioning, animals prefer to spend more time in the drug-paired compartment. Afterwards, their preference can be extinguished. Hence, the extinguished CPP can be robustly reinstated by the non-contingent administration of a priming dose of the drug as well as by exposure to stressful stimuli, revealing drug-relapse (Blanco-Gandía et al., 2018; Prus et al., 2000; Sanchis-Segura & Spanagel, 2006). A priming dose refers to a new exposure to a formerly abused substance, which can precipitate rapid resumption of abuse at previous or higher levels (Camí & Farré, 2003).

The neuronal substrates involved in the rewarding effects of a drug include the VTA-NAcc pathway, but also other structures that mediate learned

or conditioned responses (i.e. the hippocampus, the amygdala, PFC or ventral pallidum) (Koob et al., 1998; Robbins & Everitt, 1996; Robinson & Berridge, 2000; Self & Nestler, 1998; Shaham et al., 2000; White, 1989). Moreover, many studies have indicated that other ascending monoaminergic fibers, such as noradrenergic neurons from the locus coerulus and serotoninergic neurons from the raphe nuclei, also contribute to brain reward function (Watterson & Olive, 2014). Regarding the VTA-NAcc pathway, the release of DA provoked by drugs of abuse, and thus the activation of DR1 in NAcc seems to be necessary for the rewarding effects of drugs (Caine et al., 2007; White, 1989) as well as for triggering conditioned responses (Zweifel et al., 2009). Conversely, the activation of DR2 limits drug reward (Durieux et al., 2009).

1.1.3.2. Reinforcing properties

A positive reinforcer is a stimulus that strengthens the behaviour that produced it. Although similar, a "positive reinforcer" and a "reward" are not exactly the same. Rewarding stimuli, which are associated with desire and pleasure, may not necessarily be reinforcing, so they may not increase the behaviour preceding them. Therefore, rewarding stimuli only function as positive reinforcers if the behaviour that immediately precedes the reinforcer increases in similar situations in the future (Schultz, 2015). In this context, drugs of abuse are positive reinforcers that increase the probability of being administered again. The reinforcing properties of a drug can be tested either by the intravenous self-administration (SA) and the intracranial self-stimulation (ICSS) paradigms. The former model is generally considered the best among preclinical paradigms to investigate drug-seeking and drug-taking behaviour, allowing to assess the abuse liability of psychoactive drugs.

In brief summary, in this procedure, an indwelling intravenous catheter is surgically implanted into the jugular vein of the animal. After recovering, the animal is placed in a self-administration chamber equipped with levers or nosepokes (one active and one inactive) that are interfaced to a computer and a syringe pump, which contains the drug or the vehicle (Figure 5). Stimulus lights, a speaker and a smell can provide also visual, auditory and olfactory cues during drug-infusions. So that, when the animal presses the "active" lever or nosepoke, it results in a computer-controlled drug intravenous infusion and simultaneous brief presentation of the visual, auditory or olfactory cues. Conversely, if the animal presses the "inactive" one, no response is obtained. Therefore, SA is a form of operant conditioning where the positive reinforcer is the drug. Accordingly, the higher is the frequency with which the animal emits the operant behaviour, the more reinforcing the drug is. In fact, if left unchecked, a number of animals would overdose. As in the CPP paradigm, the animals can be subjected to an extinction session to extinguish their operant behaviour and, thereafter, reinstate the drug-seeking behaviour with a priming dose, constituting a model for drug-relapse (Kmiotek et al., 2012; Sanchis-Segura & Spanagel, 2006; Watterson & Olive, 2014).

Similarly, ICSS is the operant behaviour paradigm in which electrodes are implanted into specific regions of the reward system in the brain of the animals, which learn to activate them to deliver electrical pulses in these areas. In all ICSS procedures, lowering of ICSS thresholds indicates a facilitation of brain stimulation reward, whereas elevations in ICSS thresholds reflect the diminished reward value of the stimulation, and thus, an anhedonic state. In this sense, since drugs of abuse activate the brain reward system, acute administration of most drugs lower ICSS thresholds in experimental animals. By contrast, withdrawal from chronic administration of drugs and the consequent lack of DA in reward-related regions, promotes an animal's

choice to electrically stimulate its brain, thus, induces elevations in ICSS thresholds, indicating an anhedonic state that resembles the negative affective state of the withdrawal syndrome experienced by humans (Negus & Miller, 2014; Vlachou & Markou, 2011).

The dorsolateral striatum appears to be the central neural substrate that mediates the reinforcing behaviour. Emerging evidence also indicates that the release of DA in the striatal matrix promotes the consolidation of associations between sensorial and motor responses. Accordingly, the striatal matrix might mediate stimulus-response memory and hence, other brain regions may be involved, such as the cerebral cortex, substantia nigra and its projections to thalamic and brainstem motor areas (White, 1989).

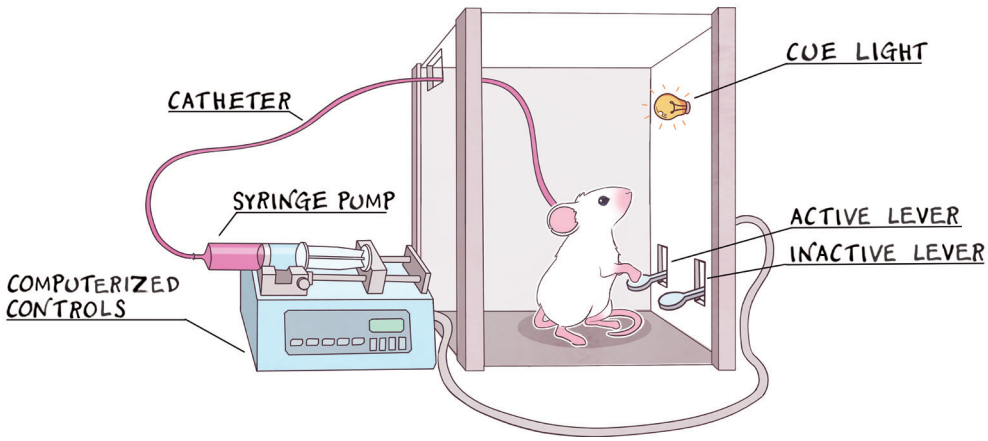

Figure 5. Self-administration paradigm. *The animal is surgically implanted a catheter into its jugular vein and placed in a self-administration chamber. During the acquisition of the behaviour, the animal can press an active or inactive lever. If it presses the active one, results in a computer-controlled drug intravenous infusion and a simultaneous brief presentation of visual, auditory or olfactory cues. The inactive lever does not lead to any response. Drugs of abuse act as positive reinforcers which make the animals want to press the active lever over and over again.*

1.1.3.3. Drug sensitization

Drug sensitization can be defined as the increase in the expected effect of a drug after repeated administration. Alternatively, sensitization can also refer to persistent hypersensitivity to the effect of a drug in a person with a history of exposure to that drug or to stress (Camí & Farré, 2003). In the present thesis, though, the term *sensitization* will be used mainly to refer to the locomotor sensitization (behavioural sensitization) to psychostimulants, that is, the process whereby repeated intermittent exposure to drugs results in a progressive and enduring increase in the motor stimulant response to the drug (Kalivas et al., 1993; Kuczenski & Segal, 1969; Pierce & Kalivas, 1997; Robinson & Berridge, 1993; Steketee & Kalivas, 2011).

The processes involved in sensitization and the neuronal substrates and pathways involved in the rewarding effects of drugs are overlapped (Robinson & Berridge, 1993, 2000). Behavioural sensitization is associated with marked and long-lasting alterations in the functional activity of the mesocorticolimbic dopaminergic system, particularly in DA and glutamate transmission in the NAcc (Hyman & Malenka, 2001; Pierce & Kalivas, 1997). Those alterations, in turn, are due to changes in the patterns of expression of certain genes in the same reward system (Nestler, 2001b), especially due to the activation of the Fos family proteins described above. In this context, the VTA is essential for the development of behavioural sensitization, whereas the NAcc seems to be necessary for its expression (Bjijou et al.,1996; Cador et al.,1995). For instance, repeated administration of stimulants increases the number of dendritic branch points and spines on medium spiny neurons in the NAcc and pyramidal neurons in the medial prefrontal cortex (mPFC) (Robinson et al., 2001). As a long-lasting phenomenon, behavioural sensitization can persist for at least one year after

cessation of drug administration (Paulson et al., 1991) and is augmented by environmental cues (Hyman & Malenka, 2001). These context cues along with the sensitization phenomena, contribute to craving and an increased risk of relapse even after long withdrawal periods (Camí & Farré, 2003).

Overall, there is compelling evidence that drugs of abuse, although having different chemical structures, and thus, diverse mechanisms of action, they all converge in inducing alterations in brain areas that mediate reward and reinforcement. These changes, in turn, may include tolerance, sensitization and/or physical dependence. In this context, the list of putative addictive substances becomes considerable.

1.1.4. Current drug situation

Drugs of abuse represent a heavy burden of disease in many countries, becoming a global problem not only at a health level, but also at a social and economic extent. According to the last data reported by the United Nations Office on Drugs and Crime (UNODC), in 2017, an estimated 275 million people, that is 5.5% of the global population aged from 15 to 64, had used drugs in the previous year. Therefore, the number of people who use drugs is 30% higher than in 2009. Such alarming data come along with over half a million deaths which are estimated to be as a result of drug use in the same year. Furthermore, some 42 million years of "healthy" life were lost as a result of drug use, including premature deaths and years lived with disability (UNODC, 2019b).

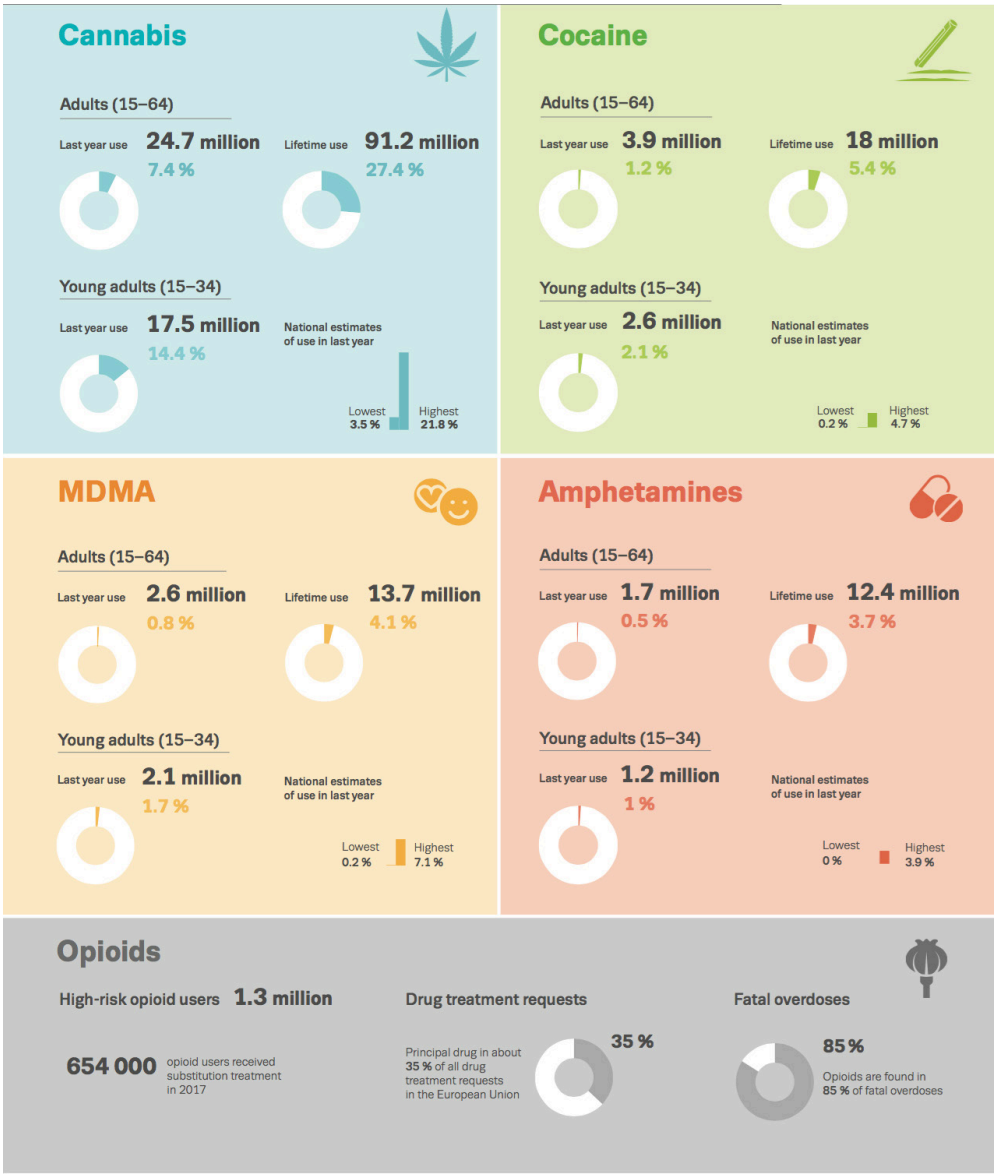

Figure 6. Estimates of drug use in the European Union during 2018. *Estimates of cannabis, cocaine, MDMA, amphetamines and opioids use, according to the age and frequency of use. Adapted from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2019)*

Currently, in modern societies, drug consumption comprises a wider spectrum of substances than in the past. At the moment, the most widely used drug worldwide is cannabis (EMCDDA, 2019; UNODC, 2019b), and, within Europe, it is followed by cocaine, ecstasy (3,4-methylenedioxymethamphetamine, MDMA), amphetamines and opioids (EMCDDA, 2019 (Figure 6). However, and favoured by globalisation and innovation, the illicit drug market is continuously changing at high speed with the constant emergence of new substances that threat public health.

Taken together, the list of the already existing psychoactive drugs is extremely extensive. Hence, the present introduction will focus exclusively on those substances that are relevant to this doctoral thesis.

1.2. Cocaine

Definition and background

Cocaine is a tropane alkaloid present in the leaves from the *Erythroxylum coca* (coca plant) that exerts powerful psychostimulant effects. For the past 4000 years, the use of coca has been greatly entrenched in the social and religious culture of many ancient civilizations in the territory that is now known as Chile and Peru. The leaves of the coca plant were, and still are, universally chewed by some indigenous communities in order to increase the state of alertness and obtain the energy and strength necessary to struggle with the hard day-to-day activities. Although the stimulant properties of coca were already known, the isolation of the cocaine alkaloid

was not achieved until 1855 by the German chemist Friedrich Gaedcke (Gaedcke, 1855).

Upon its isolation, cocaine became popular worldwide and started to be used with several purposes arising from its properties as a hunger-suppressant, invigorating, stimulant and analgesic. The drug was introduced into clinical use as a local anaesthetic for ophthalmic usage, being described also as a nerve-block, peridural, spinal and respiratory system anaesthetic (Corning, 1885). Moreover, the leaves of coca plant were officially included in the United States pharmacopoeia in 1880 as a treatment against alcohol and morphine addiction (Dugarin & Nominé, 1988). Hence, many cocaine-containing medicinal preparations appeared on the market. Besides its medical usage, the coca leaves were also included in many recipes for elaborating drinks (i.e. cocawine and Coca-Cola®), although they were eventually replaced by caffeine. All cocaine uses were spread and encouraged by the psychoanalyst Sigmund Freud, a usual consumer (Freud, 1885). By then, the social impact that cocaine caused was overwhelming, cocaine products were widely sold to "supply the place of food, make the coward brave, the silent eloquent and the render the sufferer insensitive to pain".

However, in 1914, due to its high abuse potential, the first regulatory law controlling cocaine was passed, allowing its distribution and use only under medical prescription and for therapeutic purposes. In the mid XXth century, though, cocaine consumption and addiction were substantially spread throughout the Western world, posing one of the biggest drug epidemics in history, especially in North America (Cabrera, 2000), which led to a significant limitation of its use. Nevertheless, a second full-blown epidemic was unleashed in the 1980s, especially in the United States, with the rise of crack cocaine.

Nowadays, cocaine constitutes the most abused psychostimulant and the second most consumed drug in Europe (EMCDDA, 2019), being a persistent health problem worldwide (UNODC, 2019b).

Prevalence

According to the UNODC 2019 World Drug Report, the estimate annual prevalence of the global use of cocaine in 2017 was of the 0.37% of the global population. That is, almost 30 million people used to consume cocaine in 2017. In the same year, the estimated global illicit manufacture of cocaine reached the highest values ever reported: 1,976 tons, mainly driven by increases in its manufacture in Colombia, the main manufacturer of cocaine (UNODC, 2019b). In accordance, current data on cocaine show that both the number of seizures and the volumes seized are at an all-time high (EMCDDA, 2019; UNODC, 2019b).

More concretely, in the European Union (EU), cocaine is the predominant stimulant in Southern and Western countries. Consequently, the number of new patients treated for cocaine problems has increased considerably over 35%, as well as the number of cocaine-related deaths (EMCDDA, 2019). Moreover, around 18 million people reported lifetime use of cocaine and, importantly, drug use is largely concentrated among adults rather than young adults (EMCDDA, 2019; NIDA, n.d.).

Pattern of use

Cocaine can be found as a hydrochloride salt or as a free base form. The former is the most common form, which constitutes a fine white powder, bitter to the taste, that often contains also adulterants (i.e. caffeine

or paracetamol). That powder is typically insufflated nasally (snorted, sniffed or blowed), but also ingested. Alternatively, cocaine can be turned into a solution and so injected intravenously, providing the highest blood levels of drug in the shortest amount of time (Volkow et al., 2000).

Conversely, the free base form of cocaine, known as *crack cocaine*, can be smoked. Crack is formed when mixing the hydrochloride salt with sodium bicarbonate or other weak bases (i.e. ammonium bicarbonate and ammonium carbonate). Some users convert powder cocaine into crack by themselves, mixing it with baking soda and water in a spoon and warming with a lighter.

Pharmacology: mechanism of action

Cocaine, as many psychostimulants, exerts its effects by interacting with plasma membrane transporter proteins expressed on nerve cells that synthesize the monoamine neurotransmitters DA, noradrenaline (NA) and serotonin (5-HT). To better understand the molecular mechanism of cocaine it is essential to first consider the normal physiological role of monoamine transporters.

The principal function of the monoamine transporters for dopamine (DAT), noradrenaline (NET) and serotonin (SERT) is to translocate the previously released neurotransmitters from the synaptic cleft back into the presynaptic neuron cytoplasm, a process called “neurotransmitter uptake” (reviewed by Alexander et al., 2017; Kristensen et al., 2011). Importantly, the neurotransmitter uptake is the main mechanism for terminating the action of monoamine signalling, thus, drugs of abuse that target these proteins may have significant effects on cell-to-cell transmission.

There exist several compounds whose mechanism of action is to inhibit monoamine transport function. For instance, molecules that interact at SERT (i.e. serotonin-selective reuptake inhibitors) are widely prescribed as treatment for anxiety and major depression. However, drugs that preferentially act at DAT and NET, such as cocaine, amphetamines, MDMA or methamphetamine, can have powerful psychostimulant and dependence-producing properties (Espana & Jones, 2013; Howell & Kimmel, 2008; Rothman & Baumann, 2003).

Overall, cocaine acts inhibiting preferentially DAT and NET and, with less potency, SERT. Accordingly, it is considered a sympathomimetic drug because it mimics the effects of monoamine neurotransmitters of the sympathetic CNS.

Additionally, cocaine binds and blocks the voltage-gated sodium channels in the neuronal cell membrane, thus interfering with the initiation and conduction of nerve impulses. Although this mechanism has little relevance as regards to the psychostimulant effects of cocaine, it mediates its local anaesthetic effects, as well as some side effects as cardiac arrhythmia.

Subjective user effects

Derived from its mechanism of action, cocaine produces some short-term desired sympathomimetic effects on individual users: extreme happiness (euphoria) and energy, mental alertness, loss of appetite, insomnia and increased confidence. Furthermore, some users have reported a better performing of physical and mental tasks under cocaine effects (NIDA, n.d.).

Toxicology

Cocaine acute consumption is often followed by side clinical effects such as hypersensitivity to sight, sound and touch, irritability, anxiety, paranoia, cardiac problems (constricted blood vessels with subsequent raise of blood pressure and fast and/or irregular heart beat), dilated pupils, nausea, hyperthermia, tremors, convulsions and restlessness. High doses of cocaine, in addition, can lead to bizarre, unpredictable and violent behaviour. Acute cocaine intoxications can cause death by haemorrhagic episodes, ischemic strokes or heart attacks (Havakuk et al., 2017).

Depending on the pattern of use, chronic addicts can also suffer from loss of smell, nosebleeds, malnutrition, respiratory problems, bruxism, pruritus and a higher risk for contracting infectious diseases (i.e. the human immunodeficiency virus (HIV), Hepatitis A, Hepatitis C or other sexually transmitted diseases), among many other adverse effects.

Addiction

The abuse liability of psychostimulants such as cocaine is well stablished and represents a significant public health concern (Howell & Kimmel, 2008). Cocaine is considered a highly addictive stimulant, mainly due its action inhibiting DAT. Its powerful psychostimulant, rewarding and reinforcing effects have been widely described elsewhere.

Legal status

The production, distribution and sale of cocaine is banned and illegal in most countries as regulated by the Single Convention on Narcotic Drugs

(Single Convention on Narcotic Drugs, 1961) and the United Nations Convention Against Illicit Traffic in Narcotic Drugs and Psychotropic Substances (United Nations Convention Agains Illicit Traffic in Narcotic Drugs and Psychotropic Substances, 1988). Some countries as Peru or Bolivia, though, allow the cultivation of the coca plant for traditional consumption by the local indigenous. Still, the production, sale and consumption with other purposes is prohibited.

1.3. New Psychoactive Substances

Over the last decade, the illicit drug market has changed considerably due to the emergence of New Psychoactive Substances (NPS). A NPS is defined as a new narcotic or psychotropic drug, in pure form or preparation, that is not controlled by the United Nations Drug Conventions (Single Convention on Narcotic Drugs, 1961; Convention on Psychotropic Substances, 1971), but which may pose a similar public health threat as substances already listed in these conventions (Council Decision 2005/387/JHA, 2005; Regulation (EC) No 1920/2006). It is important to point out that the term "new" does not necessarily refer to a drug synthesized for the first time, but to substances that were not available before in the drug market. In this sense, several NPS were synthesized even decades ago for different reasons than controlled drugs (Brandt, 2014; Sumnall, 2011), but their abuse was not reported until the early 2000s.

What is really new and disturbing, though, is the dramatic increase of new substances that have emerged on the drug market over the last decade, and the high speed at which they have appeared (Griffiths, 2013). As depicted

in Figure 7, the number of NPS reported on the market has been rising at an unpredictable rate until reaching a peak in 2014-2015. Fortunately, the emergence of NPS stabilized at levels comparable to 2011-2012 over the period 2015-2017, probably due to the sustained efforts made by the authorities to control them. Currently, by the end of 2018, 119 countries and territories had reported to the UNODC around 890 NPS of which more than 730 were monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), being 55 of them detected for the first time in Europe the same year (EMCDDA, 2019; UNODC, 2019a). Therefore, although the number of new substances making their debut has stabilised and it is down the peak reached in 2014-2015, the negative public health implications derived from their use (i.e. acute intoxications and deaths) remain unacceptably high (Baumann & Volkow, 2016; Logan et al., 2017).

NPS encompasses a vast array of products, including either natural herbal mixtures and synthetic or "designer" drugs. The UNODC classifies the different NPS sold in the market according to their molecular structure, defining nine different categories: synthetic cannabinoids, synthetic cathinones, aminoindanes, phencyclidine-type substances, phenetylamines, piperazines, plant-based substances, tryptamines and other substances which do not fit into the previous categories. On the other hand, when looking at their psychoactive effects, NPS can be divided into stimulants (i.e. synthetic cathinones), cannabinoids, hallucinogens, opioids, dissociative drugs and sedatives/hypnotics. In this sense, the UNOCD reported that among the NPS stated until December 2018, the majority were stimulants, followed by cannabinoids and hallucinogens (UNODC, 2019a).

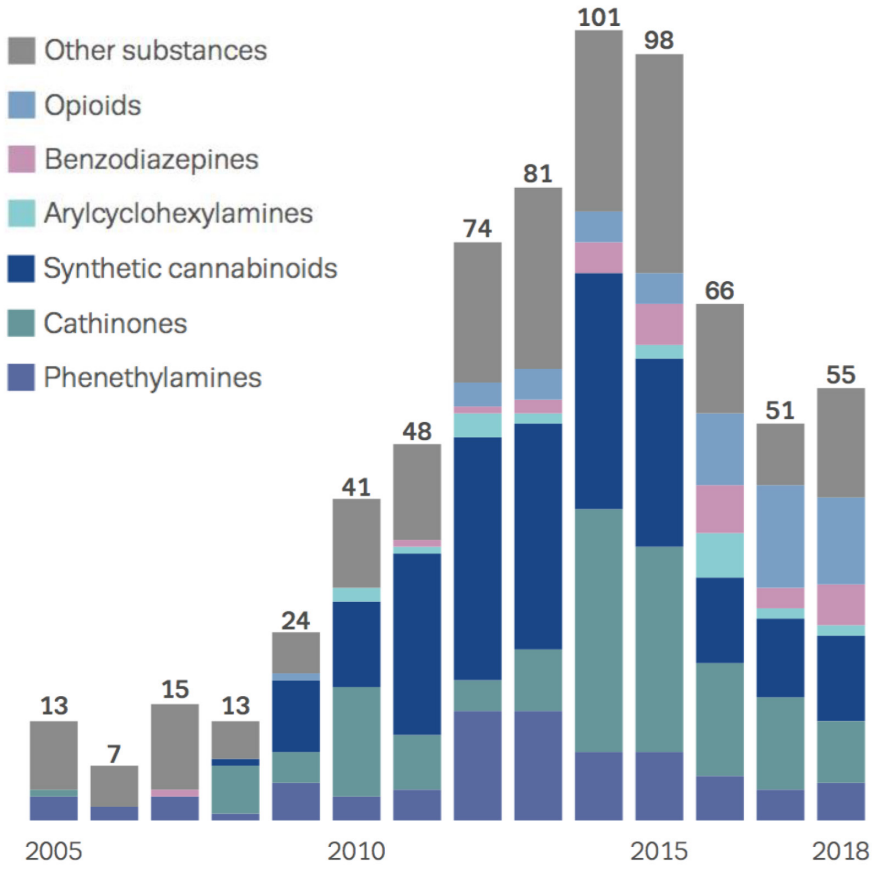

Figure 7. Number and categories of new psychoactive substances notified to the European Union Early Warning System for the first time, 2005-18. *Adapted from the European Monitoring Centre for Drugs and Drug Addiction* (EMCDDA, 2019)

NPS are usually synthesized in bulk quantities by chemical and pharmaceutical companies in China (and to a lesser extent, in India), from where they are shipped to Europe, processed into products, packaged and sold. Alternatively, substances may also be diverted by licensed manufacturers. Additionally, their production in clandestine laboratories either in Europe or elsewhere has increased in the last few years. Although restrictions

implemented over the years have considerably limited the open sale of NPS, some of them are still sold online and in physical specialized shops (such as "smart" and "head" shops), often branded as "legal high" products. Normally they are advertised with aggressive and sophisticated marketing strategies under innocuous names such as "bath salts", "plant food", "air fresheners", "herbal incenses" or "laboratory reagents", among others, featuring the label "not for human consumption", in order to circumvent consumer protection and medicine laws. Accordingly, NPS are better known as "legal highs", "bath salts" or "research chemicals". Furthermore, they can also be easily obtained in a more covert way through the *darknet* or directly via the illicit drug market where they are sold under their own name or, in some cases, intentionally mislabelled with purported ingredients differing from the actual composition (i.e. illicit drugs as heroin, cocaine or MDMA), or mixed with them (Brandt et al., 2014; EMCDDA, 2018; EMCDDA, 2019; Maurer & Brandt, 2018; Sumnall et al., 2011). Conversely, many NPS are intentionally traded as "legal" replacements to already controlled drugs such as cannabis, benzodiazepines or cocaine, since they produce similar effects as these well-known illicit drugs and are synthesized avoiding the international control (EMCDDA, 2016; EMCDDA, 2018; UNODC; 2019a).

Nonetheless, little is known about the pharmacology, toxicology and social harms of these new substances, hindering its prevention and treatment, and thus, posing a threat to public health. Moreover, since NPS are not controlled under the International Drug Control Conventions, their legal status can differ widely from country to country (UNODC, 2019a). Until 2018, over 60 countries have already implemented legal responses to control NPS. However, the speed and sophistication at which the market reacts to control measures, along with the great diversity of available products, have proved challenging for the conventions and for current drug policymakers

and lawmakers. In this sense, the new European legislation retains the three-step approach to respond to NPS: early warning, risk assessment and control measures, which are definitely needed to counter this new drug problem (EMCDDA, 2019).

1.3.1. Synthetic cathinones

Synthetic cathinones represent the second largest group of NPS monitored by the EMCDDA, just after synthetic cannabinoids. The first cathinones were reported in 2004 and, by the end of 2017, a total of 130 different synthetic cathinones had been identified (EMCDDA, 2018). Moreover, they represented the second most frequently seized group of NPS and the most seized NPS by quantity (1.9 tonnes) in 2016 (EMCDDA, 2016).

Synthetic cathinones are synthetic derivatives of the plant-derived stimulant cathinone, a potent alkaloid found in the shrub *Catha edulis* (Khat) leaves (Banks et al., 2014; Kelly, 2011; Sørensen, 2011). In fact, if attending its molecular structure, cathinone is the β-keto analogue of amphetamine (so-called β-keto-amphetamine) because of the presence of a ketone group at the β-carbon of the phenethylamine (Figure 8).

The chemical structure of cathinone can be considered as the prototype from which a range of derivatives have been developed (Figure 9). In this sense, synthetic cathinones differ from each other by ring-substitutions (R_1), substitutions on the α-carbon (R_2), variations of the length of carbon substitutions off the α-carbon (R_3) and/or amino-substitutions (N-alkylation, R_4, R_5). As a result of all these possibilities, synthetic cathinones make up a broad range of substances.

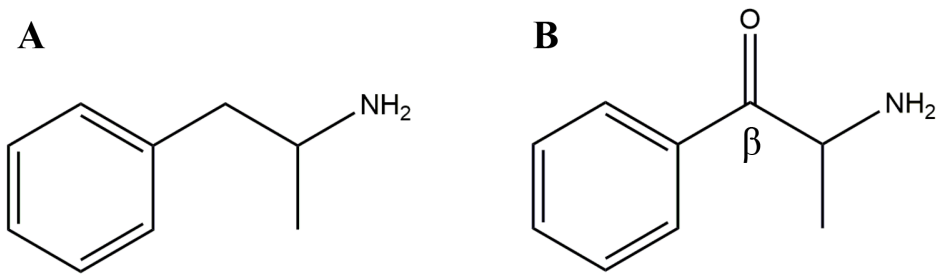

Figure 8. Chemical structure of amphetamine (A) and cathinone (B). *Cathinone is the β-keto analogue of amphetamine.*

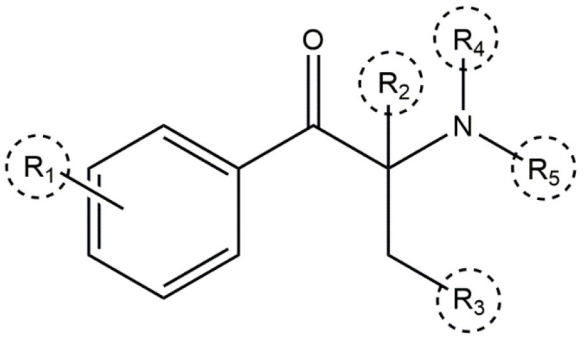

Figure 9. Generic structure considered the prototype from which synthetic cathinones are developed, differing among them in the R substituents. *Adapted from Kelly, 2011.*

Synthetic cathinones broke into the drug market at a time when the availability, and thus purity, of established drugs like MDMA and cocaine decreased in many areas (Brunt et al., 2011; Measham et al., 2010). The resultant disillusionment and necessity amongst users were a key motivation

for the displacement from illegal drugs to a first generation of substituted cathinones, which produce psychostimulant effects similar to classic stimulants, but were initially legal, easily accessible via head shops and the Internet, and cheaper than the classic ones (Brunt et al., 2011; Carhart-Harris et al., 2011; Measham et al., 2010; Winstock et al., 2011). Hence, cathinone derivatives started to be sold as legal alternatives to cocaine, MDMA or amphetamines (Brandt et al., 2010; Prosser & Nelson, 2012; Shanks et al., 2012; Spiller et al., 2011), but also as adulterants of the same replaced drugs (Brandt et al., 2014; Brunt et al., 2011; Ross et al., 2011).

The first cathinone derivative to be reported to the EMCDDA was methylone (Figure 10) (popularly known as *Explosion* or *Top Cat*), the *β*-keto analogue of MDMA (Bossong et al., 2005). Shortly after, in 2007, mephedrone (Figure 10) (slang names: *meow-meow, miaow, M-CAT* and *White Magic*) emerged also in the recreational drug market (Gustavsson and Escher, 2009; Winstock et al., 2010). Along with methylone and mephedrone, a first-generation of synthetic cathinones appeared on the drug market at an unprecedented rate, including flephedrone (Archer, 2009), ethylone, butylone and 3,4-methylenedioxypyrovalerone (MDPV) (Figure 10) (Brandt et al., 2010; Meyer & Maurer, 2010), among many others.

Synthetic cathinones are typically found in powder form, which is usually self-administered by insufflation (snorting), but also as capsules or tablets to be ingested orally. Alternatively, the powder can also be diluted in water/juice or wrapped in cigarette paper and swallowed (known as "bombing"). Less commonly, rectal or eye insertion, as well as intravenous, subcutaneous and intramuscular injection have also been reported (EMCDDA, 2019; Prosser & Nelson, 2012; Zawilska & Wojcieszak, 2013).

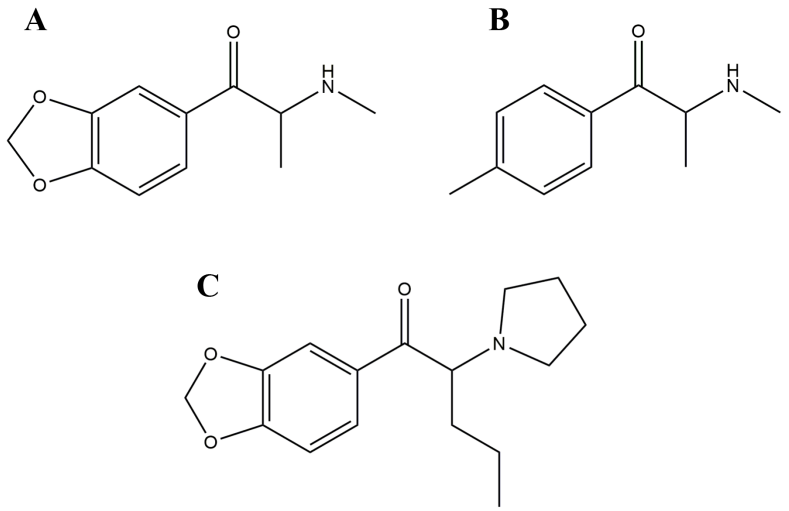

Figure 10. Chemical structure of methylone (A), mephedrone (B) and MDPV (C). *They are the three most popular first-generation synthetic cathinones.*

Nowadays, the substitution of an established illicit drug (usually stimulants or heroin) by a NPS is still a pattern of use reported among high-risk drug users (Sande, 2016; Tarján et al., 2017). Synthetic cathinones are sold as preparations of single cathinones, but more frequently, as mixtures of two or more different drugs, along with adulterants (i.e. caffeine or lidocaine) (Brandt et al., 2010; Davies et al., 2010; Zawilska & Wojcieszak, 2013; Zuba & Byrska, 2013). Therefore, synthetic cathinones use, as the rest of NPS, mainly occurs in a context of polydrug use, so they are often used in combination with other stimulants, opioids, benzodiazepines, alcohol or tobacco, either in simultaneous use or on different sessions (EMCDDA, 2010a; EMCDDA, 2017b). Concretely, concurrent use of synthetic cathinones and other stimulants (amphetamines, cocaine or MDMA) is especially prevalent among abusers (Carhart-Harris et al., 2011; EMCDDA, 2010a) and unfortunately, emerged evidence suggests that synthetic cathinones enhance the toxicity of some stimulants (Angoa-Pérez et al., 2013) and thus,

the rate of fatalities (Aromatario et al., 2012; Marinetti & Antonides, 2013; Maskell et al., 2011; Schifano et al., 2012). Moreover, in most cases, NPS are not reported to be the primary drug used by high-risk drug users, but the secondary or tertiary drug, for example, when the preferred substance is not available or to heighten the effect of the other drugs (Aromatario et al., 2012; EMCDDA, 2017b; EMCDDA, 2010a; Prosser & Nelson, 2012).

As cathinone derivatives and therefore, due to their close structural similarity to amphetamines, synthetic cathinones were predicted to act dysregulating the central monoamine system. Effectively, synthetic cathinones exert their effects by inhibiting the function of monoamine transporter proteins DAT, NET and SERT, expressed on nerve cells and other cell types in the central and peripheral nervous system (Eshleman et al., 2013; Simmler et al., 2013; Simmler et al., 2014). In this sense, synthetic cathinones are classified in two types depending on how they inhibit the transporter function (Figure 11): (1) *Blockers, cocaine-like*: those that bind to the transporter and inhibit its uptake function (i.e. MDPV (Baumann et al., 2013)); and (2) *Releasers, amphetamine-like:* those that bind to the transporter but act as substrates, thus they are translocated through the transporter channel into the neuronal cytoplasm. There, they provoke the release of intracellular neurotransmitters stored in vesicles to the cytosol and from there, to the synapse by reverse transport (Reith et al., 2015; Sitte & Freissmuth, 2015) (i.e. methylone and mephedrone (Baumann, 2014; Hadlock et al., 2011; López-Arnau et al., 2012; Martínez-Clemente et al., 2012)). Furthermore, they can act as mixed or "hybrid" reuptake inhibitors and releasing agents at the same time (Saha et al., 2019).

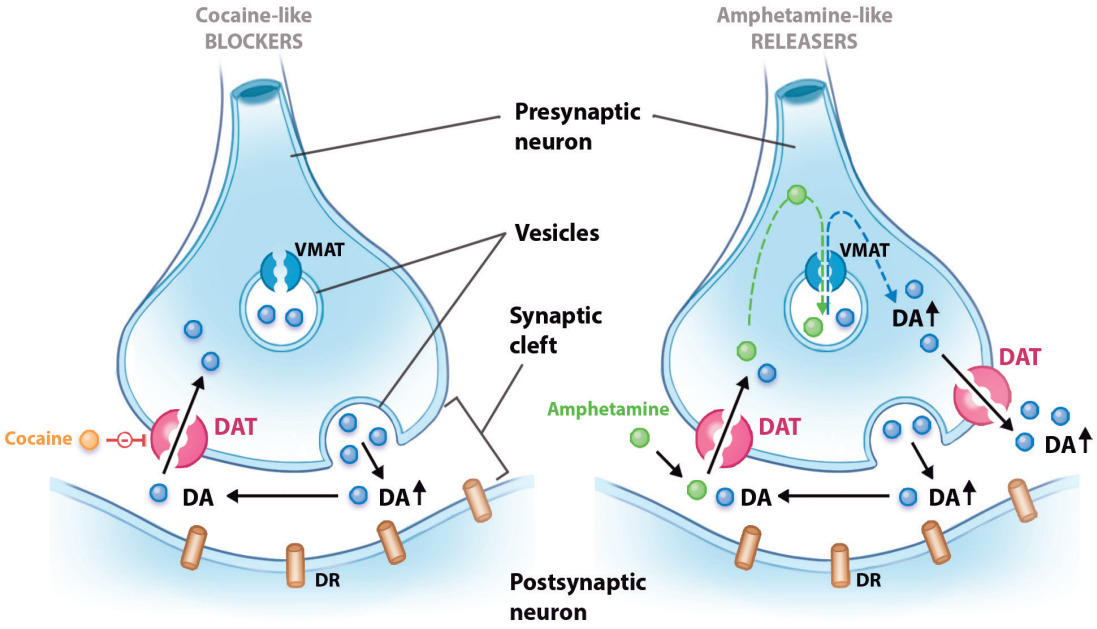

Figure 11. Mechanism of action of cocaine and amphetamine. *Cocaine acts mainly as a DAT blocker, thus inhibiting DA uptake. By contrast, amphetamine acts as a substrate, enters into the terminal and triggers efflux of the neurotransmitter. Synthetic cathinones are divided in two main groups according to their mechanism of action: blockers (cocaine-like) or releasers (amphetamine-like). VMAT: vesicular monoamine transporter.*

Taken together, regardless their mechanism of action, synthetic cathinones reduce the effectiveness with which DA, NA and 5-HT are cleared from the synapse following release, producing an increase of the extracellular monoamine concentrations in the brain, thereby enhancing cell-to-cell signalling throughout the CNS. The resulting overstimulation of postsynaptic DA, NA and 5-HT receptors in the brain and periphery results in their psychological, behavioural and toxic effects.

Self-reported data from users of synthetic cathinones indicate that individuals experience subjective effects like those produced by common illicit stimulant drugs such as amphetamine, methamphetamine, cocaine and MDMA, with which they share the mechanism of action. The main desired effects reported are increased energy, alertness, empathy, openness, concentration, euphoria and increased libido.

However, as all drugs of abuse, the desired effects come along with considerable adverse physiological effects occurring at a central and peripheral level, which arise from the hyperstimulation of the sympathetic nervous system induced by the inhibition of the monoamine transporters. Cardiac, psychiatric and neurological signs and symptoms are the most common adverse clinical effects reported in users who require medical care. More specifically, the effects include cardiovascular complications (palpitations, hypertension, tachycardia, myocardial infarction, chest pain), agitation, cerebral edema, shortness of breath, nausea, vomiting, anorexia, erectile dysfunction, bruxism, mydriasis, blurred vision, diaphoresis, hyperreflexia, etc. Also, prominent adverse neuropsychiatric effects including aggressiveness, hallucinations, paranoia, psychosis and anxiety have been reported (German et al., 2014; Kelly, 2011; Logan et al., 2017; Prosser & Nelson, 2012; Spiller et al., 2011; UNODC, 2013; Winstock et al., 2011).

In addition, because cathinones interact with monoamine transporters and thus, increase extracellular concentration of monoamine neurotransmitters in the brain (i.e. DA), they may also exert considerable psychostimulant, rewarding and reinforcing effects. Indeed, the fact that they stimulate dopamine transmission predicts that they may possess high abuse liability (Howell & Kimmel, 2008; Wise, 2008).

As mentioned above, synthetic cathinones are not under the control of the International Drug Control Conventions, which may make their legal status vary between countries and change rapidly. Only cathinone, methcathinone, cathine, amfepramone and pyrovalerone are cathinone derivatives internationally controlled (Convention on Psychotropic Substances, 1971). The legal and scientific processes by which specific cathinones became under control in the EU or the United States of America (USA) are often based on if these substances have an abuse potential that is comparable to controlled substances already featured. Data and arguments in favour of controlling substances can include, for instance, their actual or relative potential for abuse/dependence, scientific evidence of their pharmacological and behavioural effects, and their hazardous effects either on the user or on the social environment, among others (DEA, n.d.; EMCDDA, 2009). In this sense, very few preclinical or clinical studies about the pharmacology, toxicology and physiological effects of synthetic cathinones had been performed prior to their widespread abuse (i.e. Cozzi et al., 1999; Meltzer et al., 2006). However, owing to public health risk posed by bath salts, the governments of many countries started to pass laws to render synthetic cathinones illegal and, in parallel, reports of synthetic cathinone use, abuse, toxicity and death began to surface. Thus, although some cathinone derivatives are still legal and can be sold without penalty, others have been banned. Fortunately, the first-generation of synthetic cathinones, including mephedrone, methylone and MDPV, have been listed in the 1971 Convention (UNODC, 2017). In EU Member States, mephedrone is submitted to control measures, and some other cathinone derivatives are seized by control or equivalent legislation in various countries (EMCDDA, 2010b; EMCDDA, 2014b). Furthermore, all three are controlled substances in the USA (Bonson et al., 2019; DEA, 2011).

Nevertheless, the "legal highs" phenomenon seems that is going to persist anyway: when one of these drugs is banned and illegal, the drug market suppliers respond by launching or producing new unregulated alternatives, often structurally related, and they do it at high speed. In this sense, a new wave of cathinone derivatives (second-generation) has appeared recently in the market to replace those drugs that are already controlled, for example, α-pyrrolidinovalerophenone (α-PVP) and pentedrone (Brandt et al., 2010; Shanks et al., 2012). Moreover, the lack of control and data on these new substances make it challenging for law enforcement to clamp down on their manufacture, trafficking and distribution. The scientific and patent literature, along with the ingenuity of chemists involved in the trade of new drugs, means that thousands more are possible. Therefore, the introduction of "replacement" cathinones is expected to further continue (Henderson, 1988).

Overall, there exist a vast array of synthetic cathinones available on the market, either legally and illegally. However, this doctoral thesis is especially focused on one of them: MDPV, since it started at a time when MDPV was one of the newest and most used synthetic cathinones, but barely known and described. In addition, the dissertation finally extends also to newer synthetic cathinones belonging to the second-generation, including α-PVP and pentedrone. All three substances under study are depicted in Figure 12.

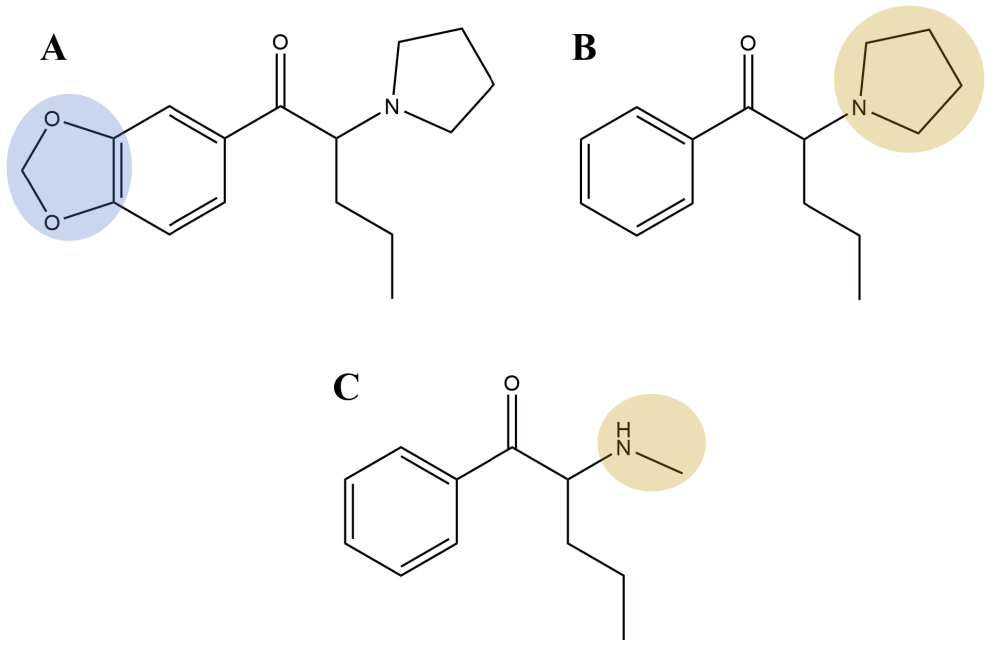

Figure 12. Chemical structure of MDPV (A) and two second-generation synthetic cathinones: α-PVP (B) and pentedrone (C). *MDPV is the 3,4-methylenedioxy counterpart of α-PVP, which in turn, differs from pentedrone only in the N-substituent: contains a pyrrolidine ring instead of a methyl.*

1.3.1.1. MDPV

Definition and background

3,4-methylenedioxypyrovalerone (MDPV) (Figure 12) is the 3,4-methylenedioxy counterpart of pyrovalerone. On the streets, MDPV is also known as *MDPK*, *MTV*, *Magic*, *Super coke*, *Peevee* or *PV*, among others (Energy control, "MDPV", n.d.; WHO, 2014; Karila et al., 2018).

It was first synthesized in the 1960s by a team at Boehringer Ingelheim and patented along with other related pyrrolidine-ketones as a stimulant of the CNS (US3478050A, 1969). Nonetheless, it appears that there has not been further development of these products.

Prevalence

MDPV belongs to the first-generation of cathinones and appeared in the European drug market in 2008, when was firstly reported to the EMCDDA (EMCDDA, 2014a). When appeared, MDPV was considered as one of the most abused synthetic cathinones and the main ingredient of "bath salts", along with mephedrone and methylone (Johnson & Johnson, 2014; Zuba & Byrska, 2013). However, currently, because of its illegal status and thus, its replacement by the second-generation cathinones (i.e. α-PVP), its prevalence is more limited, although still existent.

Pattern of use

MDPV is typically supplied as a powder and used nasally (insufflated and sniffed), ingested orally (swallowing) or injected intravenously. However, it has also been reported as a tablet, capsule or liquid form, and, less frequently, it is also administered by rectal insertion, sublingual absorption, smoked, or by subcutaneous and intramuscular injection (Energy control, "MDPV", n.d.; EMCDDA, 2014a; WHO, 2014).

Effects of MDPV appear with doses as low as 3-5 mg. Common doses of MDPV reported by users range from 5 to 11 mg (per insufflation) and from 8 to 15 mg (per oral ingestion). Therefore, single use doses of MDPV are typically stated to be 5-20 mg. Extremely, users have reported taking

repeated doses per session, reaching doses of 200 mg/session (EMCDDA, 2014a), with the subsequent risks of overdosing, several acute toxicity and even death.

Pharmacology: mechanism of action

MDPV is a powerful psychostimulant that acts similarly to cocaine, blocking selectively the DAT and NET, and very weakly, SERT. Accordingly, MDPV predominantly acts as a transporter *blocker* rather than as a *releaser* (Baumann et al., 2013; Eshleman et al., 2013; Simmler et al., 2013). Furthermore, MDPV appears to be from 10 to 50-fold more potent, effective and selective as a DAT blocker than cocaine (Baumann et al., 2013; Simmler et al., 2013), and induces a rapid and reversible functional upregulation of DAT more powerfully and lasting than cocaine (López-Arnau et al., 2019).

Subjective user effects

Information from user self-reports and clinical data on individuals suggest that the desired effects of MDPV resemble those seen with other simulants such as cocaine and amphetamine-type stimulants (EMCDDA, 2014a), and are individual-, dose- and route of administration-dependent (Prosser & Nelson, 2012). Concretely, recreational users report that the desirable subjective effects of MDPV include euphoria, mood lift, talkativeness and increased sociability, increased self-esteem, mental and physical stimulation, increased creativity, productivity and motivation, empathy, alertness, motor excitation, sexual arousal and increased libido (Energy control, "MDPV", n.d.; "MDPV, DanceSafe," n.d.; WHO 2014; Karila et al., 2018).

Toxicology

By 2014, a total of 525 non-fatal intoxications associated with MDPV had been reported to the Early Warning System by eight Member States, besides a total of 108 deaths in which MDPV was detected in post-mortem biological samples and/or implicated in the cause of death (EMCDDA, 2014b).

Reported adverse effects do not only concern acute toxicity, but also chronic toxicity. Symptoms of toxicity include cardiac complications (hypertension, tachycardia, arrhythmia, raised blood pressure), neurological effects (restlessness, consciousness alterations, involuntary body movements, nystagmus, mydriasis, blurred vision, headache, myoclonus, tightened jaw muscles, grinding teeth, confusion), psychiatric effects (agitation/aggression, paranoid psychosis, delirium, hallucinations, hyperactivity, residual depressed mood, disorganized thoughts, suicidality, anxiety), hyperthermia, loss of appetite, disturbed sleep patterns, diaphoresis, liver damage and convulsions, amongst many others. Cases of more severe toxicity include hyperpyrexia, rhabdomyolysis, stroke, cardiomyopathy, acute coronary syndrome, cerebral edema, acute renal failure, respiratory arrest, self-mutilation, coma and death (Karila et al., 2018; Regunath et al., 2012; Ross et al., 2012; Warrick et al., 2013; WHO, 2014). Also, it cannot be underestimated, the risks associated to certain practices such as MDPV injection or sexual risk-taking (i.e. contracting infections such as Hepatitis B, Hepatitis C, HIV or other sexually-transmitted diseases) (EMCDDA, 2019; Karila et al., 2018).

Addictive potential

MDPV induces a robust stimulation of DA transmission by increasing its extracellular concentrations in the NAcc (Baumann et al., 2013). Indeed, there is compelling evidence that MDPV effectively increases locomotion as well as exerts potent rewarding and reinforcing effects in rodents (Aarde et al., 2013; Berquist et al., 2016; Buenrostro-Jáuregui et al., 2016; Gannon et al., 2017, 2018; Gregg et al., 2016; Horsley et al., 2018; King et al., 2014; López-Arnau et al., 2017; Novellas et al., 2015; Simmons et al., 2018; Watterson et al., 2014; Watterson and Olive, 2014), predicting a serious potential for abuse liability (Howell & Kimmel, 2008; Watterson et al., 2013; Wise, 2008). The development of craving, tolerance, dependence and withdrawal syndrome has been reported after frequent use of high doses of MDPV (Andrabi et al., 2015).

Legal status

Apart from its use as an analytical standard and research chemical in experiments investigating its pharmacological and toxicological profile, MDPV has no known legitimate alternative uses (EMCDDA, 2014b; WHO, 2014). Since 2014, MDPV became a drug subjected to control measures across the EU (EMCDDA, 2014b) and in 2015 was definitively included in the Convention on Psychotropic Substances of 1971 by the UNODC (UNODC, 2017). In the USA, it was federally listed on the Controlled Drugs and Substances Act by the Drug Enforcement Administration (DEA) (Bonson et al., 2019; DEA, 2011).

1.3.1.2. α-PVP

Definition and background

α-PVP (α-pyrrolidinovalerophenone), popularly known as *flakka, gravel, grind, crystal love* or *snow blow,* is a synthetic cathinone that belongs to the second generation. α-PVP shares structural features with pyrovalerone and MDPV: α-PVP is simply the desmethylpyrovalerone analogous and, at the same time, it is structurally identical to MDPV but without the 3,4-methylenedioxy group (Figure 12), thus they have many characteristics in common (Glennon & Young, 2016; Marusich et al., 2014), reason why it was considered to emerge as a replacement to MDPV.

As many synthetic cathinones, α-PVP synthesis was firstly described in a British patent (GB 927475, 1963) as a CNS stimulant, followed by numerous other European and USA patents dating from 1963 to 1967. However, its mechanism of action was not elucidated until later (Meltzer et al., 2006).

Prevalence

α-PVP was formally notified to the EMCDDA system in 2011 by France. Concretely, the notification related to the seizure of approximately 5 kg of a white powder containing not only α-PVP, but also pentedrone (EMCDDA, 2015). Nowadays, α-PVP has become quite popular on the clandestine market (Ackerman, 2015; Storrs, 2015). It has been detected in all 28 Member States of the EU and more than 750 kg of α-PVP in powder has been seized in Europe since 2011 (EMCDDA, 2015).

Pattern of use

α-PVP is commonly found as a white powder and administered nasally (insufflation), ingested orally or injected intravenously (EMCDDA, 2015). It has also been detected in other forms as tablets, powder-filled capsules, liquids and jelly gums (Zawilska & Wojcieszak, 2017), and reported to be administered by alternative routes: sublingual absorption, rectal insertion, smoked, injected intramuscularly and mixed routes (oral plus injection) (Energy control, "Alfa-PVP," n.d.; Zawilska & Wojcieszak, 2017).

There is limited information from user websites about what doses are typically used. Self-reported experiences have noted that threshold oral dosages required to induce psychoactive effect may be 1-2 mg, while strong effects are reported with oral doses of 20-25 mg. Importantly, re-dosing is a recurrent pattern among α-PVP users (EMCDDA, 2015).

Pharmacology: mechanism of action

The preliminary results obtained by Meltzer et al., 2006 were soon after confirmed and extended by other authors (Cameron et al., 2013; Eshleman et al., 2017; Kolanos et al., 2015; Marusich et al., 2014). All these *in vitro* studies support that α-PVP acts as a monoamine transporter blocker and does not induce transporter-mediated efflux. Concretely, it inhibits DA and NA uptake, with almost negligible action at SERT. Furthermore, it blocks the transporters DAT and NET with very similar potencies to those of MDPV. Emerging evidence suggests that the 3,4-methylenedioxy group of MDPV, absent in α-PVP, imparts somewhat enhanced action at SERT (Eshleman et al., 2017).

Subjective user effects

Limited information from acute intoxications and self-reported user experiences suggests that the effects of α-PVP might be broadly similar to other psychostimulant drugs (EMCDDA, 2015): mental and motivational stimulation, enhanced energy, euphoria, empathy, openness, creativity, productivity, alertness and increased libido (Energy control, "Alfa-PVP," n.d.; Marusich et al., 2016; Zawilska & Wojcieszak, 2017). Furthermore, some users reported slight differences as regards to MDPV such as a faster high but with less potency and duration of the effects (Energy control, "Alfa-PVP," n.d.).

Toxicology

α-PVP has been involved in an increased number of, not only acute intoxications but also fatalities over the past few years, raising concerns in the medical field. Between 2011 and 2015, 205 acute intoxications associated with α-PVP were reported by eight EU Member States. Furthermore, between 2012 and 2015, a total of 116 deaths were associated with its consumption (EMCDDA, 2015). Clinical features described in those cases were: cardiovascular effects (tachycardia, hypertension, vasoconstriction), neurological effects (restlessness, myoclonus, convulsions or seizures, confusion, loss of consciousness), psychiatric effects (agitation, anxiety, insomnia, hallucinations, aggression, bizarre behaviour, delirium), general effects (hyperthermia, diaphoresis, lack of appetite, dehydration), rhabdomyolysis...etc. (Adams & Fagenson, n.d.; Crespi, 2016; EMCDDA, 2015; Franantonio et al., 2015; Giese et al., 2015; Grapp et al., 2016; Marinetti & Antonides, 2013; Marusich et al., 2014, 2016). Besides other complications derived from the intravenous injection of the drug (i.e. Hepatitis A, Hepatitis C, HIV...) (Giese et al., 2015). In fact, although people use α-PVP for its

potential euphoric high, symptoms are known to easily escalate into extreme agitation, paranoia, aggression, insomnia and delusions (Crespi, 2016).

Addictive potential

Information from animal studies suggests that α-PVP induces a robust stimulation of DA transmission by increasing its extracellular concentrations in the striatum (Wojcieszak et al., 2018), predicting a serious potential for abuse liability (Howell & Kimmel, 2008; Wise, 2008). Furthermore, the stimulatory effect of α-PVP in the CNS is mediated, at least in part, by the DR1 and DR2 (Kaizaki et al., 2014). The behavioural features associated with α-PVP include increased locomotor activity, as well as powerful rewarding and reinforcing effects in rodents (Aarde et al., 2015; Gatch et al., 2015a; Giannotti et al., 2017; Javadi-Paydar et al., 2018a; Kaizaki et al., 2014; Marusich et al., 2014, 2016; Nelson et al., 2017; Wojcieszak et al., 2018).

Legal status

As MDPV, α-PVP is now controlled under the United Nations Convention on Psychotropic Substances of 1971 (UNODC, 2017) and has been federally listed on the Controlled Drugs and Substances Act by the DEA (Bonson et al., 2019; DEA, 2017).

In accordance, α-PVP and its enantiomers are used only in scientific research as well as analytical reference materials in clinical and forensic case work. No further indications of α-PVP are approved nor legitimate (EMCDDA, 2015).

1.3.1.3. Pentedrone

Definition and background

Pentedrone (α-methylamino-valerophenone) is another second-generation synthetic cathinone, structurally related to α-PVP (Figure 12): both molecules only differ in their N-substituent group: a methyl or a pyrrolidine ring, respectively. It is popularly called as *penta*, *pentakristály* or *kristály* (WHO, 2016).

Prevalence

Together with α-PVP, pentedrone was formally notified to the EMCDDA system in 2011 by France because of a seizure of approximately 5 kg of a white powder containing both, α-PVP and pentedrone (EMCDDA, 2015). Since then, its use spread widely, being one of the most commonly seized cathinones in 2015 (EMCDDA, 2017a), often found mixed with other drugs such as MDPV and α-PVP (EMCDDA, 2014a; Zuba & Byrska, 2013). Pentedrone has been found in human urine samples increasingly over the past 4 years (Uralets et al., 2014).

Pattern of use

As the rest of synthetic cathinones, pentedrone is commonly found as powder and nasally snorted, ingested orally, smoked or injected intravenously (WHO, 2016).

Dose ranges used for recreational purposes are 40-100 mg by insufflation, 80-150 mg by oral ingestion, 10-20 mg by inhalation (smoked), and 30-60 mg by intravenous route (WHO, 2016).

Pharmacology: mechanism of action

Pentedrone, as its relatives, α-PVP and MDPV, acts as a DAT and NET inhibitor, with less activity towards SERT. Available data point out that pentedrone acts as a cocaine-like *blocker*, instead of a *releaser* (Eshleman et al., 2017; Simmler et al., 2014).

Subjective user effects

There are no published data on the psychological and behavioural effects of pentedrone in humans. However, because of its mechanism of action, desired effects resembling those seen and previously described with other stimulants such as α-PVP, MDPV and cocaine can be expected.

Addictive potential

The psychostimulant, rewarding and reinforcing effects of pentedrone have been demonstrated in rodents (Gatch et al., 2015b; Hwang et al., 2017; Javadi-Paydar et al., 2018b; Kim et al., 2014; Naylor et al., 2015). Furthermore, drug selectivity at DAT vs SERT is a key determinant of drug abuse potential for monoamine transporter substrates and inhibitors, such that a high DAT/SERT ratio is associated with high abuse potential, whereas potency at SERT > DAT will have low abuse liability (Negus & Banks, 2017; Negus & Miller, 2014). In this sense, pentedrone has a DAT/SERT ratio >10, which is even higher than that of methamphetamine. Overall, evidence point to a high addictive potential.

Toxicology

Several forensic caseworks of fatal intoxications involving pentedrone consumption have been reported (Liveri et al., 2016; Sykutera et al., 2015), although always in combination with other drugs like α-PVP and MDPV, that is in a poly-drug use context (WHO, 2016). Additionally, one case report has referred the development of acute psychosis after pentedrone use (Segrec et al., 2016). No further literature is available on the clinical effects and toxicological symptoms.

In animal studies, it has been described that high doses of pentedrone (70 mg/kg) produce severe convulsions, being lethal at 100 mg/kg (Hwang et al., 2017).

Legal status

Pentedrone is not controlled worldwide, so pentedrone products are legally marketed in head shops and *online* in several countries. However, in the USA, it has been listed on the Controlled Drugs and Substances Act by the DEA (Bonson et al., 2019; DEA, 2017). No therapeutic, industrial or medical uses have been described for pentedrone (WHO, 2016).

1.4. Contextual framework: starting point

The present thesis, which largely studies the neurochemical and psychopharmacological properties of MDPV, had its starting point in a previous work carried out by the same research group (López-Arnau et al., 2017). This study, in fact, was the first author's contact and contribution to

the study of MDPV. Although it can be found in the *Annex III section*, it will also be briefly set out below to place the reader in the contextual framework from which it was necessary to further continue the research, thus giving rise to the present doctoral thesis.

That study arose from different statements that have already been explained in more detail above in this introduction:

In recent years, the illicit drug market has changed considerably due to the emergence of NPS, such as synthetic cathinones, being MDPV one of the most abused and the main ingredient of bath salts. Despite the invasion of NPS, cocaine remains being the most abused psychostimulant worldwide. MDPV and cocaine share the same mechanism of action: they are mainly DAT blockers, a feature that explains their powerful psychostimulant, reinforcing and rewarding effects, as well as their high abuse liability. In fact, MDPV is known to be 10-to 50-fold more potent than cocaine.

Nevertheless, the pattern of use of MDPV and cocaine substantially differs: while MDPV use is considered as a transient trend among adolescents and young adults, cocaine is generally consumed in adulthood. A plausible reason for that trending could be the ease of access and lower price of the cathinone derivative. Importantly, experimentation as well as the process of addiction, most often starts in adolescence (Wagner & Anthony, 2002), a period during which the brain undergoes important development changes (Spear, 2013). Therefore, normal development processes might result in higher risk for drug use at this stage of the lifecycle than others. Indeed, drug exposure during adolescence is associated with more chronic and intensive use and greater risk of a substance use disorder than if it initiates in the adulthood. In this sense, normal adolescent-specific behaviours may

increase the propensity to experiment with legal and illegal drugs, which, in turn, may reflect the incomplete development of brain regions involved in the processes of executive control and motivation (Koob & Volkow, 2016).

Considering this, it resulted relevant and necessary to determine whether the use of MPDV during adolescence could lead to an increased sensitivity and subsequent vulnerability to cocaine abuse in adulthood. In this way, this study could give us valuable knowledge about the long-lasting consequences of an early and repeated exposure to MDPV on the responses of adult mice to cocaine, as well as a potential identification of a factor that could increase vulnerability to cocaine abuse, which, in that case, it would have to be carefully evaluated.

To achieve that, adolescent male Swiss CD-1 mice were treated with MDPV (1.5 mg/kg, subcutaneously (s.c.) twice daily, for 7 days). After 21 days, when animals reached adulthood, they were tested with cocaine, using locomotor activity, CPP and SA paradigms to test the psychostimulant, rewarding and reinforcing effects of cocaine, respectively. In parallel, DR2 density and the expression of c-Fos and ΔFosB was determined in the striatum at both time points: in adolescence (just after drug treatment), and in adulthood (before the first cocaine exposure).

From this study we could finally conclude that MDPV increased most of the behavioural responses related to cocaine effects, including locomotor sensitization, reward and the strength of cocaine as a reinforcer. In addition, these behavioural abnormalities were associated with an accumulation of ΔFosB and subsequent repression of c-Fos, providing a possible mechanism by which molecular changes induced by MDPV can persist for weeks after withdrawal and supporting the enhancing effects of MDPV on cocaine abuse

liability. Therefore, the results suggested that consumption of MDPV during adolescence induces long-lasting adaptative changes leading to a higher response to cocaine in adulthood, predisposing to a higher vulnerability to abuse. From a clinical point of view, this feature represents a basic step that must be carefully studied since it provides new knowledge about factors involved in the vulnerability to cocaine addiction. In this sense, MDPV use could be considered as a risk factor.

From these findings, the following objectives emerged (see section *2. Objectives).*

2 OBJECTIVES

The general aim of the present doctoral thesis has been to contribute to increasing scientific knowledge regarding novel psychoactive substances, especially MDPV, a cocaine-like psychostimulant (Part I), but also about the second-generation synthetic cathinones structurally related to MDPV (Part II). Each approach has had different objectives:

Part I

The general aim of Part I has been to study the neurochemical and psychopharmacological effects of MDPV, thereby determining the neuroadaptive changes underlying its abuse, its behavioural effects and its addictive properties. In order to pursue this aim, several specific objectives have been set up:

- To determine the behavioural effects induced by a repeated exposure to MDPV.

- To elucidate the neuroadaptive changes underlying MDPV sensitization and the abnormal related behaviours, focusing especially on the DR1 – cAMP intracellular signalling pathway triggered in the striatum. To compare them with what is already described for cocaine.

- To investigate the possible cross-sensitization between MDPV and cocaine.

- To describe and compare the changes induced by either acute or repeated exposure to MDPV or cocaine on the transcription of

plasticity-related genes in early abstinence, focusing principally on the BDNF – TrkB signalling pathway.

- To assess and compare the role of the BDNF – TrkB pathway in the development of behavioural sensitization and CPP acquisition to MDPV and cocaine.

- To assess the rewarding properties of MDPV and study the relationship between MDPV and cocaine in the reinstatement of the CPP paradigm.

- To elucidate potential changes in the expression of several plasticity- and epigenetic-related factors underlying MDPV and cocaine place conditioning after the CPP paradigm, as well as in response to a new acute drug exposure during extinction.

Part II

Part II has intended to go beyond the study of MDPV and thus, it extends to the study of new second-generation synthetic cathinones structurally related to MDPV. For this purpose, the following aims have been proposed:

- To characterize the *in vitro* pharmacology of amino-valerophenone derivatives structurally related to α-PVP and pentedrone, which only differ in their amino-substituent, thereby determining the role of the amino-terminal group in their interaction with monoamine transporters.

- To establish a quantitative structure-activity relationship (QSAR) between the molecular and physicochemical properties of the amino-substituent and their pharmacological profile.

- To assess the psychostimulant and rewarding effects of these compounds in mice.

3 METHODS AND RESULTS

Part I

3,4-Methylenedioxypyrovalerone (MDPV)

CHAPTER 1

3.1. Neuroadaptive changes and behavioural effects after a sensitization regime of MDPV

Adapted from: **Duart-Castells, L**[1]., López-Arnau, R[1]., Buenrostro-Jáuregui, M[2]., Muñoz-Villegas, P[1]., Valverde, O[3]., Camarasa, J[1]., Pubill, D[1]., Escubedo, E[4]. Neuroadaptive changes and behavioural effects after a sensitization regime of MDPV

Neuropharmacology 2019; 144:271-281. doi: 10.1016/j. neuropharm.2018.10.005. Epub 2018 Oct 12.

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3.1.1. ABSTRACT

Over the last decade the recreational drug market has become considerably more complex due to the apparition of new psychoactive substances (NPS), including synthetic cathinones (i.e. 3,4-methylenedioxypyrovalerone, MDPV). MDPV is a cocaine-like psychostimulant as it shares a similar pharmacological profile: it selectively blocks the dopamine transporter (DAT), being from 10 to 50-fold more potent than cocaine. Furthermore, in a previous study of our lab, we found out that repeated exposure to MDPV during adolescence induces long-lasting adaptative changes that lead to an increased response to cocaine in adulthood, predisposing to a higher vulnerability to its abuse.

In the present work, we keep with the investigation and further study the neuroadaptive changes underlying drug sensitization. Hence, we intended to elucidate the potential changes in specific neuronal biomarkers induced by such sensitization regime of MDPV that could explain the increased cocaine effects. Additionally, we tested other behavioural alterations induced by the drug.

Adolescent male Swiss CD-1 mice were treated with MDPV (1.5 mg/kg s.c., twice daily for 7 days) and all evaluations were performed shortly after treatment (2 h or 24 h) as well as after 21 days, when animals reached adulthood.

Regarding the behavioural testing, repeated exposure to MDPV induced an enduring anxiety-related behaviour that was mainly apparent long after treatment. Moreover, MDPV increased aggressiveness in mice, shortly after the drug was withdrawn. Finally, exposure to MDPV also

promoted a faster habituation to the open field, interpreted as a tendency for novelty-seeking and risk-taking behaviour. This feature co-occurred with an induction of ΔFosB in the orbitofrontal cortex, the raise of which is thought to be linked not only to risky behaviours but to behavioural sensitization as well.

MDPV induced an overexpression of plasticity-related factors (Arc and CDK5) and TH, which came along with a decrease in DAT and DR1 populations, pointing to a hyperdopaminergic status. Moreover, the implication of such hyperdopaminergic condition in MDPV-induced aggressiveness cannot be ruled out. Importantly, shortly after treatment, the DR2:DR1 ratio was altered towards DR1, but reverted during withdrawal. An initial and transient oxidative effect without glial activation was also observed after MDPV exposure. Finally, and by contrast to cocaine, besides the different modulation of NKκB and GluA2 expression, the regulatory autoloop between G9a and ΔFosB was not observed either, suggesting the existence of additional regulatory signals modulating ΔFosB expression.

In conclusion, despite the fact that MDPV and cocaine share the same mechanism of action and MDPV induces sensitization to the locomotor effects of cocaine, the intracellular signalling pathways triggered by the drugs notably differ.

3.1.2. INTRODUCTION

Synthetic cathinones (i.e. mephedrone, methylone and 3,4-methylenedioxypyrovalerone (MDPV)) are often used as replacements to other stimulants such as amphetamines, cocaine or ecstasy. Among them,

pyrrolidine derivatives such as MDPV are more lipophilic and thus, more able to cross the blood–brain barrier. MDPV shows cocaine-like properties and selectively inhibits the dopamine (DAT) and noradrenaline transporters, being 10- to 50-fold more potent than cocaine as a DAT blocker (Baumann et al., 2013; Simmler et al., 2013). Furthermore, MDPV exerts powerful rewarding and reinforcing effects relative to cocaine at one-tenth doses, suggesting a significant abuse risk based on its potency and its subjectively positive effects (Aarde et al., 2013; Watterson et al., 2012).

Repeated administration of psychostimulants induces psychomotor sensitization in rodents. This phenomenon has been largely associated with dynamic changes in neural processes induced by repeated drug exposure and has been proposed as a model of an initial stage of addiction in humans, which contributes to drug craving (Kalivas and Stewart, 1991; Robinson and Berridge, 1993). In this sense, cocaine exposure triggers complex adaptations in the brain that are mediated by dynamic patterns of gene expression, which are further translated into enduring changes, many of them related to drug sensitization (Schmidt et al., 2013).

In a previous study, we found out that repeated exposure to MDPV (1.5 mg/kg, twice daily, for 7 days) during adolescence, induced behavioural sensitization not only to itself but also to cocaine locomotor effects. In this context, the aim of the present study was to test other behavioural abnormalities induced by such sensitization regime of MDPV, as well as to evaluate changes in specific neural biomarkers that could explain the increased cocaine effects. Hence, this study provides valuable knowledge that leads to a better understanding about the effects of chronic MDPV exposure and its neurotoxicological potential.

With these purposes, we evaluated anxiety, habituation and aggressiveness of mice at different time-points after MDPV exposure, using the elevated plus maze (EPM), the open field (OF) and the resident intruder test (RIT) paradigms, respectively. In parallel, molecular determinations were carried out in the striatum. As some data indicate that the induction of ΔFosB within the orbitofrontal cortex (OFC) plays a role in the deficit in impulse control mediated by cocaine (Winstanley et al., 2009) we also assessed the induction of ΔFosB in this brain area.

In ventral striatum (VS), we mostly focused on the dopamine receptor 1 (DR1) intracellular signalling pathway, which involves the transcription factor ΔFosB. We studied related-markers as cyclin-dependent kinase-5 (CDK5), the activity-regulated cytoskeleton-associated protein (Arc), the AMPA glutamate receptor subunit 2 (GluA2) and the nuclear factor kappa B (NFκB).

To be activated, CDK5 has to associate to their regulatory subunits, p35 or p39, both regulated by ΔFosB (McClung et al., 2004; Nikolic et al., 1996). The complex p35/CDK5 is a neuroplasticity mediator, which is required for neurite growth (Patrick et al., 1999). On the contrary, p25, a proteolytic fragment of p35, causes dysregulation of CDK5 kinase activity. For instance, the complex p25/CDK5 hyperphosphorylates Tau, which reduces Tau's ability to associate with microtubules (Kelz et al., 1999). Altogether, when CDK5 is activated by p35, it takes part in physiological processes as neuroplasticity, while CDK5/p25 is related with neurotoxic and neurodegenerative processes. Arc is an early gene which is rapidly induced by cocaine. GluA2 is another target gene of ΔFosB, the expression and surface/intracellular ratio of which are also under homeostatic regulation (Boudreau et al., 2007). Finally, we determined G9a expression,

a histone-lysine N-methyltransferase considered an important mechanism for epigenetic regulation during the development of cocaine addiction state (Maze et al., 2010).

In the dorsal striatum (DS) we assessed the expression of proteins related with dopaminergic neurotransmission. It is known that repeated exposure to classical psychostimulants induces various synaptic adaptations, many of them related to sensitization. These neuroplastic processes include up- or down-regulation of DR1 and DR2, changes in subunits of G proteins, increased adenylyl cyclase activity and increased tyrosine hydroxylase enzyme (TH) activity or dopamine (DA) transport (Bibb et al., 2001). Accordingly, TH, DAT, DR1 and DR2 protein levels were assessed following MDPV exposure. Finally, lipid peroxidation is one of the major sources of free radical–mediated injury that directly damages membranes and generates several secondary products, being 4-hydroxy-2-nonenal (HNE) one of the most abundant. Therefore, 4-HNE is considered a robust marker of oxidative stress and a toxic compound for several cell types (Perluigi et al., 2012). Its assessment, jointly with that of the glial fibrillary acidic protein (GFAP), provided information about a putative neurotoxic effect of MDPV.

3.1.3. MATERIALS AND METHODS

Animals

All animal care and experimental protocols in this study were approved by the Animal Ethics Committee of the University of Barcelona, under the supervision of the Autonomic Government of Catalonia. All procedures

involving mice were in accordance with the European Community Council Directive (2010/63/EU for animal experiments). All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines. CD-1 mouse strain was selected for its optimal sensitivity to the reinforcing and psychostimulant effects of cocaine (McKerchar et al., 2005). Animals were housed six per cage (polycarbonate with wood-derived bedding) at 22 ± 1 °C under a 12 h light/dark cycle with free access to food and drinking water. Male Swiss CD-1 mice (Charles River, Spain) at the beginning of periadolescence (PND 41–44) were used. Animals were randomly assigned to an experimental group. During the behavioural manipulations, researchers were not aware of the treatment that each animal previously received.

Materials

Pure racemic MDPV · HCl was synthesized and characterized in our laboratory as previously described (Novellas et al., 2005). Cocaine was provided by the Spanish National Institute of Toxicology. Both MDPV and cocaine solutions were prepared in 0.9% NaCl (saline, pH=7.4) immediately before administration.

The protease and phosphatase inhibitors cocktail was purchased from Abcam (Cambridge, UK). BS^3 [bis(sulfosuccinimidyl)suberate] was obtained from ThermoFisher Scientific (Rockford, USA) and [^{3}H]WIN 35428 was from Perkin Elmer (Boston, USA). All the other reagents were of analytical grade and purchased from several commercial sources.

Drug administration protocol and experimental design

Mice were treated with MDPV (1.5 mg kg^{-1}, s.c.) or saline (5 ml·kg^{-1} s.c.), two doses in one day (4 h apart), for seven consecutive days, and housed in their cages until reaching adulthood (PND 69–72, 21 days). Behavioural tests including resident-intruder test (RIT), elevated plus maze (EPM) and the open field (OF) paradigms were performed during the withdrawal period as described below (Figure 13A). Different lots of animals were used for biochemical and behavioural experiments. The dose of MDPV used and the treatment schedule followed are based on our previous study (López-Arnau et al., 2017).

Elevated plus maze (EPM)

Either 48 h (W2) or 21 days (W21) after treatment, anxiety-related behaviour was tested using the EPM paradigm. Briefly, the maze was elevated 42 cm above the ground and consisted of two open and two closed arms (30×6 cm) which radiated from a central platform (6×6 cm). Testing was conducted under low room lighting conditions (30 LUX). Mice were placed on the centre, facing an open arm, and allowed to explore for 5 min. Their behaviour was recorded by a zenithal camera connected to a computerized tracking system (Smart 3.0 software, PanLab SL, Spain). The time spent in the centre of the maze was discarded. Results were expressed as the total time spent in closed and open arms.

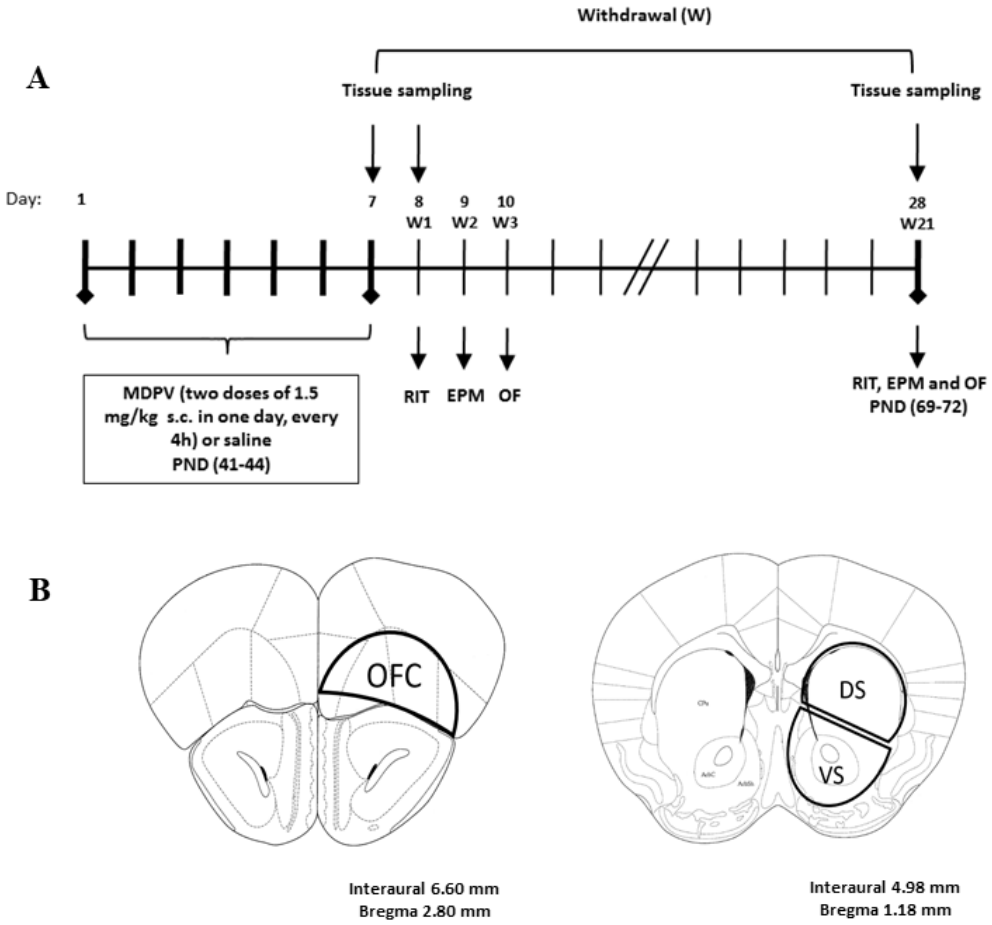

Figure 13. Drug administration protocol and experimental design. *Animals were treated with MDPV or saline two doses in one day (4 h apart) during 7 consecutive days (1-7). The RIT, EPM and OF paradigms were performed 24 h (W1, Day 8), 48 h (W2, Day 9) and 72 h (W3, Day 10) after treatment, respectively, as well as after 21 days of withdrawal (W21, Day 28). Brain samples were collected 2 h (Day 7), 24 h (W1, Day 8) and 21 days (W21, Day 28) after treatment (A).* ***Schematic diagram that illustrates the dissection of the orbitofrontal cortex (OFC), dorsal striatum (DS, nucleus caudate/putamen, CPu) and ventral striatum (VS, including nucleus accumbens shell and core, AcbSh/AcbC) according to the atlas of Paxinos and Franklin*** *(Paxinos and Franklin, 2004) (B).*

Open field (OF)

Either 3 (W3) or 21 days (W21) after the end of the treatment, mice were evaluated in a circular open arena sized 100 cm in diameter. Previously, mice underwent two consecutive habituation sessions (10 min, days W1 and W2). The floor of the circular arena was virtually divided into two zones, namely the centre (70 cm) and the periphery. The time spent in these two zones was recorded during 10 min with the same computerized system cited above. The fraction of total exploratory time spent in the central zone, the number of entries in centre, the latency of the first entrance to the centre, the central vs. total walking ratio, the locomotion and mean speed of animals were measured.

Resident intruder test (RIT)

Mice were tested for offensive aggressive behaviour using the resident intruder paradigm either one (W1) or 21 days (W21) post-treatment, as described (Koolhaas et al., 2013), with minor modifications. Briefly, each mouse (resident) was housed with a female for at least one week before the test day. This fact facilitated the development of territoriality and prevented social isolation. Females were previously sterilized by ligation of the oviducts, so they were regularly receptive without becoming pregnant and developing maternal aggression. On the experiment day, females were removed from the residential cages 1 h before the test. During the test, an unfamiliar male (intruder) was introduced into the home cage and the resident-intruder interactions were video-recorded for 10 min. The resident was scored for two general measures of offensive aggression: latency to the first attack and number of attacks. If any signs of suffering or wounds were observed, the

animals were immediately separated, and the experiment was terminated. However, no early ending was required in any case.

Tissue samples preparation

Mice were killed by cervical dislocation 2 h (Day 7) or 24 h after the treatment (Day 8, W1), as well as after 21 days (Day 28, W21), for the analysis of several factors including Arc, GluA2, CDK5, p35/p25, phospho-Tau (Thr205)/Tau, NFκB, G9a, TH, DAT, DR1, DR2, ΔFosB, 4-HNE and GFAP. The OFC, VS (including NAcc) or DS, when appropriate, were quickly dissected out and stored at −80 °C until use. Particularly, for the dissection of the OFC, brains were rapidly removed and placed in a mouse brain acrylic matrix (Alto, Agnthos, Sweden) placed on ice. Two double edge blades were used to obtain a 1 mm thick slice (from 2 to 3 mm anterior to bregma) which contained the region of interest. The anatomical boundaries for each brain subregion are depicted in Figure 13B. In order to reduce the number of animals, the VS was used to study the signalling pathways related with neuroplasticity and those considered involved in the rewarding and sensitizing effects of drugs, while the DS was reserved to assess the neuroadaptations that take place in the dopaminergic transmission and the possible neurotoxic effects induced by repeated exposure to MDPV.

Tissue samples for Western blot analysis were processed as described (Pubill et al., 2013) with minor modifications. Briefly, tissue samples were homogenized at 4 °C in 20 vol of lysis buffer (20 mM Tris-HCl, pH=8, 1% NP40, 137 mM NaCl, 10% glycerol, 2 mM EDTA) containing the protease and phosphatase inhibitor cocktail. The homogenates were shaken and rolled for 2 h at 4 °C and centrifuged at 15,000 ×g for 30 min at 4 °C. Aliquots of resulting supernatants (total lysate) were collected and stored

at −80 °C until use. Protein content was determined using the Bio-Rad Protein Reagent (Bio Rad, Inc., Spain).

Dopamine transporter density

The density of the DA transporter in striatal membranes was measured using [^{3}H]WIN 35428 binding assays as described previously (López-Arnau et al., 2015). The crude membrane preparation used in the experiments collected both the synaptosomal membrane and the endosomal fraction.

Total RNA extraction and gene expression determination

Total RNA isolation from VS was carried out by means of a TRI reagent-Chloroform based extraction protocol. RNA content in the samples was measured at 260 nm and sample purity was determined by the A260/280 ratio in a NanoDrop™ ND-1000 spectrophotometer (Thermo-Fisher Scientific). The isolated mRNA was reverse-transcribed by a Reverse Transcription Polymerase Chain Reaction (RT-PCR) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the Veriti® thermal cycler (Applied Biosystems, Foster, CA, USA). Briefly, complementary DNA (cDNA) was synthesized in a total volume of 20 μL by mixing 1 μg of total RNA and the appropriate volumes of each reagent. The cDNA product was used for subsequent real-time PCR amplification using the Step One Plus™ Real-Time PCR System (Applied Biosystems, USA) with 25 ng of the cDNA mixture and the assays-on-demand from Applied Biosystems Mm00479619_g1 for Arc, Mm0113261_m1 for G9a and Mm00607939_s1 for Actb as an endogenous control. Fold-changes in gene expression were calculated using the standard comparative Cycle threshold (Ct) method (ΔΔCt) (Livak and Schmittgen, 2001).

Surface receptor cross-linking with BS^3

21 days after the last injection of MDPV or saline, mice were killed by cervical dislocation. Accumbal tissue from each mouse was processed for cross-linking assays as described (Boudreau et al., 2007).

Western blotting and immunodetection

A general Western Blotting and immunodetection protocol was used. Briefly, for each sample, 10–25 μg of protein was mixed with sample buffer (0.5M 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 or onto a 4–15% gradient Tris-HCl gel (Bio-Rad, Hercules, CA) for the surface receptor cross-linking experiments. Proteins were electrophoresed and subsequently transferred to polyvinylidene fluoride sheets (PVDF) (Immobilon-P; Millipore, USA). PVDF membranes were blocked for 1 h at room temperature with 5% defatted milk in Tris-buffer plus 0.05% Tween-20 and incubated overnight at 4 °C with the corresponding primary antibodies. After washing, membranes were incubated for 1 h at room temperature with the corresponding peroxidase-conjugated anti-IgG antibody. Immunoreactive protein was visualized using a chemoluminiscence- based detection kit following the manufacturer's protocol (Immobilion Western, Millipore) and a BioRad ChemiDoc XRS gel documentation system (BioRad, Inc., Madrid, Spain). Scanned blots were analysed using a BioRad Image Software and dot densities were expressed as a percentage of those taken from the control. As a protein load control, immunodetection of β-tubulin (1:2500, Sigma Aldrich) or GAPDH (1:5000, Merck Millipore) were used. The whole list of antibodies used for these experiments is contained in Table S1 (supplementary material).

Data analysis

Data from biochemical analyses were normalized with 100% defined as the mean of the technical replicates in the control group. Data were expressed as mean ± standard error of the mean (SEM). Data from qPCR experiments were expressed as fold-change variations. Differences between groups were compared using one or two-way analysis of variance (ANOVA) or Student's test for independent samples where appropriate. Significant differences ($P < 0.05$) were analysed using the Bonferroni post hoc test for multiple comparison measures only when F achieved the necessary level of statistical significance ($P < 0.05$) and there was no significant variance in homogeneity. Statistic calculations were performed using GraphPAD Prism 6.0 software.

3.1.4. RESULTS

Behavioural effects after a sensitization regime of MDPV

The EPM test was performed in order to assess anxiogenic or anxiolytic effects induced by the drug withdrawal. The cathinone derivative did not induce an anxiety-related behaviour 48 h after treatment, since both the control and the MDPV groups displayed a similar behaviour (saline: open arms 53.90 ± 8.28 s; closed arms: 126.91 ± 10.58 s, $t_{10}=3.879$, $P < 0.01$; MDPV: open arms 41.33 ± 8.62 s; closed arms 121.59 ± 18.30 s, $t_{8}=3.023$, $P < 0.05$). Nevertheless, after 21 days of withdrawal (W21), when EPM was carried out in a different batch of animals, the saline group did not show differences between the time spent in the open and closed arms (open

arms 81.78 ± 13.27 s, closed arms 111.62 ± 8.04 s, t_6=1.915, n.s), while MDPV-treated mice presented an anxious-related behaviour, thus they spent significantly more time in the closed arms than in the open ones (open arms 57.16 ± 11.45 s, closed arms 118.38 ± 16.85 s, t_7=2.315, $P < 0.05$) (Figure 14A). Two-way ANOVA analysis of the time spent in the open arms, which is the main parameter for measuring anxiety-like behaviour, revealed a significant effect of MDPV treatment ($F_{1,33}$=4.280, $P < 0.05$) and time ($F_{1,33}$=5.429, $P < 0.05$) (W2: saline n=11 and MDPV n=9; W21: saline n=7 and MDPV n=8).

In the OF (Figure 14B, Table 1), the behaviour of both groups started to diverge on day W3 until reaching statistical significance on day W21 (two-way ANOVA: variable treatment $F_{1,13}$=5.687, $P < 0.05$, variable time $F_{1,13}$=34.560 $P < 0001$; interaction $F_{1,13}$=5.974, $P < 0.05$; saline n=7 and MDPV n=8). Specifically, MDPV-treated mice spent more time in the central area of the arena, considered aversive, compared with the control group. In addition, MDPV-exposed mice exhibited a higher central vs. total walking ratio, a shorter latency in the first entrance to the centre, and a higher but not significant number of entries in the centre. Overall, no significant differences between MDPV and saline-treated mice regarding to the speed and total distance travelled in every trial were found.

Increased aggressiveness was demonstrated in the RIT. Two-way ANOVA analysis of the latency to the first attack showed a significant effect of treatment ($F_{1,17}$=4.456, $P < 0.05$) and time within withdrawal ($F_{1,17}$=7.303, $P < 0.05$). The same statistical analysis applied to the number of attacks exhibited a significant effect of time ($F_{1,17}$=8.215, $P < 0.05$) and the interaction *treatment x time* ($F_{1,17}$=10.730, $P < 0.01$) (saline n=9 and MDPV n=10). Post-hoc analysis revealed that MDPV-treated mice showed a shorter

latency time to first attack and a higher number of attacks compared to the saline group 24 h after treatment (W1) ($P < 0.05$; in both cases) (Figure 14C and D). However, when the test was performed after 21 days, the saline group showed a shorter latency to first attack compared to W1 ($P < 0.05$), probably because adults often show a greater territorial behaviour than adolescents. Also, MDPV-treated animals reduced the number of attacks if compared with W1 ($P < 0.001$), which were no longer different from those of saline. Therefore, MDPV induced an aggressive behaviour shortly after the drug withdrawal, an effect that disappeared over withdrawal.

Table 1. Behavioural parameters registered in the OF test, both 3 and 21 days after treatment. *The corresponding F values of the two-way ANOVA analysis are shown below. *P < 0.05 compared with saline W21 group or #P < 0.05 compared with MDPV W3 group using Bonferroni post hoc test.*

Treatment-withdrawal day	Mean speed (cm/s)	Locomotion (cm)	Central vs. total walking ratio	Latency 1st entrance to the centre (s)	Entries in centre
Saline W3 n=7	5.57 ± 0.18	3343.72 ± 108.06	0.10 ± 0.014	29.02 ± 6.19	24.00 ± 3.09
MDPV W3 n=9	5.40 ± 0.64	3098.83 ± 338.05	0.17 ± 0.02	6.72 ± 2.87	45.87 ± 8.37
Saline W21 n=7	5.06 ± 0.29	3035.96 ± 171.56	0.16 ± 0.02	35.95 ± 16.31	35.00 ± 4.92
MDPV W21 n=9	5.22 ± 0.76	3360.10 ± 418.18	0.26 ± 0.03 *#	6.13 ± 2.78*	60.87 ± 1.74#
Two-way ANOVA analysis					
F (variable treatment (d.f.), P) Paired for t	$F_{1, 13} = 0.0009$ n.s.	$F_{1, 13} = 0.0082$ n.s.	$F_{1, 13} = 5.975$ P < 0.05	$F_{1, 13} = 10.35$ P < 0.01	$F_{1, 13} = 4.203$ P=0.0611
F (variable time (d.f.), P) Paired for t	$F_{1, 13} = 0.2629$ n.s.	$F_{1, 13} = 0.0372$ n.s.	$F_{1, 13} = 27.59$ P < 0.001	$F_{1, 13} = 0.1170$ n.s.	$F_{1, 13} = 11.63$ P < 0.01

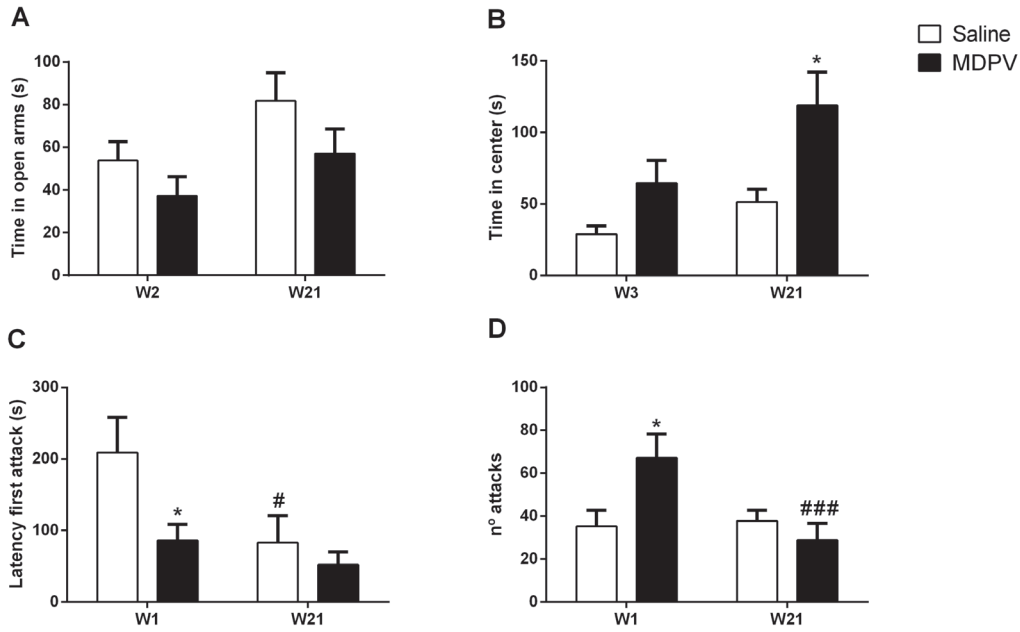

Figure 14. Behavioural effects after repeated exposure to MDPV. *EPM results: Bars represent the time spent in the open arms either 48 h (W2) and 21 days (W21) after treatment (A). OF results: Bars represent the time spent in the centre of the arena 72 h (W3) and 21 days (W21) after treatment (B). RIT results: Bars represent both the latency to the first attack and the number of attacks after 24 h (W1) and 21 days (W21) of withdrawal (C,D). Results are expressed as mean ± SEM. *P < 0.05 vs saline group. #P < 0.05 and ### P < 0.001 vs treatment-matched W1.*

Neuroadaptive changes induced by a sensitization regime of MDPV

Once the treatment with MDPV was finished, the expression of several factors in the striatum was determined by Western blot, qPCR and radioligand binding assays.

Expression of factors related to neuroplasticity and the sensitizing and reinforcing effects of drugs within the VS

Arc levels were significantly increased 2 h after MDPV exposure (t_{10}=2.567, $P < 0.05$, n=6 per group) (Figure 15A). However, its expression declined over withdrawal until being lower than that of the control group on W21 (t_8=2.71, $P < 0.05$, n=5 per group) (Figure 15B). As an attempt to explain such unexpected results, mRNA levels encoding Arc were quantified by qPCR in the VS of saline and MDPV-treated mice, on W21. Interestingly, MDPV-treated mice presented significantly higher levels of Arc mRNA (Figure 15B, inset) (t_{12}=2.281, $P < 0.05$, saline n=8 and MDPV n=6).

Some validated target genes for ΔFosB, such as CDK5, GluA2, and NFκB, were also assayed.

MDPV-treated mice showed a significant increase of CDK5 expression (t_{10}=4.783, $P < 0.001$, n=6 per group) (Figure 15C). However, such increase was not accompanied by a pathological activation of the protein as the ratio p35/p25 was not significantly different between groups of treatment (t_{10}=0.276, n.s.; n=6 per group). Accordingly, the levels of phospho-Tau (Thr 205)/Tau were not altered either by MDPV exposure (t_{10}=1.192, n.s., n=6 per group) (Figure 15E and F). Moreover, CDK5 overexpression was also evident after withdrawal (W21) (t_8=1.871, $P < 0.05$, n=5 per group) (Figure 15D). The increase at the two different time-points was of the same magnitude, suggesting a stable and enduring overexpression of this protein over withdrawal.

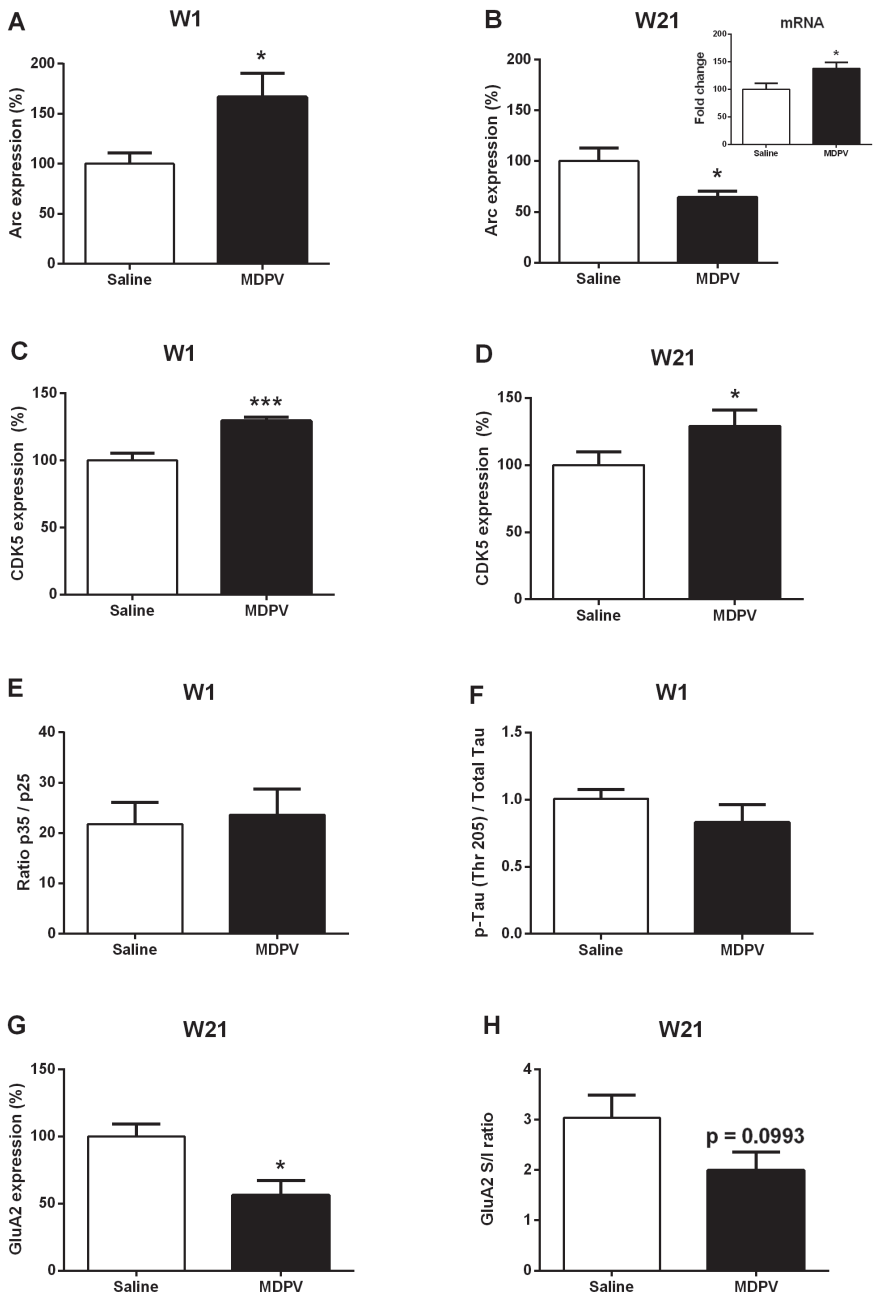

Figure 15. Effect of repeated exposure to MDPV on the expression of factors related to neuroplasticity and the sensitizing and reinforcing effects of drugs in VS: *Arc protein expression 24 h (A) and 21 days after treatment (B). mRNA levels encoding Arc (B, inset).*

*CDK5 expression 24 h (C) and 21 days after treatment (D). p35/p25 ratio 24 h after treatment (E). p-Tau (Thr 205)/total Tau ratio 24 h after treatment (F). GluA2 expression 21 days after treatment (G). GluA2 surface / intracellular ratio 21 days post-treatment (H). Results are expressed as mean ± SEM. *P < 0.05 or ***P < 0.001 compared with its corresponding saline group.*

Another ΔFosB target assayed was NFκB, the expression of which remained unaffected shortly after treatment with MDPV (saline: 100 ± 4.78%; MDPV: 101.51 ± 5.29%, t_{10}=0.212, n.s., n=6 per group).

No changes in GluA2 expression were detected on W1 (saline: 100 ± 8.74%; MDPV: 106.70 ± 14.16%, t_{10}=0.402, n.s., n=6 per group). Nevertheless, on W21, GluA2 protein levels halved (t_8=3.090, P < 0.05, saline n=5 and MDPV n=4) and an internalization of this subunit was apparent, although it did not reach statistical significance (t_{10}=1.817, n.s., n=6 per group) (Figure 15G and H).

G9a expression was also determined through quantification of its coding mRNA. Our results showed a significant increase in G9a transcription 24 h after MDPV exposure (t_8=4.229, P < 0.01, n=5 per group) (Figure 16).

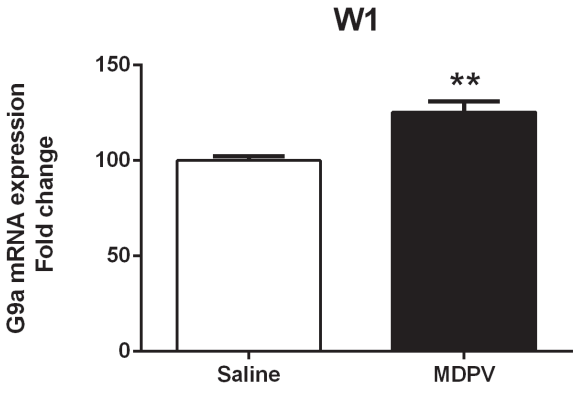

Figure 16. Effect of repeated exposure to MDPV on the expression of mRNA encoding G9a methyltransferase in VS 24 h post-treatment. *Results are expressed as mean ± SEM. **P < 0.01 vs saline group.*

Expression of factors related with dopaminergic transmission within the DS

The levels of DR1 were significantly increased in MDPV-treated mice 24 h after treatment (t_{10}=3.289, $P < 0.001$, n=6 per group) (Figure 17A). As reported previously (López-Arnau et al., 2017), DR2 levels at the same time were significantly lower in MDPV-treated mice (saline: 100 ± 7.68%, MDPV: 73.70 ± 6.59%), therefore, the ratio DR2:DR1 was altered towards DR1. Nevertheless, after withdrawal (W21) a significant decrease of around 25% of the DR1 population was evident, (t_8=2.931, $P < 0.05$, n=5 per group) (Figure 17B), while DR2 levels remained unchanged (data not shown). Therefore, the DR2:DR1 reverted.

TH, the key enzyme for the biosynthesis of DA, was up-regulated 24 h after dosing (t_{10}=2.573, $P < 0.05$, n=6 per group) and such overexpression persisted during withdrawal (t_8=2.316, $P < 0.05$, n=5 per group) (Figure 17C and D).

Finally, we measured the [^{3}H]WIN 35428 bound to a crude membrane preparation as a measure of DAT density in dopaminergic terminals. Although no changes were observed shortly after MDPV exposure (W1) (t_9=0.355, n.s., saline n=5 and MDPV n=6), DAT protein levels were significantly decreased after 21 days (W21) (t_{14}=2.484, $P < 0.05$, n=8 per group) (Figure 17E and F).

Neurotoxic effects of MDPV in DS

In order to assess any possible neurotoxic effect induced by the repeated exposure to MDPV, 4-HNE and GFAP were measured. 24 h after treatment, 4-HNE was increased by almost 200% (Saline: 100 ± 19%; MDPV: 296.51

± 35.16%, t_{10}=4.889, P < 0.001, n=6 per group), while no changes were observed in GFAP expression (Saline: 100 ± 14%; MDPV: 100.89 ± 13.13%, t_{10}=0.046, n.s., n=6 per group). Considering the fact that GFAP response could be delayed in time, we re-assess both proteins on W21. However, no alterations on GFAP expression were observed either (saline: 100 ± 27.78%; MDPV: 92.42 ± 16.84%, t_8=0.233, n.s., n=5 per group). Furthermore, 4-HNE levels had returned to normal (saline: 100 ± 9.06%; MDPV: 84.77 ± 5.62%, t_{10}=1.429, n.s., n=6 per group).

Effect of repeated exposure to MDPV on ΔFosB expression within the OFC

Our findings from the OF paradigm encouraged us to determine ΔFosB protein levels within the OFC, the induction of which is known to play a key role in the deficit in impulse control mediated by cocaine (Winstanley et al., 2009). Student's t-test of the results showed that MDPV exposure significantly induced ΔFosB overexpression, which was evident 21 days after treatment (saline: 100 ± 19.65%; MDPV: 175.76 ± 15.84%, t_9=3.04, P < 0.05; saline n=5 per group and MDPV n=6 per group).

3.1.5. DISCUSSION

In the present work, we studied the behavioural and neuroadaptive changes induced by a sensitizing MDPV exposure. The most important findings from the behavioural experiments are that repeated exposure to MDPV induces a long-lasting anxiety-related and risk-taking behaviours and increases aggressiveness shortly after drug withdrawal. At the same time, the intracellular responses that MDPV triggers are similar to those of cocaine, although some differences must be highlighted.

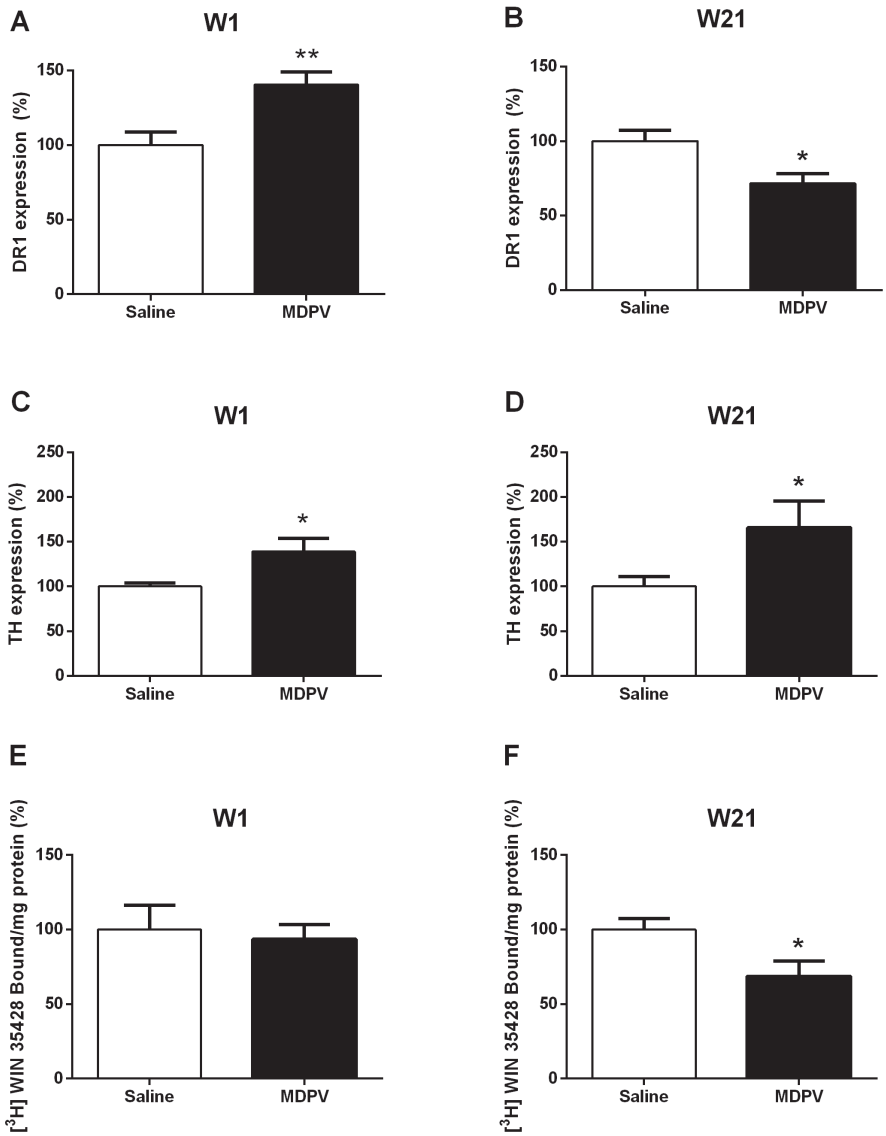

Figure 17. Effect of repeated exposure to MDPV on the expression of factors related with dopaminergic transmission in DS: *DR1 expression 24 h (A) and 21 days after treatment (B). TH expression 24 h (C) and 21 days after treatment (D). DAT density 24 h (E) and 21 days after treatment (F). DAT density was measured as [³H]WIN 35428 bound. Results are expressed as mean ± SEM. *P < 0.05 or **P < 0.01 vs its corresponding saline group.*

Initially, animals were tested for behavioural abnormalities induced by repeated drug exposure using the EPM, OF and RIT paradigms. Regarding EPM, the repeated exposure to MDPV caused an anxiogenic-like effect that was apparent mainly long after treatment, when animals had already become adults. Such anxiety-like behaviour encouraged us to test other behavioural abnormalities in the OF, a simple test that provides an initial screen for locomotion, anxiety and emotional-related behaviours (Bailey and Crawley, 2009). After MDPV treatment, mice ventured more frequently into the central area, where they stayed longer, without presenting changes in their locomotor activity (distance travelled and mean speed). This conduct is considered atypical because it involves leaving a defensive zone to enter in a more exposed one, so it opposes to the rodents' natural aversion to brightly lit open areas. Moreover, it reflects a non-anxious related behaviour. However, considering the results obtained in the EPM paradigm, we cannot attribute this atypical behaviour to an anxiolytic effect of MDPV. Additionally, repeated exposure to the OF is a method for assessing habituation to an increasingly familiar environment. Thus, our results can be interpreted as a faster habituation to a new environment, favouring a risky behaviour (Clément et al., 1995). Consistent with our findings, the same pattern of behaviour in the OF has also been observed with other drugs, for instance, short after a long-term treatment with MDMA (Abad et al., 2013; Mechan et al., 2002), which, in addition, also induced a lasting anxiety-like response in the EPM (Rodríguez-Arias et al., 2011). Therefore, it seems that after repeated exposure to MDMA or MDPV, animals adapt faster to the repeated social isolation resulting from the physical separation from cage mates when performing the OF test, and the stress created by the brightly lit, unprotected novel environment.

Based on case studies, MDPV, as other cathinone derivatives, seems to induce an aggressive behaviour in humans (James et al., 2011; Murray et al., 2012; Penders et al., 2012). To our knowledge, this is the first study in the literature reporting that MDPV exposure can increase aggressiveness in mice, thereby we evidenced that territorial aggression was increased shortly after MDPV withdrawal. In humans, it is suggested a role of DR2 in pathological aggressive behaviour. Chen et al. (2005) observed a correlation between the *dr2* and *dat* gene polymorphisms with pathological violence in adolescents, in a blinded clinical trial, linking hypo-activation of striatal and prefrontal areas to disease severity. Therefore, we cannot rule out the possibility that the changes in dopaminergic neurotransmission evidenced early after MDPV exposure could contribute to the aggressiveness induced by MDPV withdrawal. Moreover, this behavioural effect declined upon withdrawal, when the DR2:DR1 ratio recovered and synaptic DA clearance by DAT was reduced.

Another purpose of the present study was to investigate changes in specific neuronal biomarkers after a sensitization regime of MDPV which could explain the increased sensitivity to cocaine effects long after MDPV exposure. In this sense, the activation of immediate early genes, such as *Arc*, by psychomotor stimulants, has been interpreted primarily as a key step influencing long-term plasticity in neurons (Nestler, 2001). In our study, MDPV-treated mice showed an early overexpression of Arc, which significantly reversed after withdrawal. As an attempt to elucidate the reason for such decline, we quantified mRNA levels encoding Arc and we observed that, interestingly, they were significantly increased. Therefore, a discrepancy between Arc mRNA and protein levels at the same time became evident. However, such inconsistency has also been described after subchronic and chronic cocaine exposures (Fumagalli et al., 2006). We can

reasonably attribute this controversy to a reduced Arc mRNA turnover or to an inhibition of protein synthesis. Alternatively, we cannot rule out the possibility that the increased mRNA levels were indeed accompanied by an increase in protein levels which could not be detected because of the specific targeting and therefore, trafficking, of the protein to synapses after long stimulation periods. Overall, our results regarding Arc are relevant as this protein is considered a reliable index of activity-dependent synaptic modifications (Larsen et al., 2005), and its overexpression has also been reported after chronic cocaine exposure (Fumagalli et al., 2006). Furthermore, Arc has been associated to the altered morphology of dendrites and spines observed after cocaine exposure. In this sense, we can suggest that the increase in Arc expression herein reported may alter the morphology of these structures in striatum, thereby setting the stage of drug addiction.

As previously reported (López-Arnau et al., 2017), repeated exposure to MDPV provokes an accumulation of ΔFosB in VS, observed either 24 h after treatment (≈290% expression) and after 21 days (≈135%), which has been associated to MDPV-induced behavioural sensitization. Additionally, the relationship between the induction of ΔFosB within the OFC and an increase in risk-taking behaviour and locomotor sensitization to cocaine has been demonstrated (Winstanley et al., 2009). In our study we also observed an increase of ΔFosB protein levels within the OFC which, in fact, was more than double of that registered in the VS. Therefore, we cannot rule out the possibility that such increase of ΔFosB levels in the OFC is involved in both behavioural abnormalities observed after MDPV exposure: behavioural sensitization and increased risk-taking behaviour. Hence, we extend knowledge suggesting that behavioural sensitization to MDPV and cocaine might be associated not only to the accumulation of ΔFosB in VS, but also within the OFC.

It is known that ΔFosB is involved in close to one quarter of all the genes influenced by chronic cocaine exposure in the NAcc. It functions as a transcriptional activator or as a repressor, depending on the duration and the degree of its expression (Nestler, 2008). CDK5, one of its target genes, is an example of a gene that is induced by chronic, but not acute, cocaine administration (Bibb et al., 2001). Moreover, the activation of CDK5 is not only under positive control of ΔFosB but it is also regulated by extracellular signal-regulated kinase (ERK), the phosphorylation of which is increased in the NAcc by drugs of abuse through a DR1-dependent mechanism (Valjent et al., 2004). In our study, repeated exposure to MDPV significantly increased CDK5 protein levels and, apparently, such increment was not accompanied by an aberrant and pathological activation of the protein nor by an alteration of the levels of phospho-Tau at Thr 205, since p35/p25 and phosphor-Tau (Thr 205)/Tau ratios were no different from those of the control group. In fact, p35 levels were considerably higher than those of p25, suggesting a correct activation of the kinase. Moreover, it is noteworthy that unlike ΔFosB, whose levels declined over time, the expression of CDK5 remained stable during the whole withdrawal, that is for at least 3 weeks after drug cessation. Therefore, we can suggest that the hyperdopaminergic state observed in mice after repeated exposure to MDPV might activate the mitogen-activated protein kinase (MAPK/ERK) pathway which would contribute to the stable induction and expression of CDK5 besides ΔFosB. On the other hand, it is known that CDK5 controls dopamine neurotransmission through the modulation of DARPP-32, a protein phosphatase- 1 inhibitor. In this way, CDK5 mediates cellular responses to cocaine-induced changes in dopamine signal transduction and cytoskeletal reorganization (Bibb et al., 2001). Therefore, it is considered a key element for the plasticity observed after chronic cocaine administration. Benavides and Bibb (2004) suggested

a model of the cellular signalling pathways under control of CDK5, whose activation takes place downstream of the overactivation of DR1 after cocaine administration. Given that MDPV has the same pharmacological mechanism of action as cocaine, we might expect a similar chain of events to those suggested for cocaine regarding CDK5 activity.

ΔFosB also regulates the AMPA receptor GluA2 subunit (Kelz et al., 1999; McClung and Nestler, 2003). GluA2 protein levels are not affected by cocaine exposure, but an internalization of this subunit during withdrawal in cocaine-sensitized animals has been described (Boudreau et al., 2007). In the present study, repeated non-contingent administration of MDPV did not produce any effect on GluA2 subunit expression, as it has been described for cocaine. However, and by contrast to cocaine, GluA2 subunit decreased substantially over withdrawal, and an apparent internalization of the subunit was suspected, even though it did not reach statistical significance. We may speculate that this adaptation of the GluA2-subunit is the result of the likely decreased glutamatergic neurotransmission present after withdrawal from repeated MDPV exposure, as it has been observed after repeated cocaine exposure (Baker et al., 2003). Likewise, the glutamate system has been associated with the reinforcing and psychostimulant properties of MDPV (Gregg et al., 2016).

Epigenetic changes have been revealed as critical mechanisms contributing to drug-induced plasticity by regulating gene expression. G9a specifically catalyses the dimethylation of lysine 9 of histone 3 (H3K9me2). Maze et al. (2010) showed that acute cocaine increases G9a levels in the NAcc. In contrast, they found a down-regulation of G9a 24 h after repeated cocaine administration, consistent with an overexpression of some of its target genes such as Arc (Oey et al., 2015) and ΔFosB (Maze et al., 2010).

In fact, as ΔFosB accumulates, it represses G9a and thereby potentiates its own further induction. In our work, an overexpression of G9a was observed in VS 24 h after treatment. In this sense, we could say that most probably the overexpression of genes known to enhance synaptic plasticity, which are at the same time G9a target genes (i.e. *arc, fosB, cdk5* or *nfκb*) might have been limited or even blocked by such G9a overexpression, a feature that could explain, at least partly, the aforementioned differences observed in gene expression induced by repeated exposure to cocaine or to MDPV. Furthermore, as MDPV-treated animals showed increased ΔFosB protein levels in the same brain area, alternative mechanisms must modulate G9a expression, aside from ΔFosB.

In parallel, parameters related to dopaminergic neurotransmission were studied in DS. Repeated cocaine exposure is known to induce short-term changes such as increased TH expression in NAcc (Rodriguez-Espinosa and Fernandez-Espejo, 2015). Similarly, MDPV exposure also induced an increase of TH protein levels that was evident not only shortly after treatment, but also after 21 days of abstinence, suggesting a long-lasting adaptive change in gene expression that can be mediated by several mechanisms (Kumer and Vrana, 1996), as it occurs with CDK5.

There is compelling evidence that increased dopamine uptake through its specific transporter (DAT) results in oxidative damage via the cytosolic oxidation of the neurotransmitter (Masoud et al., 2015). We did not observe changes 24 h after MDPV treatment, but a decrease in total DAT density 21 days after the last drug injection. In this sense, such reduction could be explained, at least partly, as a mechanism to guarantee a resilience response to avoid terminal injury by oxygen radicals derived from high DA intracellular levels.

In vivo, chronic cocaine use is accompanied by an immediate change in the DR2:DR1 ratio signalling towards the DR1 (Thompson et al., 2010). In the present study, DR1 population initially increased while DR2 was reduced in MDPV treated mice. Hence, the DR2:DR1 ratio altered towards the DR1. Nevertheless, after three weeks of abstinence, DR1 population decreased substantially and DR2 availability increased, returning to normal. These changes observed long after treatment ran in parallel with the increased TH levels and the lower removal of DA from synapses via DAT, pointing to a hyperdopaminergic status. Possibly, all these effects are due to adaptive neuroplastic changes associated to MDPV abuse and the withdrawal syndrome.

Given the hyperdopaminergic status induced after repeated MDPV exposure and because increased DA uptake can result in oxidative damage, 4-HNE, a robust marker of oxidative stress (Perluigi et al., 2012) and GFAP, which indicates glial activation, were assessed to investigate a putative neurotoxic effect of MDPV. Shortly after drug exposure we found that 4-HNE tripled its levels, suggesting an important oxidative effect generated by DA or possibly by the reactive quinones generated from MDPV metabolism (Baumann et al., 2017). Nonetheless, such oxidative stress did not seem to be severe enough to trigger an astroglial response. Furthermore, it was transient, so disappeared over time without leaving relevant consequences.

In conclusion, repeated exposure to MDPV induces an anxiety-related behaviour, increases aggressiveness shortly after treatment and promotes a quick habituation to new environments, interpreted as a risk-taking behaviour. We speculate that the accumulation of ΔFosB within the VS and the OFC can contribute to both behavioural abnormalities: locomotor sensitization and the risky-behaviour. Other neuroadaptive changes that persist long after

withdrawal probably also contribute to MDPV and cocaine sensitization: Arc and CDK5 overexpression, changes in DR2:DR1 ratio, high levels of TH and a low DA clearance by DAT, which point to a disorder of DA imbalance promoted by MDPV (i.e. long-lasting hyperdopaminergic status). Although MDPV and cocaine share the same mechanism of action, the intracellular responses that they trigger notably differ. Thus, even though repeated exposure to MDPV resulted in an accumulation of ΔFosB, as it occurs after cocaine exposure, they modulate the expression of G9a, NFκB and GluA2 differently. Hence, there might be other independent regulatory signals modulating ΔFosB and its target genes.

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3.1.7. SUPPLEMENTARY MATERIAL

Table S1. Commercial sources and dilution of primary and secondary antibodies used in the Western blot experiments. *Brain region and time point of the measure are specified for each target.*

PROTEIN	BRAIN AREA	TIME	COMMERCIAL SOURCE PRIMARY ANTIBODY AND WORKING DILUTION	COMMERCIAL SOURCE SECONDARY ANTIBODY AND WORKING DILUTION
Arc	VS	2 h Post-treatment (Day 7); Day 28 (W21)	Abcam, Cambridge, UK (1:1000) # ab23382	Anti-mouse IgG GE Healthcare, Buckinghamshire, UK (1:2500)
GluA2	VS	24 h Post-treatment (Day 8, W1); Day 28 (W21)	Santa Cruz Biotechnology, USA (1:1000) #N-19 sc-7611 Neuromab Antibodies Inc (1:1000) #75-002	Anti-goat IgG Santa Cruz Biotechnology, USA (1:5000) Anti-mouse IgG GE Healthcare, Buckinghamshire, UK (1:2500)
CDK5	VS	24 h Post-treatment (Day 8, W1); Day 28 (W21)	Santa Cruz Biotechnology, USA (1:1000) # J-3 sc-6247	Anti-mouse IgG GE Healthcare, Buckinghamshire, UK (1:2500)
P35/P25	VS	24 h Post-treatment (Day 8 W1)	Cell Signaling Technology, Inc (1:1000) #2680	Anti-rabbit IgG GE Healthcare, Buckinghamshire, UK (1:5000)
P-Tau (Thr 205)	VS	24 h Post-treatment (Day 8 W1)	ThermoFisher Scientific, USA (1:1000) #44738G	Anti-rabbit IgG GE Healthcare, Buckinghamshire, UK (1:5000)
Tau	VS	24 h Post-treatment (Day 8 W1)	ThermoFisher Scientific, USA (1:1000) # AHB0042	Anti-mouse IgG GE Healthcare, Buckinghamshire, UK (1:2500)
NFκB	VS	24 h Post-treatment (Day 8, W1)	Cell Signaling Technology, Inc (1:1000) #8242	Anti-rabbit IgG GE Healthcare, Buckinghamshire, UK (1:5000)
TH	DS	24 h Post-treatment (Day 8, W1); Day 28 (W21)	Transduction Laboratories, Lexington, Y, USA (1:5000) #612300	Anti-mouse IgG GE Healthcare, Buckinghamshire, UK (1:2500)
DR1	DS	24 h Post-treatment (Day 8 W1); Day 28 (W21)	Sigma Aldrich, St. Louis, Mo, USA (1:1000) #D2944	Anti-rat IgG GE Healthcare, Buckinghamshire, UK (1:5000)
GFAP	DS	24 h Post-treatment (Day 8 W1); Day 28 (W21)	Dako, Cambridge, UK (1:1000) #Z0334	Anti-rabbit IgG GE Healthcare, Buckinghamshire, UK (1:5000)
4-HNE	DS	24 h Post-treatment (Day 8 W1); Day 28 (W21)	Abcam, Cambridge, UK (1:1000) # ab46545	Anti-rabbit IgG GE Healthcare, Buckinghamshire, UK (1:5000)
ΔFosB	OFC	Day 28 (W21)	Abcam, Cambridge, UK (1:500) # ab11959 [83B1138]	Anti-mouse IgG GE Healthcare, Buckinghamshire, UK (1:2500)

CHAPTER 2

3.2. Role of the BDNF – TrkB signalling pathway in the development of behavioural sensitization to MDPV and cocaine

Adapted from: **Duart-Castells, L**[1]., López-Arnau, R[1]., Vizcaíno, S[1]., Camarasa, J[1]., Pubill, D[1]., Escubedo, E[2]. 7,8-Dihydroxyflavone blocks the development of behavioural sensitization to MDPV, but not to cocaine. Differential role of the BDNF – TrkB pathway

Biochem Pharmacol. 2019; 163:84-93. doi: 10.1016/j.bcp.2019.02.004. Epub 2019 Feb 6.

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3.2.1. ABSTRACT

3,4-Methylenedioxypyrovalerone (MDPV) is a blocker of the dopamine transporter (DAT) that exerts powerful psychostimulant, rewarding and reinforcing effects. We have recently reported that MDPV increases vulnerability to cocaine abuse. However, the intracellular signalling pathways triggered by cocaine and MDPV notably differ: although repeated exposure to both drugs induces an accumulation of ΔFosB, other related-factors are not altered by MDPV exposure. Therefore, additional pathways might be affected.

In this context, several studies have suggested a key role for the brain-derived neurotrophic factor – tropomyosin receptor kinase B (BDNF – TrkB) pathway in behavioural abnormalities induced by psychostimulants, including locomotor sensitization. To gain more in-depth knowledge of these mechanisms, the present study started by investigating for the first time the cross-sensitization between MDPV and cocaine. Afterwards, we aimed to elucidate the role of the BDNF – TrkB pathway in the development of locomotor sensitization to both drugs.

Mice were treated with MDPV (1.5 mg/kg s.c.) or cocaine (10 or 15 mg/kg, i.p.) once daily for 5 days. After 10 days of withdrawal, animals were challenged with cocaine (8 mg/kg) or MDPV (1 mg/kg). For biochemical determinations, MDPV (1.5 mg/kg) or cocaine (15 mg/kg) were administered acutely or repeatedly and BDNF, DR3 and G9a transcription levels, as well as pro- and mature BDNF (mBDNF) protein levels, were determined.

Our results demonstrated that repeated administration of MDPV or cocaine cross-sensitized to their locomotor effects. Furthermore, only MDPV

induced early changes in BDNF transcription. However, although MDPV increased cortical BDNF mRNA, the accumbal levels of the mature protein decreased. Interestingly, such decline was involved in the development of locomotor sensitization, thus the pre-treatment with 7,8-Dihidroxyflavone (7,8-DHF, 10 mg/kg i.p.), a TrkB agonist, blocked the development of sensitization to MDPV but not to cocaine, for which no changes in BDNF expression were observed at early withdrawal. Therefore, the BDNF – TrkB signalling pathway plays a key role in the development of MDPV-induced sensitization.

To sum up, a cross-sensitization between MDPV and cocaine was evidenced, so both drugs enhance the abuse liability to each other. Moreover, our findings suggest that decreased BDNF – TrkB signalling in the nucleus accumbens has an important role in the behavioural sensitization to MDPV, pointing TrkB agonists as potential therapeutic drugs to prevent behavioural abnormalities such as MDPV sensitization.

3.2.2. INTRODUCTION

During the last decade the illicit drug market has changed considerably due to the emergence of New Psychoactive Substances (NPS) which include synthetic cathinones. The popularity of cathinones as recreational drugs has been increasing since they broke into the drug market, so that they are used as substitutes for other classical psychostimulants such as cocaine or ecstasy.

3,4-Methylenedioxypyrovalerone (MDPV) is one of the most popular synthetic cathinones and one of the main ingredients of the so-called "bath salts" [1,2]. As many psychostimulants, MDPV produces some of its

neurochemical effects by interacting with the transporters of monoamine neurotransmitters. In the same way as cocaine, MDPV increases dopamine (DA) levels in the synaptic cleft by inhibiting its uptake. Animal studies indicate that MDPV is 10–50-fold more potent as a dopamine transporter (DAT) blocker than cocaine [3,4]. Moreover, it has also been demonstrated that MDPV exerts powerful psychostimulant, rewarding and reinforcing effects related to cocaine at one tenth-doses [5], pointing to a high abuse liability and thus a presumable upward consumption of this substance in the next years, probably favoured by its affordable cost. Therefore, new findings about MDPV and its relationship with addiction are of special interest. Moreover, research on this topic becomes important considering the potential health and social consequences associated with newly emerging molecular variants of this drug.

Behavioural sensitization to psychostimulants is the process whereby repeated intermittent exposure to drugs results in a progressive and enduring increase in the motor stimulant response to the drug [6–10]. Furthermore, behavioural sensitization is a long-lasting phenomenon, shown to persist for at least one year after cessation of drug administration [11]. Hence, repeated drug exposure may induce dynamic changes in neural processes, which may influence susceptibility to drug abuse and relapse by increasing the reinforcing value of acute drug administration [9,12]. It is also known that the ventral tegmental area (VTA) is essential for the development of behavioural sensitization, whereas the nucleus accumbens (NAcc) seems to be necessary for its expression [13,14].

There is convincing evidence that the brain-derived neurotrophic factor (BDNF) along with its specific receptor, tropomyosin receptor kinase B (TrkB), have a key role in the behavioural abnormalities observed in rodents

after psychostimulant administration [15–17]. BDNF present in NAcc and dorsal striatum is chiefly, but not exclusively, supplied by anterograde axonal transport from cortical pyramidal neurons in frontal cortex [18-21].

Research done over the last two decades has reported BDNF to be involved in the long-term neuronal adaptations leading to functional modifications in the synapses associated with cocaine abuse and related behaviours [17,22,23]. Moreover, it is also involved in the behavioural abnormalities and neurotoxicity induced by methamphetamine consumption [24]. Hence, the BDNF – TrkB signalling may be a potential therapeutic target for treating drug addiction.

Transcription of the *bdnf* gene is under control of not only transcriptional factors but also of epigenetic mechanisms including chromatin remodelling and DNA methylation [25]. G9a is a histone 3 lysine 9 (H3K9me2)-specific dimethyltransferase that acts as a negative regulator of BDNF signalling through TrkB [26]. It is also known that repeated exposure to cocaine induces long-lasting epigenetic changes in the brain, modifying the chromatin structure of DNA via histone acetylation, phosphorylation, and/or methylation [27,28]. For instance, Maze et al. [29] demonstrated that H3K9me2 is substantially reduced in the NAcc after chronic cocaine exposure due to decreased levels of G9a. In this sense, it has been identified an essential role for H3K9me2 and G9a in cocaine-induced structural and behavioural plasticity [29].

At the same time, BDNF, synthesized in either VTA neurons or neurons originating from the cortex, controls dopamine receptor 3 (DR3) expression [30]. Interestingly, hyperresponsiveness to drug-associated cues and context-dependent behavioural sensitization might be related to hypersensitive

postsynaptic dopaminergic receptors. Among them, DR3 seems to be a key target since it is highly expressed in the shell of NAcc, in which DA release is mainly triggered by drugs [31]. Furthermore, DR3 expression controls behavioural sensitization and BDNF [30,32], which at the same time controls DR3 expression and enhances the conditioned reward and locomotor activity induced by cocaine [33].

Drug-induced locomotor sensitization has been described for both cocaine and MDPV [34–37]. The fact that different addictive drugs produce the same effects implies that they may exert some of them through the same neural mechanisms. Furthermore, cross-sensitization between addictive drugs has also been described [38,39]. For this reason, the present study aims to investigate for the first time the cross-sensitization between MDPV and cocaine, as well as to determine and compare the changes induced by either acute or chronic exposure to MDPV or cocaine on the transcription of plasticity genes in early abstinence. Thus, this work is mainly focused on the role of BDNF – TrkB signalling pathway in the development of behavioural sensitization to MDPV and cocaine, and also its target gene, *dr3*, and the regulator histone methyltransferase G9a.

3.2.3. MATERIALS AND METHODS

Animals

Male adolescent (PND 41-44) Swiss CD-1 mice (Charles River, Spain) were used for all experiments due to its optimal sensitivity to the reinforcing and psychostimulant effects of cocaine. Animals were randomly assigned to

an experimental group and housed six per cage in temperature-controlled conditions (22 ± 1 °C) under a 12 h light/dark cycle and had *ad libitum* access to standard food and water. During the behavioural manipulations, researchers were not aware of the treatment that each animal had received. All the procedures adhered to the guidelines of the European Community Council (2010/62/EU) and ARRIVE and were approved by the Animal Ethics Committee of the University of Barcelona. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Materials

Pure racemic MDPV · HCl was synthesized and characterized in our laboratory as described [40]. Cocaine was provided by the Spanish National Institute of Toxicology. MDPV and cocaine solutions for injection were prepared in 0.9% NaCl (saline, pH=7.4) immediately before administration.

Both TrkB ligands 7,8-Dihydroxyflavone (7,8-DHF) and ANA-12 (N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were prepared as a micro-suspension in phosphate-buffered saline containing 0.5% (w/v) carboxymethyl cellulose and 0.1% (v/v) Tween-80.

Specific ELISA mBDNF and proBDNF Kits were purchased from Biosensis (Thebarton SA, Australia), the protease and phosphatase inhibitor cocktail from Abcam (Cambridge, UK) and the organic solvents ethanol, chloroform and isopropanol, from Scharlab (Barcelona, Spain). The rest of reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Drug administration protocol and experimental design

For cross-sensitization experiments animals followed the administration regime depicted in Figure 18.

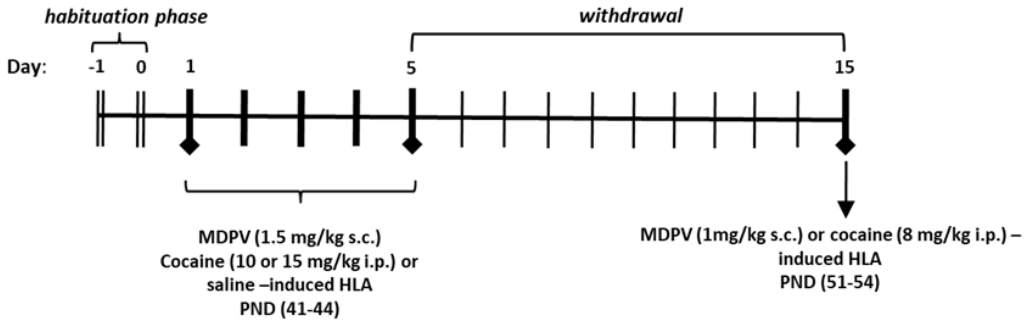

Figure 18. Drug administration protocol and experimental design. *After two days of habituation (days –1 and 0), mice were treated with saline (5 ml/kg), MDPV (1.5 mg/kg s.c.) or cocaine (10 mg/kg or 15 mg/kg i.p.), once daily for five consecutive days (days 1–5) and their horizontal locomotor activity (HLA) was recorded. 10 days later (day 15), all mice were challenged with MDPV (1 mg/kg s.c.) or cocaine (8 mg/kg i.p.) and their HLA was recorded.*

A moderate dose of MDPV (1.5 mg/kg, s.c.) eliciting hyperlocomotion was chosen for this study. This dose, in mice, is equivalent to a dose of approximately 8 mg in humans [40,41], which is in the middle range of the doses most commonly used by consumers [42]. The doses of cocaine (10 and 15 mg/kg, i.p.) have been chosen on the basis that MDPV is at least 10-fold more potent than cocaine *in vivo* and *in vitro* [3–5,43]. No higher doses were used since those we selected predominantly induce locomotor activity (e.g., horizontal forward locomotion, even though some occasional licking were observed), whereas higher doses could produce non-ambulatory stereotypies,

such as head bobbing, sniffing or licking that could have interfered the analysis because of the competition between non-ambulatory behaviours and locomotor activity.

The sensitization procedure consisted of three phases over 17 days: habituation, treatment and challenge. In the habituation phase (the two days prior treatment, days −1 and 0), mice were handled for 10 min, injected with saline (5 ml/kg) and immediately placed for 45 min in a black Plexiglass open field arena. After the habituation phase, mice were injected with saline (5 ml/kg), MDPV (1.5 mg/kg s.c.) or cocaine (10 mg/kg or 15 mg/kg i.p.) once daily for five consecutive days (days 1–5). Animals were placed in the open field arena immediately after each saline or drug injection and their horizontal locomotor activity (HLA) was recorded. Ten days after the final administration (day 15), all mice were challenged with cocaine (8 mg/kg i.p.) or MDPV (1 mg/kg s.c.) and their HLA was registered again.

For biochemical determinations, acute and chronic exposure regimes were designed. In acute experiments, a single dose of saline (5 ml/kg), MDPV (1.5 mg/kg s.c.) or cocaine (15 mg/kg i.p.) were injected and animals were sacrificed 1 h or 2 h post-administration for the subsequent study of different markers. The chronic administration protocol was the same as the one used for the cross-sensitization experiments (Figure 18) but sacrificing the animals 2 h, 24 h or 10 days post-treatment (day 15).

Horizontal locomotor activity (HLA) measurement

Animals were given their treatment (saline, MDPV or cocaine solutions) and immediately placed in a black Plexiglass open field arena (25×25×40 cm) under low-light conditions and white noise. HLA was video-monitored for

30 min using a specific tracking software (Smart 3.0 Panlab, Barcelona, Spain). All HLA experiments were performed during the resting-phase of the animals (between 8:00 am and 2:00 pm).

Effects of 7,8-DHF on the development of behavioural sensitization after repeated exposure to cocaine or MDPV

To evaluate the sensitizing effects of MDPV and cocaine under a previous treatment with 7,8-DHF, mice were assigned to one of the following four groups: vehicle + saline; vehicle + MDPV or cocaine; 7,8-DHF + saline and 7,8-DHF + MDPV or cocaine. 7,8-DHF (10 mg/kg, i.p.) was administered 30 min prior to the saline or drug injection, to ensure constant levels of the agonist throughout all the procedure. The treatment schedule used was the same as for the cross-sensitization experiments, but the HLA was only recorded after the challenge with MDPV or cocaine, on day 15. The 7,8-DHF dose as well as the administration schedule were selected according to those used in multiple studies in the literature [24,44].

Tissue samples preparation

Mice treated according to the administration protocols described above were sacrificed by cervical dislocation, 1 h or 2 h after an acute dose and 2 h, 24 h or 10 days after the repeated regime, for the analysis of different factors including: G9a, BDNF and DR3. NAcc, ventral striatum (VS) containing NAcc, or medium prefrontal cortex (mPFC), when appropriate, were quickly dissected out and stored at −80 °C until use. Particularly, for the dissection of the NAcc and the mPFC, brains were rapidly removed and placed in an ice-cold mouse brain acrylic matrix (Alto, Agnthos, Sweden). Two double edge blades were properly inserted to obtain a 2 mm (2 mm

anterior to bregma) or 1 mm (from 2 to 3 mm anterior to bregma) thick slices, respectively, which contained the regions of interest [45]. The NAcc was micropunched away from the rest of the brain tissue.

Tissue samples for ELISA analysis were processed following the instructions provided by the kit manufacturer. Briefly, NAcc samples were thawed and homogenized through sonication at 4 °C in 100 volumes of RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% (v/v) NP-40 and 0.5% (w/v) sodium deoxycholate, pH=7.5–8) containing a protease and phosphatase inhibitor cocktail. Once homogenized, samples were kept on ice for 30 min. Sample sonication and cooling with ice were performed twice. Homogenates were centrifuged at 14,000 ×g for 30 min at 4 °C and the resulting supernatants (total lysates) were collected and kept at −80 °C until use. Protein content was determined using the Bio-Rad Protein Reagent (Bio Rad, Inc., Madrid, Spain).

Quantification of mBDNF and proBDNF protein levels

The determination of proBDNF and mBDNF was performed following the instructions of the corresponding ELISA kit. In brief, the kit consisted of a microplate pre-coated with mouse monoclonal anti-mature or polyclonal anti-proBDNF capture antibodies. Then, samples containing unknown amount of the target protein were added and bound to the capture antibody. After washing steps to rid the microplate of unbound substances, a biotinylated anti-mature or anti-proBDNF detection antibody, respectively, and horseradish peroxidase (HRP)-conjugated streptavidin were added for detection. The addition of a substrate (3,3,5,5-tetramethylbenzidine, TMB) yielded a coloured reaction product that was directly proportional to the concentration of mature or proBDNF present in samples and protein standards.

Total RNA extraction and gene expression determination

Total RNA isolation from ventral striatum or mPFC was carried out by means of a TRIsure™ reagent-Chloroform based extraction protocol. TRIsure™ (Bioline, Meridian Bioscience Inc., UK) monophasic solution of phenol and guanidinium isothiocyanate facilitates disruption of cells during homogenization and effectively inhibits DNase and RNase activity. Simultaneously, solubilizes biological material and denatures protein. After solubilization, the addition of chloroform causes phase separation, where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase [46]. RNA content in the samples was measured at 260 nm, and sample purity was determined by the A260/280 ratio in a NanoDrop™ ND-1000 (Thermo Scientific).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the Veriti® thermal cycler (Applied Biosystems, Foster, CA, USA). Briefly, complementary DNA (cDNA) was synthesized in a total volume of 20 μL by mixing 1 μg of total RNA and the appropriate volumes of each kit reagent.

Quantitative real-time polymerase chain reaction (qPCR) was performed using the StepOnePlus™ Real-Time PCR System and the Taqman one-step PCR Master Mix (Applied Biosystems, Foster, CA, USA). In brief, 25 ng of total cDNA was added per 20-μl reaction mixture containing sequence-specific primers and Taqman probes from Applied Biosystems Mm0113261_m1 for G9a, Mm04230607_s1 for BDNF, Mm00432887_m1 for DR3 and Mm00607939_s1 for β-actin. The β-actin transcript level was used to normalize differences in sample loading and preparation. The

reaction conditions were as follows: the holding stage was initiated by a 2-min incubation at 50 °C and 10 min of polymerase activation at 95 °C, followed by a cycling stage of 40 cycles at 95 °C for 15 s and 60 °C for 60 s. All reactions were performed in triplicate. Fold-changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method (comparative Ct method) for each experimental sample [47]. All primer-probe combinations were optimized and validated for relative quantification of gene expression. All experiments included a no-template control, and real-time PCR reactions also included a no-reverse transcriptase (no-RT) control for which no amplification products were seen, indicating that cDNA was amplified rather than residual genomic DNA.

Conditioned place preference (CPP) test

The place conditioning protocol used was non-biased as described previously [48]. CPP was performed in three phases: preconditioning, conditioning and test. During the preconditioning phase (day 1), mice had free access and roam among the three compartments of the apparatus for 15 min. The time spent in each compartment was recorded by computerized monitoring software (Smart 3.0 Panlab, Barcelona, Spain). During the conditioning phase (days 2–5; sessions 1–8), mice were given 7,8-DHF (10 mg/kg i.p.), 30 min before the s.c. administration of MDPV (1.5 mg/kg) or saline. After the MDPV injection, animals were immediately placed into one of the two conditioning compartments for 20 min (sessions 1, 3, 5 and 7). On the alternate sessions (2, 4, 6 and 8), mice were placed in the other compartment for 20 min after being given a saline injection. Two conditioning sessions per day were performed, separated by a 5-hours period. Control animals received saline every session. The preference test (day 6) was

conducted as the preconditioning phase. A preference score was expressed in seconds and calculated for each animal as the difference between the time spent in the drug-paired compartment in the test minus the time spent in the same compartment in the preconditioning phase.

Data acquisition and statistical analysis

Data were expressed as mean ± SEM. Differences between groups were compared using one- or two-way ANOVA where appropriate. One-way ANOVA was used when analysing differences due to the factor "treatment" in the experiment, whereas two-way ANOVA was performed when two different factors were analyzed: *"treatment × time"*. The α error probability was set at 0.05. Significant differences ($P < 0.05$) were analysed using the Tukey's post hoc test for multiple comparisons measures where appropriate only if F achieved the necessary level of statistical significance ($P < 0.05$) and no significant variance in homogeneity was observed. Every set of results was tested in the calculator QuickCalcs of GraphPad software, which performs Grubbs' test (extreme studentized deviate method), to determine whether any of the values in the set was a significant outlier from the rest. Statistic calculations were performed using GraphPAD Prism 6.0 software.

3.2.4. RESULTS

Cross-sensitization between MDPV and cocaine

To evaluate the ability of cocaine to sensitize to the motor stimulant effects of MDPV and vice versa, a cross-sensitization experiment was performed

following the administration regime displayed in Figure 18, in which two different doses of cocaine were tested (10 mg/kg and 15 mg/kg, i.p.).

During the treatment phase, HLA was measured daily and immediately after each saline or drug injection. MDPV induced an acute hyperlocomotion that, unlike cocaine, significantly increased with repeated daily exposure (Figure 19). Accordingly, two-way ANOVA revealed an effect of the day ($F_{4,368}$=8.681, $P < 0.001$), treatment ($F_{3,92}$=131.3, $P < 0.001$) and the interaction between both factors ($F_{12,368}$=1.931, $P < 0.05$) (n=24/group). Moreover, MDPV-treated mice showed a significantly higher locomotor activity at a dose of 1.5 mg/kg during the treatment compared to cocaine at both doses tested (10 and 15 mg/kg).

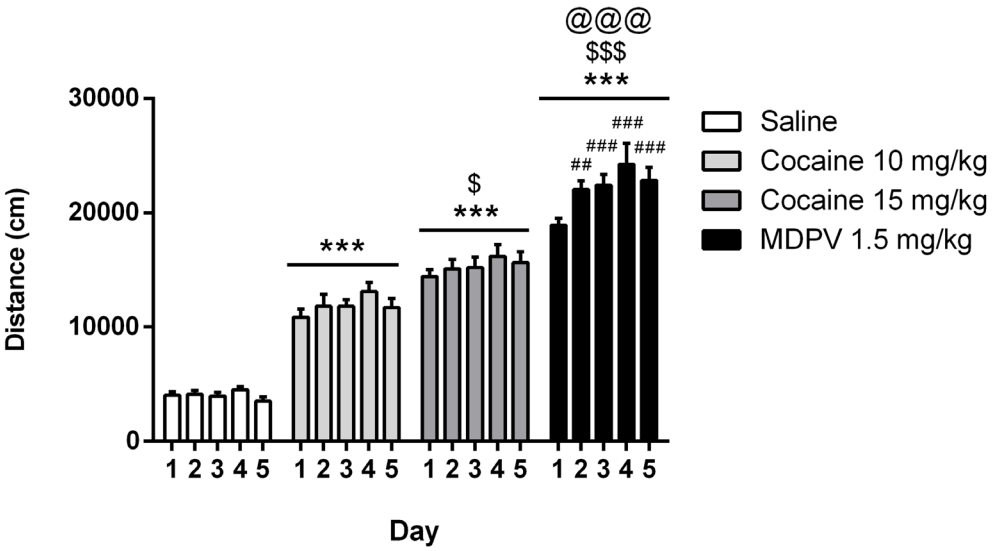

Figure 19. Horizontal locomotor activity (30 min) induced by saline, cocaine (10 mg/kg or 15 mg/kg, i.p.) and MDPV (1.5 mg/kg, s.c.) administration during the five days of treatment. *Bars represent mean ± SEM of the distance travelled after each injection. ***P < 0.001 vs the saline-matched day. $P < 0.05 and $$$P < 0.001 vs the cocaine 10 mg/kg-matched day. @@@P < 0.001 vs the cocaine 15 mg/kg-matched day. ##P < 0.01 and ###P < 0.001 vs MDPV day 1.*

When analysing the HLA after a challenge with MDPV (1 mg/kg, s.c.) or cocaine (8 mg/kg i.p.) on day 15 after withdrawal (Figure 20A and 20B), a higher locomotor response was evidenced in both MDPV and cocaine-treated mice compared to the control group. Accordingly, one-way ANOVA reported an effect of treatment for both challenge drugs (MDPV challenge: $F_{3,44}=8.697$, $P < 0.001$, n=12/group; cocaine challenge: $F_{3,43}=14.15$, $P < 0.001$, saline, cocaine 15 mg/kg and MDPV-treated groups, n=12/group; cocaine 10 mg/kg group, n=11). On the other hand, no differences were found between MDPV and cocaine-pretreated mice after the challenge with MDPV. However, it is noteworthy that, after the challenge with cocaine, MDPV-pretreated mice showed a higher locomotor response compared to cocaine 10 mg/kg-pretreated mice ($P < 0.05$) but not to those pretreated with cocaine 15 mg/kg. Therefore, repeated administration of cocaine 15 mg/kg or MDPV 1.5 mg/kg developed equivalent locomotor sensitization. Thus, the dose of 15 mg/kg of cocaine was chosen for the subsequent experiments.

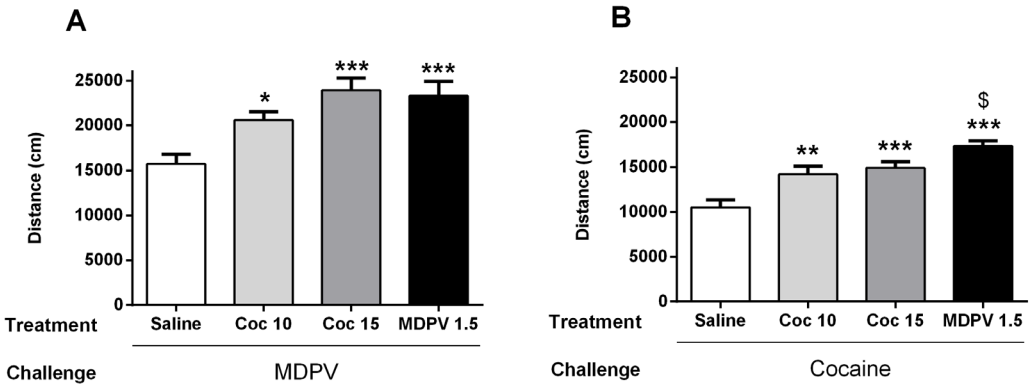

Figure 20. Effect of cocaine (10 mg/kg (Coc 10) or 15 mg/kg (Coc 15), i.p.) and MDPV (1.5 mg/kg (MDPV 1.5), s.c.) repeated treatment on the horizontal locomotor activity (30 min) induced by a challenge with MDPV (A) or cocaine (B). *Bars represent mean*

*± SEM of the distance travelled after a single MDPV (1 mg/kg, s.c.) or cocaine (8 mg/kg, i.p.) injection 10 days after treatment. **P < 0.01 and ***P < 0.001 vs saline group. $P < 0.05 vs cocaine 10 mg/kg.*

Effect of MDPV and cocaine on G9a methyltransferase, BDNF and DR3 expression

To further investigate the role of the BDNF – TrkB pathway in the development of behavioural sensitization, we assessed and compared the changes occurred in related genes after both an acute and a repeated exposure to MDPV and cocaine.

After an acute dose of MDPV or cocaine

G9a methyltransferase mRNA in VS was determined 1 h and 2 h post-injection. Two-way ANOVA revealed an effect of treatment ($F_{2,29}$=8.166, $P < 0.01$) and time ($F_{1,29}$=14.02, $P < 0.001$) with interaction between both factors ($F_{2,29}$=8.090, $P < 0.01$) (Figure 21A). 1 h after an acute dose of MDPV (1.5 mg/kg) or cocaine (15 mg/kg), the levels of G9a mRNA were similarly increased in cocaine ($P < 0.05$) and MDPV ($P < 0.01$)-treated mice compared to the saline group. However, this overexpression rapidly declined within 2 h. Importantly, this decline was more marked for the MPDV-treated mice than for those treated with cocaine, so significant differences were found between both groups at 2 h ($P < 0.01$) as well as among the MDPV-treated mice when comparing between the two time-points ($P < 0.001$) (saline 1 h and MDPV 1 h groups, n=5/group, cocaine 1 h, saline 2 h and MDPV 2 h groups, n=6/group; cocaine 2 h group, n=7).

In addition, when assessing the expression of the transferase in the mPFC 2 h post-injection, no changes were observed neither by MDPV nor

by cocaine administration (saline group, n=5; cocaine and MDPV groups, n=7/group) (Figure 21B).

In the same brain area, one-way ANOVA of BDNF mRNA levels 2 h post-administration yielded a significant effect of treatment ($F_{2,16}$=23.73, $P < 0.001$) (Figure 21C). Particularly, an acute MDPV injection induced a significant overexpression of this factor ($P < 0.001$), whereas no changes by cocaine were observed (saline group, n=5; cocaine and MDPV groups, n=7/group).

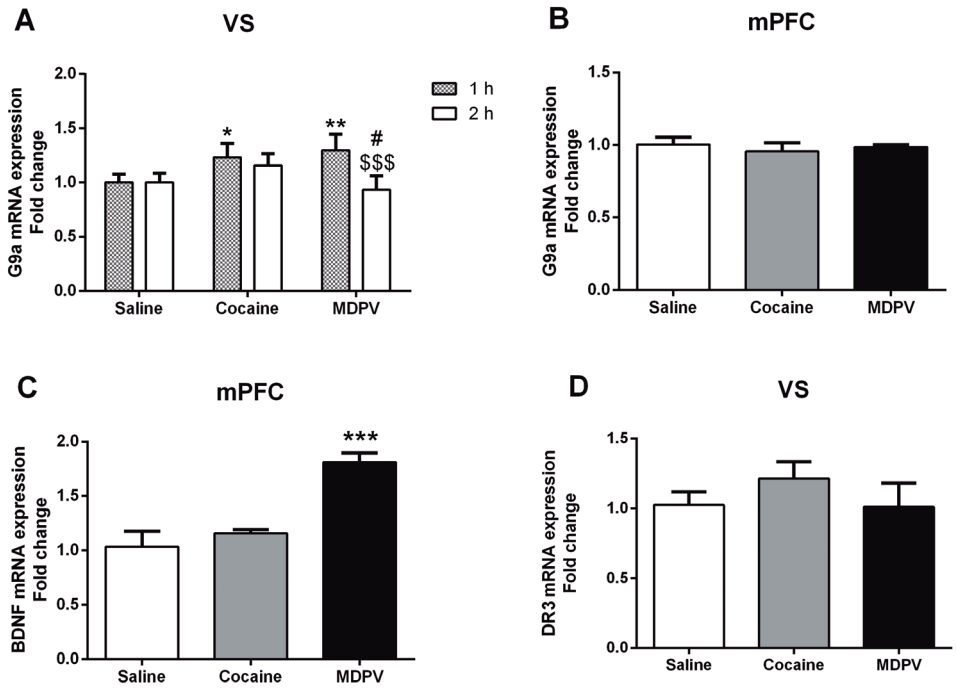

Figure 21. Effect of a single dose of MDPV (1.5 mg/kg, s.c.) or cocaine (15 mg/kg, i.p.) on the mRNA levels encoding G9a in VS 1 h and 2 h post-injection (A), G9a in mPFC 2 h post-injection (B), BDNF in mPFC 2 h post-injection (C), and DR3 in VS 2 h post-injection (D). *Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 vs saline group. #P < 0.05 vs cocaine 2 h. $$$ P < 0.001 vs MDPV 1 h.*

Since cortical BDNF controls DR3 expression in the NAcc, the mRNA levels encoding this receptor were also assessed 2 h post-administration in VS (containing NAcc). Nevertheless, no changes in DR3 mRNA levels were evidenced in this brain area (Figure 21D) (saline group, n=6; cocaine and MDPV groups, n=7/group).

After a repeated exposure to MDPV or cocaine

G9a gene expression was determined 2 h and 24 h after the last drug injection. Maze et al [29] described the effect of repeated cocaine exposure on G9a expression in NAcc after 24 h but, because we did not find changes at this time point, we assessed its levels also 2 h after drug administration.

As shown in Figure 22A, two-way ANOVA (n=6/group) reported a significant effect of treatment ($F_{2,30}$=3.811, $P < 0.05$), time ($F_{1,30}$=28.60, $P < 0.001$) and the interaction between both factors ($F_{2,30}$=9.743, $P < 0.001$). Particularly, repeated cocaine exposure upregulated G9a in VS 2 h post-treatment ($P < 0.01$), whereas MDPV did not alter significantly its expression. However, when measuring G9a mRNA 24 h post-treatment, no changes were observed for cocaine, pointing to a transient overexpression of the methyltransferase, which had returned to control values at 24 h of withdrawal. Regarding MDPV, despite no effect was observed after two hours, a significant reduction of G9a expression over time was evidenced when comparing between both time-points ($P < 0.05$).

We did not detect any effect induced by MDPV or cocaine on G9a transcript in mPFC 2 h post-treatment (n=6/group) (Figure 22B). In parallel, *bdnf* and *dr3* gene expression were determined 2 h post-treatment in mPFC and VS, respectively. One-way ANOVA (n=6/group) revealed a significant

effect of treatment ($F_{2,15}$=7.590, P < 0.01). Repeated exposure to MDPV, but not to cocaine, increased BDNF expression (P < 0.05) (Figure 22C). At the same time, a similar pattern of expression was observed for DR3, although the differences observed did not reach statistical significance (n=6/group) (Figure 22D).

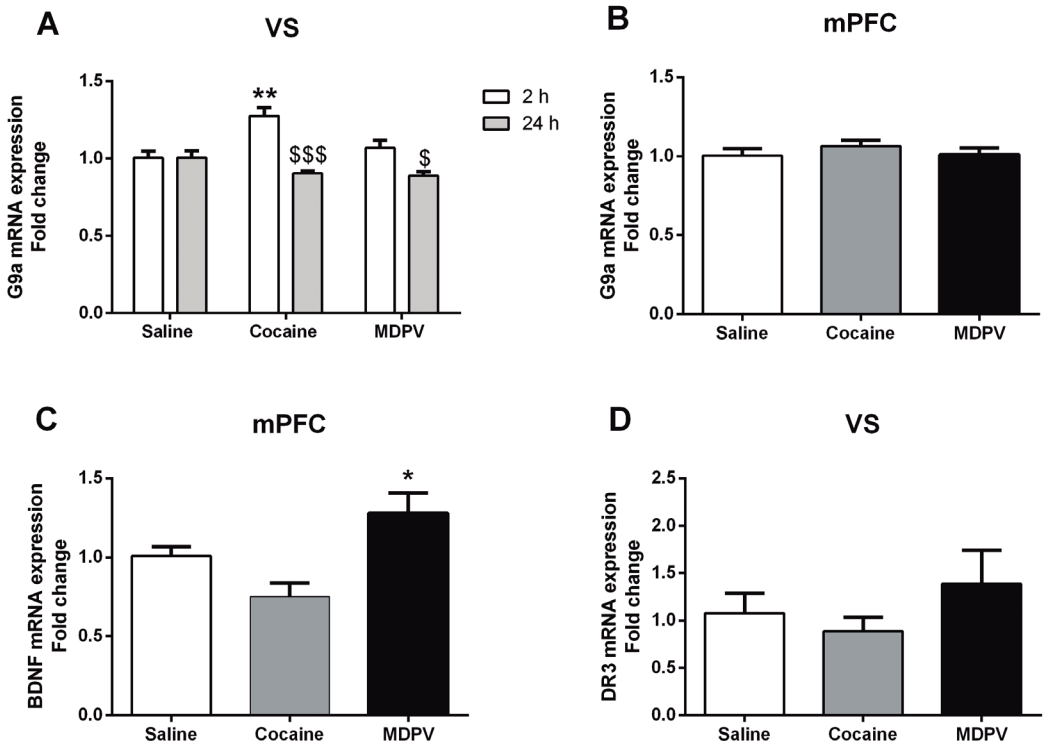

Figure 22. Effect of MDPV (1.5 mg/kg, s.c.) and cocaine (15 mg/kg, i.p.) repeated exposure on the mRNA expression encoding G9a in VS 2 h and 24 h post-treatment (A), G9a in mPFC 2 h post-treatment (B), BDNF in mPFC 2 h post-treatment (C), and DR3 in VS 2 h post-treatment (D). *Results are expressed as mean ± SEM. *P < 0.05 and **P < 0.01 vs saline group. $P < 0.05 and $$$P < 0.001 vs 2 h-matched drug.*

Given the fact that those fluctuations in BDNF transcription after drug exposure may not necessarily affect overall protein expression, pro- and mBDNF protein levels were assessed in dissected tissue from NAcc. Such protein levels were determined 2 h after treatment, but also 24 h and 10 days after the last administration of both substances.

Despite the observed increment in *bdnf* gene transcription induced by MDPV in mPFC, neither MDPV nor cocaine treatment altered proBDNF levels in NAcc (n=6/group) (Figure 23A). Surprisingly, two-way ANOVA revealed a significant effect of time ($F_{2,44}$=4.782, $P < 0.05$) and treatment ($F_{2,44}$=7.650, $P < 0.01$) in mBDNF expression, although no interaction between both factors was evidenced. Therefore, despite no differences were observed in the propeptide levels, mBDNF was decreased shortly after a repeated MDPV exposure ($P < 0.05$), even though its levels were restored after 10 days of withdrawal. In cocaine-treated-mice, no changes were observed at any of the time-points assessed until 10 days of withdrawal (saline 2 h and day 10, cocaine 2 h and 24 h, MDPV 2 h and day 10 groups, n=6/group; saline 24 h and MDPV 24 h groups, n=5/group; cocaine day 10 group, n=7) (Figure 23B).

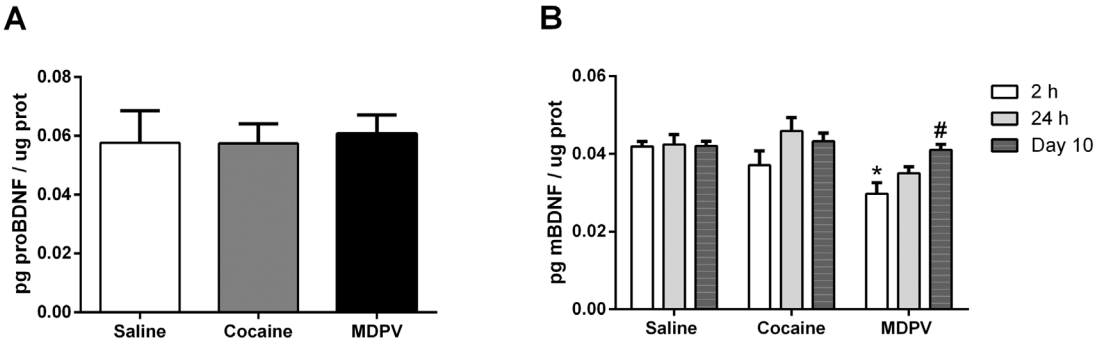

Figure 23. Effect of MDPV (1.5 mg/kg, s.c.) and cocaine (15 mg/kg, i.p.) repeated exposure on protein levels in NAcc of proBDNF 2 h after exposure (A), and mBDNF 2 h, 24 h and

10 days after exposure (B). *Results are expressed as mean ± SEM. *P < 0.05 vs saline group. #P < 0.05 vs MDPV 2 h.*

Effect of 7,8-DHF on the development of locomotor sensitization after repeated administration of MDPV or cocaine

Given the decrease of mBDNF protein levels observed 2 h after a repeated exposure to MDPV, we sought to determine if this alteration was involved in the development of locomotor sensitization to the drug. With this purpose, 7,8-DHF, a TrkB receptor agonist, was administered 30 min prior to every drug injection (pre-treatment).

From the experiments with MDPV (Figure 24A), one-way ANOVA revealed a significant effect of treatment ($F_{3,32}$=19.64, P < 0.001) (n=8/group). As we expected, post-hoc analysis showed that exposure to MDPV (1.5 mg/kg) significantly increased (P < 0.0001) acute MDPV (1 mg/kg)-induced locomotion 10 days after treatment, compared to its control group (vehicle + saline treated). Interestingly, pre-treatment with 7,8-DHF significantly blocked (P < 0.001) such development of sensitization. Moreover, control (saline) animals pretreated with 7,8-DHF or the vehicle showed a similar locomotor response, which evidences the null effect of the agonist on the locomotor activity by itself.

To prove that the effect of 7,8-DHF in the development of locomotor sensitization to MDPV was mainly due to its ability to bind and selectively activate TrkB, ANA-12, an antagonist of this receptor, was administered previously to the flavone. One way- ANOVA ($F_{3,31}$=20.15, P < 0.001) revealed that both groups exposed to MDPV (vehicle + MDPV and ANA-12 + 7,8-DHF + MDPV) developed behavioural sensitization (25,592 ± 716 cm,

P < 0.001 and 21,343 ± 1,192 cm, P < 0.01, respectively, vs saline-treated groups). This result suggested that ANA-12 prevented the binding of 7,8-DHF to TrkB and thereby its further activation, which seems to be involved in the effects induced by the agonist. No changes in the locomotor activity of control (saline) animals pretreated with ANA-12 + 7,8-DHF were observed.

On the other hand, in the experiments with cocaine, no effect of 7,8-DHF was evidenced in the development of behavioural sensitization (Figure 24B). One-way ANOVA ($F_{3,42}$=16.33, P < 0.001) evidenced that locomotor sensitization was similarly developed in both cocaine-treated groups, regardless they were pretreated or not with 7,8-DHF (vehicle + saline, 7,8-DHF + saline, vehicle + cocaine groups, n=12/group; 7,8-DHF + MDPV group, n=10).

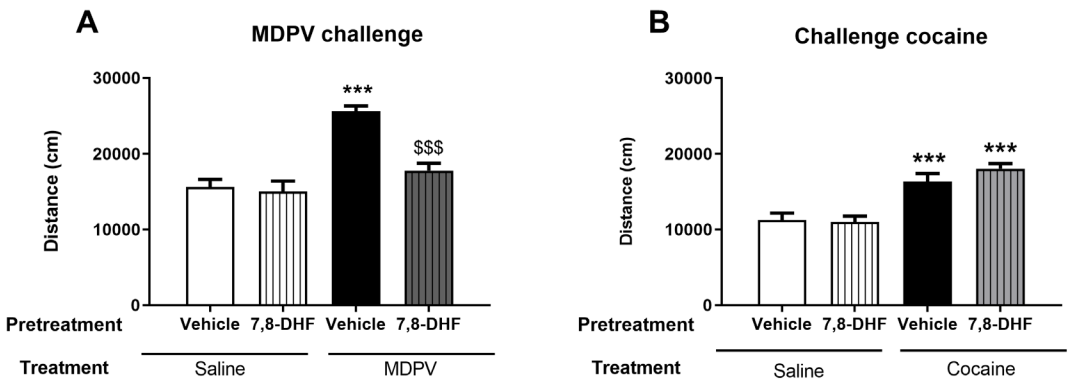

Figure 24. Effect of 7,8-DHF (10 mg/kg, i.p.) pre-treatment on the development of locomotor sensitization to MDPV 1.5 mg/kg (A) or cocaine 15 mg/kg (B). *Bars represent mean ± SEM of the distance travelled after an MDPV (1 mg/kg s.c.) or cocaine (8 mg/ kg, i.p.) challenge given 10 days after treatment. ***P < 0.001 vs saline-treated groups. $$$P < 0.001 vs vehicle+MDPV group.*

Effect of 7,8-DHF on the place conditioning induced by MDPV

The CPP paradigm was used to study whether 7,8-DHF could modify the conditioning properties of MDPV, aside from the development of locomotor sensitization.

The percentages of time spent in both compartments during the preconditioning phase were 51.48 ± 2.45% and 48.57 ± 2.45% ($P > 0.05$), indicating a total lack of preference for either side. 4 mice from the saline and MDPV groups were withdrawn from the experiment due to an initial preference for one of the compartments (> 65% of the total session time). One more animal from the MDPV group was withdrawn after the test day because it was a significant outlier (Saline and 7,8-DHF groups, n=12/group; MDPV group, n=11 and 7,8-DHF + MDPV group, n=13). On the test day (day 6, postconditioning), one-way ANOVA revealed a significant effect of treatment ($F_{3,44}=16.50$, $P < 0.001$). Accordingly, repeated administration of MDPV (1.5 mg/kg, s.c.) induced a preference for the MDPV-paired compartment ($P < 0.001$) which was not modified by the previous administration of 7,8-DHF. Furthermore, 7,8-DHF did not exert any conditioning or aversive effect by itself (Figure 25).

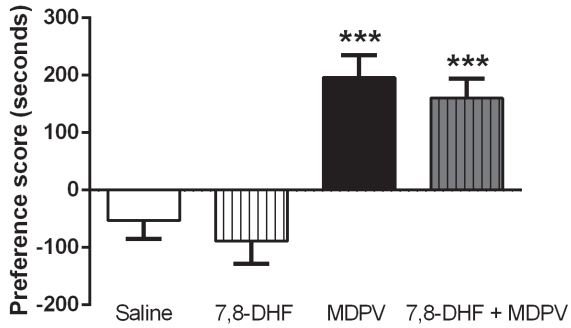

Figure 25. Effect of 7,8-DHF on MDPV (1.5 mg/kg s.c.)-induced conditioned place preference. *The x-axis represents the treatment group, and the y-axis represents the preference score (difference between the time (s) spent in the drug-paired compartment on the test and preconditioning day). ***P < 0.001 vs saline group.*

3.2.5. DISCUSSION

In a previous work, we reported that exposure of adolescent mice to MDPV sensitizes to cocaine effects and induces a higher vulnerability to cocaine abuse in adulthood [48]. Cocaine abuse represents a heavy burden of disease in many countries, becoming a global problem. Any factor that increases the vulnerability to cocaine abuse must be carefully evaluated. In this sense, the present study examines more closely the common pathways involved in the effects of MDPV and cocaine, using the same dose of MDPV previously described, and also mice at the beginning of periadolescence. Despite the risks associated with new cathinones use, little is known about their consequences, especially among adolescents, although the exposure pattern in this age group is very similar to that observed among adults [49].

Based on their pharmacological mechanism, we tested the possible cross-sensitization between cocaine and MDPV. Following our schedule, during the induction of sensitization, only MDPV elicited a hyperlocomotion that significantly increased with repeated daily exposure, whereas cocaine did not. Even so, regarding to the expression of sensitization (challenge day, 10 days after withdrawal), we observed that the induction of sensitization by cocaine 15 mg/kg was equivalent to that induced by MDPV 1.5 mg/kg. Additionally, our results demonstrate that repeated administration of MDPV or cocaine sensitizes to cocaine or MDPV locomotor effects, respectively.

Moreover, this is the first time that it is evidenced that the challenge with MDPV triggers an enhanced locomotor activity in animals pretreated with cocaine. This is of chief significance since sensitization-like phenomena has been frequently proposed to account for relapses in drug consumption. In this line, here we demonstrate that previous consumption of one of

these psychostimulants may favour relapse when a new dose is taken after a withdrawal period and also may favour the onset of an addiction to the other drug when taken for the first time.

In previous studies [35,48] we found that, although repeated exposure to MDPV resulted in an increase of ΔFosB expression, as it occurs after cocaine exposure, other factors were not affected by the cathinone derivative. Therefore, additional pathways might be involved in MDPV effects. To gain more in-depth knowledge of these mechanisms, this study aimed to investigate the role of BDNF in the locomotor sensitization induced by MDPV and cocaine, as well as to assess the effects of a single administration of both psychostimulants in such signalling pathway.

Dynamic alteration of BDNF expression and the consequent adjustments in brain functioning and neuronal plasticity are subjected to environmental changes (i.e., drug exposure) by means of altered epigenetic programming. Differential cocaine-induced effects at specific *bdnf* promoters are mediated by distinct epigenetic mechanisms. In mammals, the dimethylation of histone H3 on lysine 9, which is catalysed by the methyltransferase G9a, is commonly associated with gene silencing [50]. It has been described that there is an increase in G9a expression in NAcc after an acute dose of cocaine, but a decrease after repeated doses, which favours the expression of genes epigenetically controlled by this methyltransferase such as ΔFosB, BDNF or Arc [29].

In the present study we found an early significant increase in G9a expression induced after an acute dose of cocaine or MDPV, which rapidly reverted. It is noteworthy that this decline was more marked after MDPV exposure. When a schedule of repeated administrations was used, we also

obtained an early very significant increase of G9a mRNA induced by cocaine, which disappeared after 24 h after treatment. Again, the signal was more transient in the case of MDPV and, in fact, the early increase did not reach statistical significance. Taken together, we hypothesize that the initial increase induced by MDPV might have taken place much earlier than when we performed the determination.

Nevertheless, in a previous work from our lab [34], an overexpression of this transferase was observed in the VS 24 h after a higher and longer MDPV exposure (1.5 mg/kg, 2 doses in a day for 7 days). All over, our findings suggest that G9a expression after drug intake may be time and dose-dependent and, moreover, its regulation runs temporarily different for cocaine than for MDPV.

Bdnf gene is differentially regulated in regions of the mesolimbic pathway during the withdrawal or abstinence period following repeated cocaine administration [51]. Therefore, although the repressive role of G9a in the NAcc, no effect of this methyltransferase has been described in mPFC after cocaine exposure, but an increased histone acetylation [52]. In this sense, we considered it was worth to investigate if the changes in the methyltransferase transcription induced by these psychostimulants were region-specific. G9a expression in mPFC was not altered neither by acute nor by repeated doses of both drugs, pointing that the changes in this histone methyltransferase produced by MDPV or cocaine administration occur in the striatum, where probably play a relevant role in the regulation of behavioural responses to these DAT blockers [53], rather than in the mPFC.

Considering the discrepancy observed in the epigenetic regulation according to the brain area (VS or mPFC), we determined the effect of cocaine

and MDPV on BDNF transcription. Given the fact that Le Foll [54] described that a single exposure to cocaine (20 mg/kg) increases BDNF mRNA expression throughout the cortex in the adult rat, but not in the NAcc, we only determined BDNF expression in mPFC 2 h after the injection of the psychostimulants. In our study, using adolescent mice and a lower dose of cocaine (15 mg/kg), we did not observe such increase in BDNF transcription. However, a clear difference between cocaine and MDPV equivalent treatments must be highlighted. A single dose of MDPV, but not of cocaine, was enough to upregulate BDNF mRNA levels. Nevertheless, the G9a methyltransferase expression in the same brain area remained unaffected. Hence, we can conclude that, shortly after an acute or repeated MDPV administration, BDNF mRNA expression in mPFC increases without being apparently under the control of G9a-mediated epigenetic mark.

In adult mice, it has also been described a close relationship between increased BDNF mRNA expression in the cortex and an early increase of DR3 mRNA in the NAcc [54]. In our study, using adolescent mice, even though MDPV generated an increase of the neurotrophin transcript, it did not cause any change in accumbal DR3 mRNA, after neither acute nor repeated exposure.

Based on changes observed in mPFC, we decided to investigate BDNF protein in the NAcc. BDNF is initially synthesized as its precursor (proBDNF) and subsequently cleaved into mature BDNF (mBDNF), which can be anterogradely transported to its target neurons [54]. In this sense, levels of both the pro (2 h post-administration) and the mature isoforms (2 h, 24 h and 10 days after administration) of this protein (proBDNF and mBDNF, respectively) were determined. Despite the early increment observed in BDNF

transcription induced by MDPV, neither MDPV nor cocaine treatment altered proBDNF protein levels in the NAcc.

We would like to emphasize once again the fact that alterations of G9a after repeated doses did not translate into any modification of the immature protein levels, so even though G9a is an important factor involved in cocaine-induced plasticity, it does not seem to exert a direct influence on the *de novo* synthesis of BDNF.

Regarding cocaine, no effect on BDNF mRNA transcription, proBDNF or mBDNF protein levels were evidenced at any time until 10 days of withdrawal. These findings agree with the lack of changes in BDNF expression observed during early cocaine withdrawal [55,56]. Indeed, changes are reported to appear after 3 weeks – 30 days of withdrawal. On the other hand, the effects of cocaine in BDNF protein synthesis generate controversy. Li and Wolf [15] summarized these effects in a long table in which only one of the 30 cites referred to a study performed in mice [57]. Zhang and co-workers exposed C57BL/6J mice to 20 mg/kg of cocaine for seven consecutive days. Under these conditions, BDNF expression was slightly induced in the NAcc and caudate putamen (CPu). The rest of the existing literature report the effects of cocaine on BDNF especially in rats, at doses higher than 15 mg/kg, and mainly after a long withdrawal. In contrast, the effects observed shortly after repeated cocaine doses are controversial when examining BDNF mRNA and protein levels [21,55]. Overall, although cocaine exposure generally leads to increases in BDNF levels in reward-related brain regions, exceptions have also been observed. So, the regional selectivity and timing of cocaine effects can vary widely depending on the experimental conditions [15].

In the same way, we demonstrated that repeated MDPV administrations induced temporal and regional-specific changes in BDNF expression, with a decoupling between transcriptional and translational processes. Conversely to the discrete up-regulation of mRNA levels in mPFC, mBDNF protein levels were reduced in the NAcc shortly after treatment, although they restored within 10 days of withdrawal. Fumagalli et al. [58] also described a decoupling between transcriptional and translational processes shortly after repeated cocaine exposure (five daily injections, 5 mg/kg), which differently modulates BDNF mRNA in PFC and protein levels in rat striatum. Concretely, cocaine increased BDNF mRNA levels 2 h after the last injection with no changes in mBDNF protein levels in the striatum. Moreover, it has been described that BDNF protein levels in the NAcc progressively increase after withdrawal from cocaine self-administration, but this gradual increase does not derive from local protein synthesis in NAcc neurons [21,56].

Considering these unexpected results, a new *in vivo* experiment was carried out in sought to determine if such decline in mBDNF levels induced by MDPV was related to locomotor sensitization. Therefore, a new sensitization experiment was carried out, but in this case, the animals were pretreated with 7,8-DHF, a BDNF receptor (TrkB) agonist. Daily administration of 7,8-DHF 30 min prior to the injection of MDPV during the induction period completely blocked the development of sensitization to MDPV. In addition, to prove that the effect of the flavone was exclusively mediated via TrkB activation, the same experiment was carried out in the presence of ANA-12, a potent and selective TrkB antagonist. ANA-12 prevented the effect of the flavone in such a way that the group ANA-12 + 7,8-DHF + MDPV developed locomotor sensitization after an MDPV challenge.

Taken together, these results suggest that the decrease in mBDNF levels, leading to decreased receptor stimulation, is involved in the sensitization developed to MDPV. In fact, the same effect of 7,8-DHF on the development of behavioural sensitization after repeated administration of methamphetamine (METH) was described by Ren et al. [24]. So, even though MDPV and methamphetamine have substantial differences regarding their mechanism of action, a decrease in BDNF protein levels has been found during early withdrawal of both substances [59]. Therefore, this suggests that METH and MDPV abusers may suffer from a severe dysfunction on the BDNF – TrkB signalling, which is importantly involved in the development of behavioural sensitization. In fact, mouse models with reduced BDNF expression exhibit a variety of alterations in the DA system [60], which indicates that BDNF has some direct influence on this system.

It is noteworthy that, regarding mBDNF levels, the differences between cocaine and MDPV observed in the *in vitro* determinations agree with the results of the *in vivo* experiments. So, the expression of cocaine-induced locomotor sensitization, for which no changes in mBDNF levels were observed, was not altered by the previous administration of the flavone. Therefore, other signalling pathways may be involved in the development of cocaine-induced sensitization despite the great similarity of its mechanism of action with that of MDPV.

Nevertheless, when assessing the rewarding properties of MDPV in the CPP paradigm, 7,8-DHF did not modify CPP acquisition, suggesting that the BDNF – TrkB modulation has a specific effect on the motor sensitization to the drug. In fact, behavioural sensitization and CPP have been proven to be modulated by different mechanisms. Associative memory develops correlation between drug-induced affective states and contextual

cues, triggering craving and some goal-directed behaviour toward drugs, like that seen in the CPP paradigm. However, the transition from recreational to pathological and compulsive drug-seeking may involve associative (place conditioning) and non-associative processes, such as sensitization, whereby the neural substrate mediating the response to the drug is directly augmented [61,62].

In summary, repeated administration of MDPV or cocaine cross-sensitizes to their locomotor effects. When using equivalent sensitization doses of both psychostimulants, only MDPV induces early changes in BDNF transcription, and this ability to activate the mPFC and increase *bdnf* gene expression is preserved after a repeated treatment. However, although MDPV increases cortical BDNF mRNA, the accumbal levels of the mature protein significantly decrease. Therefore, exposure to psychostimulants does not always affect BDNF mRNA and protein expression in the same direction, possibly due to the complex regulation of BDNF synthesis and transport. Alternatively, upregulation of BDNF mRNA levels might be a compensatory response to the primary loss of mBDNF protein. Our findings demonstrate that BDNF, but not DR3, plays a role in the development of MDPV-induced locomotor sensitization, which may influence susceptibility to drug abuse and drug relapse. Given the protective role of BDNF, we cannot rule out the possibility that neurons expressing lower levels of BDNF after repeated MDPV injections could be more vulnerable because of reduced trophic support [63].

3.2.6. REFERENCES

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CHAPTER 3

3.3. Characterization of the rewarding properties elicited by MDPV and its interaction with cocaine

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Progress in Neuro-Psychopharmacology & Biological Psychiatry 2020 [accepted for publication on January, 21st 2020]

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3.3.1. ABSTRACT

Recently, new psychoactive substances (NPS) have broken into the drug market, being initially traded as replacements to already controlled drugs such as cocaine or ecstasy. Currently, NPS use mainly occurs in a context of polydrug use. However, the substitution of an established illicit drug with an NPS is still a pattern of use reported among high-risk drug users. For instance, 3,4-methylenedioxypyrovalerone (MDPV), a synthetic cathinone generally considered a cocaine-like psychostimulant as it shares a similar pharmacological profile, is being used as an alternative to cocaine especially among adolescents and young adults.

We have recently reported a cross-sensitization between both drugs. In this sense, the aim of the present work was to further study the relationship between cocaine and MDPV but in the reinstatement of the conditioned place preference (CPP) paradigm. Accordingly, four experimental groups of male OF1 mice were designed: MDPV-MDPV, Cocaine-Cocaine, Cocaine-MDPV and MDPV-Cocaine. The first drug refers to the substance with which the animals were conditioned (MDPV 2 mg/kg or cocaine 10 mg/kg, i.p.) and the second, to the substance with which the preference was reinstated. In parallel, the expression of several factors related to the rewarding properties of drugs was determined in ventral striatum: G9a, ΔFosB, CB1, CDK5, Arc and c-Fos.

MDPV induced CPP at doses ranging from 1 to 4 mg/kg. Although 2 mg/kg MDPV produced a psychostimulant effect stronger than that of 10 mg/kg cocaine, both doses were equivalent in their rewarding properties. Nevertheless, MDPV-conditioned animals needed significantly more time to extinguish the preference. Importantly, our results showed that cocaine

and MDPV are able not only to reinstate the CPP induced by themselves but also that induced by the other drug (cross-reinstatement). Therefore, they restored drug-seeking behaviour with respect to each other although relapse into drug-taking was always higher with the conditioning drug.

Hence, some differences underlying their mechanism of action could be noted. For instance, MDPV-treated mice showed increased ΔFosB levels which correlated with its longer extinction time compared to cocaine, suggesting the activation of neuroplasticity mechanisms during at least 12 days after drug withdrawal. In addition, the neuroplasticity mechanisms that both drugs activate may notably differ since MDPV-conditioned mice were more responsive to a new cocaine exposure, implying a high vulnerability to cocaine abuse.

3.3.2. INTRODUCTION

During the last decade, synthetic cathinones such as mephedrone and 3,4-methylenedioxypyrovalerone (MDPV) have been gaining prominence among young people due to their euphoric effects, affordable price and availability (EMCDDA, 2018). Between 2005 and 2017, 130 synthetic cathinones have been notified to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) for the first time, 12 of which were detected in 2017, making them the second largest group of new substances currently under monitoring. New psychoactive substances (NPS) use mainly occurs in a context of polydrug use of illicit opioids, amphetamines, alcohol and medicinal products (Grund et al., 2016). Thus, NPS are rarely reported to be the primary drug used by high-risk drug-users, but more often they are a secondary or tertiary drug; for example, when the preferred substance is

not available or to heighten the effect of other drugs. In fact, the substitution of an established illicit drug with an NPS is a pattern of use driven by lack of availability, low purity and high prices of classically used drugs. In this sense, new synthetic stimulants are purported to be alternatives to controlled substances such as cocaine, amphetamine, and ecstasy (Volkow, 2013). For instance, the use of mephedrone as a replacement for cocaine is reported among intravenous opiate users (Sande, 2015).

Among the new synthetic cathinones, popularly known as "bath salts", MDPV is generally considered a cocaine-like psychostimulant, as it shares a similar pharmacological profile. They both exert their action mainly by blocking the transporters of dopamine and noradrenaline (Gregg et al., 2014), thus, inducing similar physiological and locomotor responses. However, some differences regarding their pharmacology and pharmacokinetics must be highlighted. *In vitro* studies indicate that MDPV, in contrast to cocaine, is a pure catecholamine-selective transporter blocker with little serotonin transporter activity (Luethi et al., 2018; Simmler et al., 2013). Moreover, it is well known that the effects of MDPV can be between 10 and 50 times more potent than those of cocaine (Baumann et al., 2013; López- Arnau et al., 2017; Simmler et al., 2013). In fact, a positive correlation between the dopamine and noradrenaline transporter inhibition potency of stimulants and the psychoactive doses and effects in humans has been described (Luethi and Liechti, 2018). Furthermore, in rodents, MDPV shows a considerably higher elimination half-life, ranging from one to six hours (depending on the experimental conditions), whereas that of cocaine does not reach an hour (Anizan et al., 2016; Baumann et al., 2017; Carmona et al., 2005; Corkery et al., 2018; Lau et al., 1991; Meyer et al., 1990; Novellas et al., 2015; Pan and Hedaya, 1998, 1999; Peters et al., 2016). Also, it is well known that cocaine metabolizes to multiple metabolites, some of which are pharmacologically

active, i.e. norcocaine (Carmona et al., 2015; Lau, 1992; Ma et al., 1997; Meyer et al., 1990; Pan and Hedaya, 1998; Zheng et al., 2019). By contrast, although MDPV also metabolizes (Baumann et al., 2017; Horsley et al., 2018; Kim et al., 2016; Novellas et al., 2015), and their metabolites have recently been shown to interact with monoamine transporters *in vitro* (Luethi et al., 2019), motor activation produced by the drug only correlates with the parent compound, but not with metabolite concentrations (Anizan et al., 2016; Baumann et al., 2017). Accordingly, these authors suggested that most probably MDPV metabolites do not contribute to the *in vivo* effects of systemically administered MDPV, especially since they exist as conjugated forms which may not cross the blood-brain barrier.

The novelty, diversity and rapid development of synthetic cathinones create several barriers against a better understanding of the neurobiological and behavioural profile of MDPV. Fortunately, in recent years there has been a growing number of preclinical studies that have reported the behavioural effects and addictive properties of this drug (Duart-Castells et al., 2019a; Gannon et al., 2017; Hicks et al., 2018). For example, it has been described that MDPV induces an anxiety-like behaviour and enhances aggressiveness in mice (Duart-Castells et al., 2019a), increases locomotor activity (Fantegrossi et al., 2013; Gatch et al., 2013) and can induce locomotor sensitization (Buenrostro-Jáuregui et al., 2016; López-Arnau et al., 2017). In addition, MDPV induces deficits in the novel object recognition test when administered in a binge pattern (Sewalia et al., 2018). Regarding its abuse liability, MDPV is orally and intravenously self-administered (Aarde et al., 2013; Gannon et al., 2017) and lowers intracranial self-stimulation thresholds (Watterson et al., 2014). Overall, its rewarding and reinforcing properties have been well demonstrated (Karlsson et al., 2014; King et al., 2014; Watterson et al., 2014).

Currently, despite the emergence of NPS in the illegal drug market, cocaine is still one of the most abused psychostimulants worldwide, especially among adults (EMCDDA, 2019; World Drug Report, 2019). However, MDPV is being used as an alternative to cocaine among adolescents and young adults because of its cocaine-like psychostimulant properties, more affordable price and greater accessibility. Nevertheless, despite the obvious risks associated with the use of new cathinones, little is known about its consequences.

In this sense, the long-term effects of MDPV on cue-induced rewarding effects and its interaction with other drugs of abuse like cocaine are largely unclear. Given that cocaine and MDPV have similar pharmacological profiles and taking into account the high prevalence of polydrug consumption, it is necessary to develop studies of drug-drug interactions to determine if there are additive or interacting effects that could modify the potential addictive effects of these drugs.

We have recently reported that exposure to MDPV during adolescence enhances the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood. Furthermore, the expression of several factors related to the effects of cocaine are influenced by MDPV treatment, which in turn, have been associated with altered behavioural responses to the drug (Duart-Castells et al., 2019a; López-Arnau et al., 2017). ΔFosB, among other biomarkers (i.e. G9a dimethyltransferase, c-Fos, the cyclin-dependent kinase-5 (CDK5)), has been attributed a crucial role. ΔFosB is a member of the Fos family of transcription factors, which promotes reward and motivation and serves as a key mechanism of drug sensitization and increased drug self-administration (Nestler, 2013). ΔFosB may accumulate in response to many types of compulsive behaviours and persists in neurons for relatively long periods

of time. In this way, ΔFosB represents a molecular mechanism that could trigger and then maintain alterations in gene expression that persist long after drug withdrawal (Nestler, 2001).

Additionally, cross-locomotor sensitization between MDPV and cocaine has been described (Duart-Castells et al., 2019b). This is of chief importance, since sensitization-like phenomenon has been frequently proposed to account for relapses in drug consumption, which naturally constitutes a major clinical problem. Environmental stimuli, stress or contact with the drug are the main triggers of relapse into drug-seeking (Blanco-Gandía et al., 2018).

The conditioned place preference (CPP) paradigm evaluates the conditioned rewarding effects of drugs of abuse, pairing a primary reinforcer with a contextual stimulus, which acquires secondary appetitive properties (Tzschentke, 2007). CPP is also a very suitable model to study reinstatement of drug-seeking, as an extinguished CPP can be reinstated by non-contingent administration of a priming dose of the drug (Aguilar et al., 2009). In this sense, MDPV has already been described to induce CPP at a wide range of doses (Gregg et al., 2016; Karlsson et al., 2014; King et al., 2015; Oliver et al., 2018), and it has also been shown to reinstate CPP after extinction of the preference (Hicks et al., 2018).

As a whole, the aim of the present work was to study the relationship between cocaine and MDPV in the reinstatement of the CPP paradigm after extinction. Additionally, the locomotor sensitizing effects of both psychostimulants were assessed. In parallel, we sought to determine potential changes in the expression of different factors involved in plasticity and epigenetic regulation that may be altered during the development of

addiction. Specifically, we studied the histone-lysine N-methyltransferase G9a, ΔFosB, the cannabinoid receptor 1 (CB1), the cyclin-dependent kinase-5 (CDK5), the activity-regulated cytoskeleton-associated protein (Arc) and c-Fos.

3.3.3. MATERIALS AND METHODS

Animals

A total of 237 male mice of the Oncins France 1 (OF1) strain were acquired commercially from Charles River (Barcelona, Spain). 147 were employed for behavioural studies and 90 for biochemical studies. OF1 mouse strain is an albino outbred strain commonly used in toxicology and pharmacology. Animals were 21 days old on arrival at the laboratory and were all housed in groups of 4/5, under standard conditions (cage size 28x28x14.5 cm), for 8 days prior to initiating the experimental schedule, at a constant temperature (21 ± 2°C), with a reverse light cycle (white lights on 19:30 - 7:30h). Food (standard diet) and water were available *ad libitum* in all the experiments (except during the behavioural tests). Mice were manipulated at the same time on each test day to minimize inter-day variability. All procedures involving mice and their care complied with the ARRIVE guidelines and national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes. The Animal Use and Care Committees of the University of Valencia and the University of Barcelona approved the present study. Animals were randomly assigned to an experimental group.

During the behavioural manipulations, researchers were not aware of the treatment that each animal had received.

Drugs and reagents

MDPV was synthesized and characterized in racemic form as HCl salt as described previously (Novellas et al., 2015). Cocaine hydrochloride was provided by the Spanish National Institute of Toxicology. Both MDPV and cocaine solutions for injection were prepared with saline (0.9% NaCl, pH=7.4) immediately before administration and administered intraperitonially.

The protease and phosphatase inhibitor cocktail was purchased from Abcam (Cambridge, UK), TRIsure™ from Bioline (Meridian Bioscience Inc., UK) and the organic solvents (ethanol, chloroform and isopropanol) from Scharlab (Barcelona, Spain). The rest of the reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental Design

An overall and more detailed description of the sets of animals and the experimental procedure related to the behavioural and biochemical studies is provided in Table S2 (supplementary material).

In experiment 1 (MDPV-induced reinstatement of the CPP), mice were conditioned with 1, 2 or 4 mg/kg MDPV and, thereafter, subjected to extinction sessions. Once the preference had been extinguished, reinstatement with priming doses of 50% of the previous dose was induced. This procedure was repeated with progressively lower doses until the preference could not be further reinstated.

In experiment 2 (Cross-reinstatement between MPDV and cocaine) four groups were employed: MDPV-MDPV, MDPV-Cocaine, Cocaine-Cocaine and Cocaine-MDPV. In the names of the groups the first drug refers to the substance with which they were first conditioned and the second to the substance with which they performed the reinstatement test. The conditioning doses were 10 mg/kg cocaine or 2 mg/kg MDPV. Animals underwent the CPP procedure and were subsequently exposed to extinction sessions. Once the conditioned preference had been extinguished, reinstatement was induced with priming doses of 50% of the previous dose. After the second extinction, this procedure was repeated with progressively lower doses until preference could not be reinstated in the MDPV-MDPV and Cocaine-Cocaine groups. However, in the MDPV-Cocaine and Cocaine-MDPV groups, after the second extinction, animals received a priming injection of the corresponding drug at the same dose used during the conditioning phase (10 mg/kg cocaine or 2 mg/kg MDPV).

In experiment 3 (Locomotor activity), a further set of animals was employed to assess locomotor activity. The treatment was the same as in experiment 2 (10 mg/kg or 2 mg/kg MDPV, once daily, for 4 days), but after 12 days of withdrawal, a challenge of MDPV 2 mg/kg or cocaine 10 mg/kg was given to mice and their locomotor activity was measured for 1 h.

Finally, in experiment 4 (Biochemical analysis) a different set of animals was exposed to the same CPP protocol, following the same procedure as in experiment 2 but with different timings of brain extraction. 24 h and 12 days after the CPP test to MDPV (2 mg/kg) or cocaine (10 mg/kg) five different factors were determined: G9a, ΔFosB, CB1 receptor, CDK5 and Arc. Because of the larger number of sessions required to extinguish MDPV than cocaine

conditioning after the CPP test, reinstatement was carried out at different times after the paradigm. To overcome this limitation and be able to evaluate how animals responded to a single dose of each drug after being conditioned to cocaine or MDPV, a priming-dose of MDPV 2 mg/kg, cocaine 10 mg/kg or saline 5 ml/kg was administered to the animals 12 days after the CPP test, thus simulating an early reinstatement-phase in animals in which preference had not yet been extinguished. 2 h after the priming injection, mRNA levels encoding G9a or immediate-early genes such as Arc and c-Fos were determined. In this way, three different conditions were employed in this experiment, subdividing the animals according to the different timings of brain extraction: 24 h or 12 days after the post-conditioning test, and the rest, 2 h after the simulated-reinstatement session, which also took place 12 days later.

Conditioning place preference (CPP) test

For Place Conditioning we employed sixteen identical Plexiglas boxes with two equal sized compartments (30.7 cm length × 31.5 cm width × 34.5 cm height) separated by a grey central area (13.8 cm length × 31.5 cm width × 34.5 cm height). The compartments have different coloured walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

Acquisition of CPP

The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, consisted of three phases and was performed as described previously (Maldonado et al., 2006; Blanco-Gandía et al., 2018). To summarize the main aspects, in the first phase, known as pre-conditioning (Pre-C), 42 PND adult mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day for 3 days. On day 3, the time spent in each compartment over a 900-s period was recorded, and animals showing a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for any compartment were excluded from the rest of the experiment. Half the animals in each group received the drug or vehicle in one compartment, and the other half in the other compartment. After assigning the compartments, no significant differences were detected between the time spent in the drug-paired and vehicle-paired compartments during the pre-conditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 h, they received an injection of cocaine or MDPV immediately before being confined to the drug-paired compartment for 30 min. Confinement was carried out in both cases by closing the guillotine door that separated the two compartments, making the central area inaccessible. During the third phase, known as post-conditioning (Post-C), the guillotine door separating the two compartments was removed (day 8) and the time spent by the untreated mice in each compartment during a 900-s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug (CPP score). If this difference is positive, then the drug has induced a

preference for the drug-paired compartment, while the opposite indicates that an aversion has been developed.

Extinction of CPP

All groups in which a preference for the drug-paired compartment was established underwent two weekly extinction sessions that consisted on placing the animals in the apparatus (without the guillotine doors separating the compartments) for 15 min. The extinction condition was fulfilled when there was a lack of significant differences between the time spent in the drug-paired compartment during the extinction session and Pre-C test values in two consecutive sessions.

Reinstatement of CPP

24 h after extinction had been confirmed, the effects of a priming dose of cocaine or MDPV were evaluated (Blanco-Gandía et al., 2018). Reinstatement tests were the same as those carried out in Post-C (free ambulation for 15 min), except that animals were tested 15 min after the administration of the respective dose of cocaine or MDPV. When reinstatement of the preference was achieved, a subsequent extinction process was carried out until the CPP was completely extinguished. Afterwards, a new reinstatement test was conducted with progressively lower doses of the drug. This procedure of extinction-reinstatement was repeated until a priming dose was confirmed to be ineffective. Priming injections were administered in the vivarium, which constituted a non-contingent place to that of the previous conditioning procedure.

Locomotor response

Locomotor response to 10 mg/kg cocaine or 2 mg/kg MDPV was assessed by evaluating the movements of mice inside the cage by means of photocell beam breaks. For this experiment, a similar schedule of drug administration to that in the CPP procedure was followed. Animals were injected with saline, 10 mg/kg of cocaine or 2 mg/kg of MDPV once daily for four days. 12 days after the last administration, a single dose of 10 mg/kg of cocaine or 2 mg/kg of MDPV was administered and motor activity was measured. The animal was placed in the box for 30 min to become habituated and was subsequently injected with the corresponding drug. Locomotor activity was recorded for 1 h and automatically measured with an actimeter (Cibertec S.A., Madrid, Spain) consisting of eight cages (33×15×13 cm), each with eight infrared lights located in a frame around the cage. In this apparatus, beams are positioned on the horizontal axis 2 cm apart, at a height just above the bottom of the cage (body level of mice). The different frames are placed 4 cm apart, and, since they are opaque, prevent animals from seeing each other while allowing them to hear and smell conspecifics being tested at the same time.

Tissue samples preparation

For the biochemical determinations, mice from experiment 4 were sacrificed by cervical dislocation 24 h and 12 days after the MDPV (2 mg/kg) or cocaine (10 mg/kg) -induced-CPP test, or 2 h after the priming-dose (saline 5 ml/kg; MDPV 2 mg/kg or cocaine 10 mg/kg), also given 12 days later. The ventral striatum containing the nucleus accumbens was quickly dissected and stored at -80ºC until use.

Tissue samples for Western blot analysis were processed as described (Pubill et al., 2013) with minor modifications. In brief, tissue samples were homogenized at 4ºC in 20 volumes of lysis buffer (20 mM Tris-HCl, pH=8, 1% NP40, 137 mM NaCl, 2 mM EDTA) with the protease inhibitor cocktail. The homogenates were shaken and rolled for 2 h at 4ºC and centrifuged at 15 000 xg for 30 min at 4ºC. Aliquots of resulting supernatants (total lysate) were collected and stored at -80ºC until use. Protein content was determined using the Bio-Rad Protein Reagent (Bio Rad, Inc., Spain).

Western blotting and Immunodetection

A general Western blotting and immunodetection protocol was used. In brief, equal amounts of protein (10 μg) for each sample were mixed with sample buffer (0.5M 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 10% acrylamide gels. Proteins were electrophoresed and subsequently transferred to fluoride (PVDF) sheets (Immobilon-P; Millipore, USA). PVDF membranes were blocked for 1 h at room temperature with 5% bovine serum albumin in Tris-buffer plus 0.05% Tween-20, and incubated overnight at 4ºC with CB1 (1:1000, Frontiers Institute, South Africa), ΔFosB (1:1000; Abcam, Cambridge, UK), Arc (1:1000; Santa Cruz Biotechnology, CA, USA) or CDK5 (1:1000; Santa Cruz Biotechnology, CA, USA) primary antibodies. After washing, membranes were incubated for 1 h at room temperature with their respective secondary peroxidase-conjugated anti-IgG antibody: donkey anti-rabbit (1:5000, GE Healthcare, USA) or sheep anti-mouse (1:2500 GE Healthcare, USA). Immunoreactive protein was visualized using a chemoluminiscence-based detection kit following the manufacturer's protocol (Immobilion Western,

Millipore) and a BioRadChemiDoc XRS gel documentation system (BioRad, Inc., Madrid, Spain). Scanned blots were analysed using a BioRad Image Software and dot densities were expressed as a percentage of those taken from the control. As a control for loading, GAPDH (1:5000; Merck Millipore, USA) antibody was used.

Total RNA extraction and gene expression determination

Total RNA isolation from ventral striatum was carried out by means of a TRIsure™ reagent- Chloroform based extracted protocol. RNA content in the samples was measured at 260 nm, and sample purity was determined by the A260/280 ratio in a NanoDrop™ ND-1000 (Thermo-Fisher Scientific).

The isolated mRNA was reverse-transcribed by a Reverse Transcription Polymerase Chain Reaction (RT-PCR) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the Veriti® thermal cycler (Applied Biosystems, Foster, CA, USA). In short, complementary DNA (cDNA) was synthesized in a total volume of 20 μl by mixing 1 μg of total RNA and the appropriate volumes of each kit reagent.

Quantitative real-time polymerase chain reaction (qPCR) was performed using the StepOnePlus™ Real-Time PCR System and the Taqman one-step PCR Master Mix (Applied Biosystems, Foster, CA, USA). 25 ng of total cDNA product was added per 20- μl reaction mixture containing sequence-specific primers and Taqman probes from Applied Biosystems Mm00479619_g1 for Arc, Mm00487425_m1 for c-Fos, Mm01132261_m1 for G9a and Mm00607939_s1 for β-actin, as an endogenous control. All reactions were performed in triplicate. Fold-changes in gene expression

were calculated using the $2^{-\Delta\Delta Ct}$ method (comparative Ct method) for each experimental sample (Livak and Schmittgen, 2001). All primer-probe combinations were optimized and validated for relative quantification of gene expression. All experiments included a no-template control, and real-time PCR reactions also included a no-reverse transcriptase (no-RT) control for which no amplification products were seen, indicating that cDNA was amplified rather than residual genomic DNA.

Data acquisition and statistical analysis

For CPP data, the time spent in the drug-paired compartment was analysed by means of a mixed analysis of variance (ANOVA) with one between variable – Group, with 3 or 4 levels (MDPV 1 mg/kg, MDPV 2 mg/kg, MDPV 4 mg/kg or MDPV-MDPV, MDPV-Cocaine, Cocaine-Cocaine, Cocaine-MDPV) – and a within variable – Days, with 2 levels (Pre-C, and Post-C). Data related to extinction and reinstatement values in the groups showing CPP were analysed by means of Student's t-tests. The time required for the preference to be extinguished in each animal was analysed by means of the Kaplan–Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. Although the mean of the group as a whole determined the day on which extinction was considered to have been achieved, preference was considered to be extinguished when a mouse spent 380 s or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the Pre-C tests performed in the study (mean=370 s). When the preference was not extinguished in an animal, it was assigned the number of days required for extinction for the group as a whole. Subsequent Bonferroni post – hoc tests were calculated when required. Analyses were performed using SPSS v24.

Data of motor activity were analysed with a two-way ANOVA with two between variables – Treatment, with 3 levels (Saline, Cocaine or MDPV); and Challenge, with 2 levels (Cocaine or MDPV).

Biochemical data were analysed using one-way ANOVA. Data from qPCR analysis were expressed as fold-change variations. Data from Western Blot analysis were expressed as mean ± SEM and were normalized with 100% defined as the mean of the replicates in the control group. The α error probability was set at 0.05. Significant differences ($P < 0.05$) were analysed using the Bonferroni post hoc test for multiple comparisons measures only if F achieved the necessary level of statistical significance and no significant variance in homogeneity was observed. Statistic calculations were performed using GraphPad Prism 6.0 software.

3.3.4. RESULTS

Experiment 1: MDPV – induced reinstatement of the CPP

Results of the MDPV-induced CPP in animals receiving the three different doses of MDPV are presented in Figure 26. The ANOVA for the time spent in the drug-paired compartment revealed an effect of the variable Day ($F_{1,39}=83.749$, $P < 0.001$; MDPV 1 mg/kg n=15/group, MDPV 2 mg/kg n=14/group and MDPV 4 mg/kg n=13/group). All groups developed place preference for the drug-paired compartment, regardless the dose of MDPV ($P < 0.001$ in all cases). The Kaplan-Meier analysis revealed no significant differences in the time required to extinguish the preference between different doses (data not shown). MDPV 1 mg/kg-treated mice required 11

sessions for the preference to be extinguished. A priming dose of 0.5 mg/kg MDPV reinstated the preference (Student's t-test, t=-2.475, 14 d.f.; $P < 0.05$), and, after 4 extinction sessions, reinstatement was not achieved with a priming dose of 0.25 mg/kg MDPV. MDPV 2 mg/kg-treated mice required 15 sessions to extinguish the preference and exhibited reinstatement with a priming dose of 1 mg/kg. After 6 extinction sessions, reinstatement of the preference with a dose of 0.5 mg/kg MDPV was obtained (Student's t-test, t=-2.834, 14 d.f.; $P < 0.01$ and t=-3.828, 14 d.f.; $P < 0.01$, respectively). After one more extinction session, no reinstatement was induced with 0.25 mg/kg MDPV. Finally, the MDPV 4 mg/kg group required 10 sessions to extinguish the preference, which was reinstated with the priming doses of 2 mg/kg ($P < 0.001$), 1 mg/kg ($P < 0.05$; after 2 extinction sessions) and 0.5 mg/kg MDPV ($P < 0.05$; after 2 extinction sessions). Again, after one extinction session, preference was not reinstated with 0.25 mg/kg.

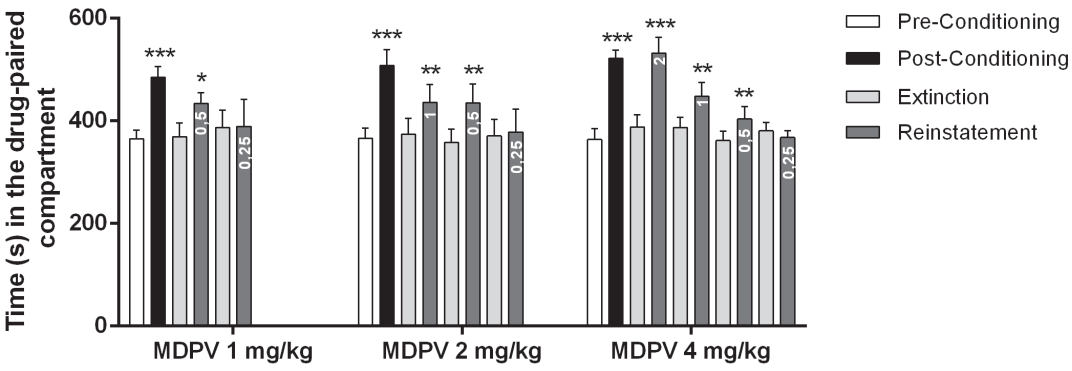

Figure 26. Effects of 1, 2 and 4 mg/kg MDPV on the conditioned place preference (CPP) test. *Bars represent the mean ± SEM time in seconds spent in the drug-paired compartment during the pre-conditioning (white), post-conditioning (black), the last extinction session (light grey) and reinstatement (dark grey). Numbers inside the bars represent the dose of MDPV used*

*in each reinstatement test. *P < 0.05; **P < 0.01; ***P < 0.001 significant difference vs pre-conditioning or the last extinction session.*

Experiment 2: Cross-reinstatement between MDPV and cocaine

The results regarding the effects of cocaine or MDPV-induced CPP are depicted in Figure 27. The ANOVA revealed a significant effect of the variable Day ($F_{1,53}$=53.082, P < 0.001; MDPV-MDPV, cocaine-cocaine and cocaine-MDPV groups, n=14/group, MDPV-cocaine n=15/group), as all the groups spent more time in the drug-paired compartment in the Post-C test than in the Pre-C test (P < 0.001). The Kaplan-Meier analysis revealed that MDPV-conditioned groups needed significantly more sessions (10.9 for MDPV-MDPV and 12.4 for MDPV-cocaine) for preference to be extinguished than those conditioned with cocaine (6.2 for cocaine-cocaine and 5.8 for cocaine-MDPV) (P's< 0.05 for MDPV-MDPV and P's< 0.01 for MDPV-cocaine) (Figure 28).

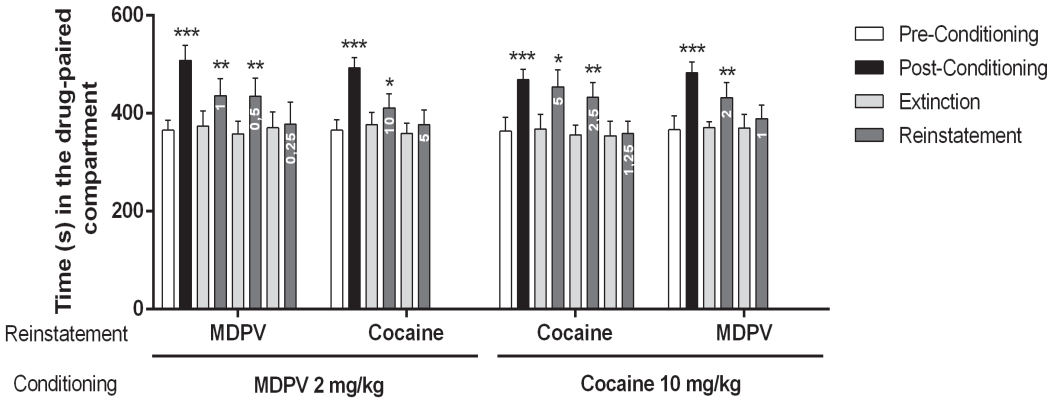

Figure 27. Cross-reinstatement between MDPV and cocaine. *Bars represent the mean ± SEM time in seconds spent in the drug-paired compartment during the pre-conditioning (white),*

*post-conditioning (black), the last extinction session (light grey) and reinstatement (dark grey). Numbers inside the bars represent the dose of MDPV or cocaine used in each reinstatement test. *P < 0.05; **P < 0.01; ***P < 0.001 significant difference vs pre-conditioning or the last extinction session.*

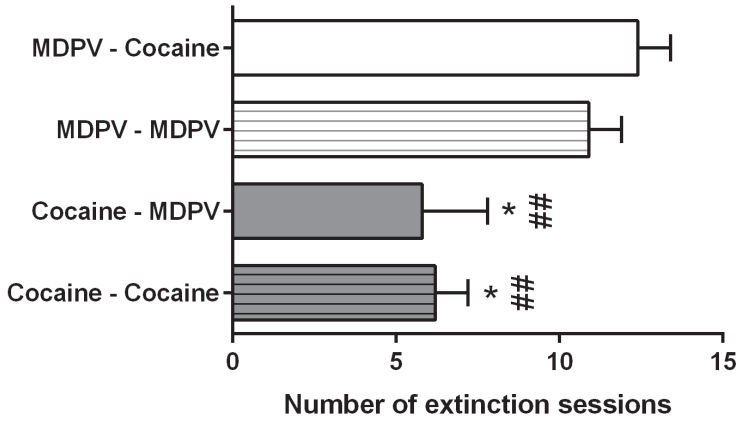

Figure 28. Number of extinction sessions required to extinct mice after the CPP test. *Bars represent the mean ± SEM number of extinction sessions needed to extinguish the preference for the drug-paired compartment after the CPP test induced by MDPV 2 mg/kg and cocaine 10 mg/kg. The first drug named refers to the one used for the conditioning, the second to the one used for the subsequent reinstatement. *P < 0.05 significant differences vs MDPV-MDPV; ##P < 0.01 significant differences vs MDPV – cocaine.*

MDPV-MDPV mice exhibited reinstatement with priming doses of 1 mg/kg (after 15 extinction sessions) and 0.5 mg/kg MDPV (after 6 extinction sessions) (Student's t-test, t=-2.834, 14 d.f.; P < 0.01 and t=-3.828, 14 d.f.; P < 0.01, respectively). Preference was reinstated in cocaine-cocaine mice with 5 mg/kg and 2.5 mg/kg cocaine (after 10 and 1 extinction sessions, respectively) (Student's t-test, t=-2.676, 14 d.f.; P < 0.05 and t=-3.288, 14 d.f.; P < 0.01 respectively). No further reinstatements were achieved.

Regarding cross-reinstatement between MDPV and cocaine, preference was not reinstated with 50% of the other drug in neither MDPV-cocaine nor cocaine-MDPV mice. However, when the complete dose was administered (either 10 mg/kg cocaine for MDPV-cocaine mice or 2 mg/kg MDPV for cocaine-MDPV mice), both groups showed reinstatement of the preference for the drug-paired compartment (Student's t-test, t=-3.135, 14 d.f.; $P < 0.01$ for cocaine-MDPV mice and t=-2.237, 14 d.f.; $P < 0.05$ for MDPV-cocaine mice).

Experiment 3: MDPV-induced cross-sensitization to cocaine motor effects

The ANOVA revealed an effect of the Challenge ($F_{1,42}=21.53$, $P < 0.001$) and the interaction Treatment x Challenge ($F_{2,42}=4.226$, $P < 0.05$) (n=8/group). As shown in Figure 29, the 10 mg/kg dose of cocaine was less effective in increasing horizontal locomotor activity (HLA) than 2 mg/kg MDPV, so in animals treated with saline, a challenge with MDPV induced a higher motor activity than that with cocaine ($P < 0.001$). In addition, the challenge of 10 mg/kg of cocaine induced a higher motor activity in animals previously treated with cocaine ($P < 0.05$) or MDPV ($P < 0.01$), when compared with the saline group. However, there were no differences among the treatment groups challenged with MDPV.

Experiment 4: G9a, ΔFosB, CB1 receptor, CDK5 and Arc expression 24 h after the CPP test

When the expression of the regulator G9a histone dimethyltransferase was determined, no changes were evident in its mRNA levels 24 h after the CPP test, which was 48 h after the last drug injection ($F_{2,15}=1.042$, $P > 0.05$; n=6/group; saline: 1 ± 0.015, MDPV: 1.053 ± 0.049 and cocaine: 0.990 ± 0.027).

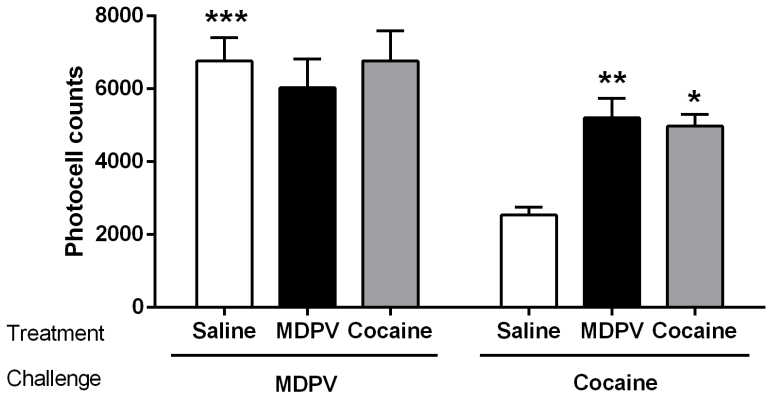

Figure 29. Locomotor sensitization to MDPV and cocaine. *Bars represent mean ± SEM of the photocell counts registered during 60 min after a MDPV (2 mg/kg) or cocaine (10 mg/kg) challenge given 12 days after treatment. Animals followed the same drug administration protocol as for the CPP experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 significant differences vs saline-cocaine group.*

Nevertheless, at the same time, ΔFosB, a protein thought to be regulated by G9a histone methyltransferase activity, was significantly increased 24 h after the MDPV-induced CPP test ($F_{2,12}$=8.8450, P < 0.01; n=5/group) with respect to the saline (P < 0.01) and cocaine groups (P < 0.05) (Figure 30A). Moreover, as this protein is a lasting factor over time, we determined its expression 12 days after CPP, at the same time as the other animals received the priming doses simulating the reinstatement. As expected, the ΔFosB increment was sustained in MDPV-conditioned mice ($F_{2,12}$=5.055, P < 0.05; n=5/group) (Figure 30B).

However, despite the major ΔFosB expression observed in MDPV-treated mice, the expression of one of its target genes, CDK5 ($F_{2,12}$=0.3948, P > 0.05; n=5/group) (Figure 31A), as well as that of the CB1

receptor ($F_{2,12}$=0.1621, P > 0.05; n=5/group) (Figure 31B), was not altered by either MDPV- or the cocaine-induced CPP 24 h after the Post-C.

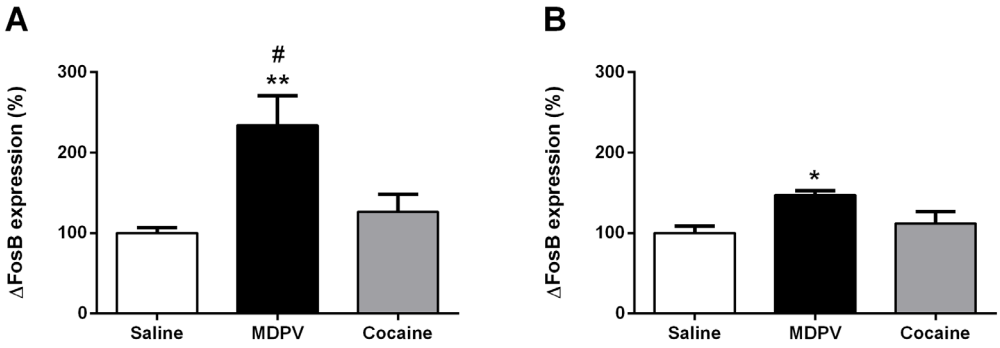

Figure 30. ΔFosB protein expression in ventral striatum. *Bars represent the mean ± SEM protein expression 24 h (A) or 12 days (B) after the MDPV or cocaine-induced CPP. *P < 0.05, **P < 0.01 significant differences vs saline, #P < 0.05 vs cocaine.*

We also assessed the levels of Arc protein, which is coded by *arc*, an immediate-early gene involved in plasticity. One-way ANOVA ($F_{2,12}$= 2.272, P > 0.05; n=5/group) revealed that Arc levels in the ventral striatum seemed decreased in both MDPV- and cocaine-treated mice, even though statistical significance was not reached (Figure 31C).

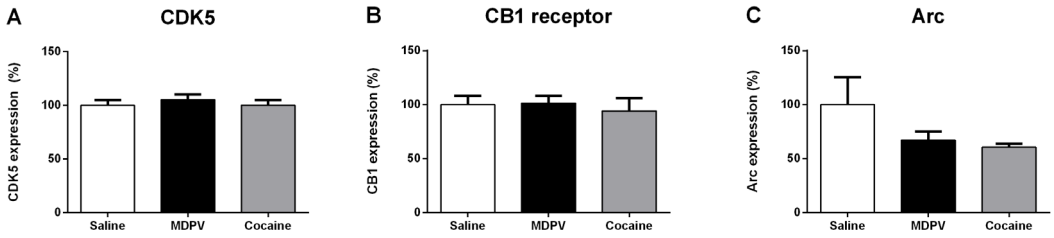

Figure 31. CDK5 (A), CB1 receptor (B) and Arc (C) protein expression in ventral striatum. *Bars represent the mean ± SEM protein expression 24 h after MDPV or cocaine-induced CPP.*

G9a, Arc, and c-Fos gene expression 2 h after a priming dose

As mentioned above, due to the higher number of sessions required to extinguish the conditioning preference induced by MDPV versus that induced by cocaine, the reinstatement tests were carried out at different times after the CPP test. To overcome this limitation and to be able to evaluate how animals responded to a single dose of each drug after being conditioned with cocaine or MDPV, a priming dose of MDPV 2 mg/kg, cocaine 10 mg/kg or saline 5 ml/kg was given 12 days after the CPP test, simulating an early reinstatement-phase before extinction. 2 h after the priming injection, the mRNA levels encoding G9a or immediate-early genes such as Arc and c-Fos were determined.

One-way ANOVA to analyse G9a mRNA expression (Figure 32A and 32B) revealed an effect of the priming drug after conditioning with MDPV ($F_{2,15}$=14.90, $P < 0.001$; n=6/group) or cocaine ($F_{2,15}$=16.81, $P < 0.001$; n=6/group). Specifically, the acute injection of cocaine caused a decrease of G9a expression in both MDPV- ($P < 0.001$) and cocaine-conditioned mice ($P < 0.05$). However, when a single dose of MDPV was administered, only the animals previously conditioned to cocaine presented an increase of G9a expression ($P < 0.05$), while no modification of its expression was observed in the mice that had previously received the same drug (MDPV).

Regarding Arc mRNA expression after the cocaine priming-dose, a different pattern of expression was observed depending on the conditioning drug. One-way ANOVA ($F_{2,15}$=8.816, $P < 0.01$; n=6/group) revealed a significant effect of cocaine only when animals were conditioned with the same drug, in such a way that cocaine decreased Arc expression compared to saline ($P < 0.05$) and MDPV ($P < 0.01$). Surprisingly, when assessing

Arc mRNA expression after a priming dose of MDPV, no changes in its transcription were observed after any conditioning treatment (Figure 32C and 32D).

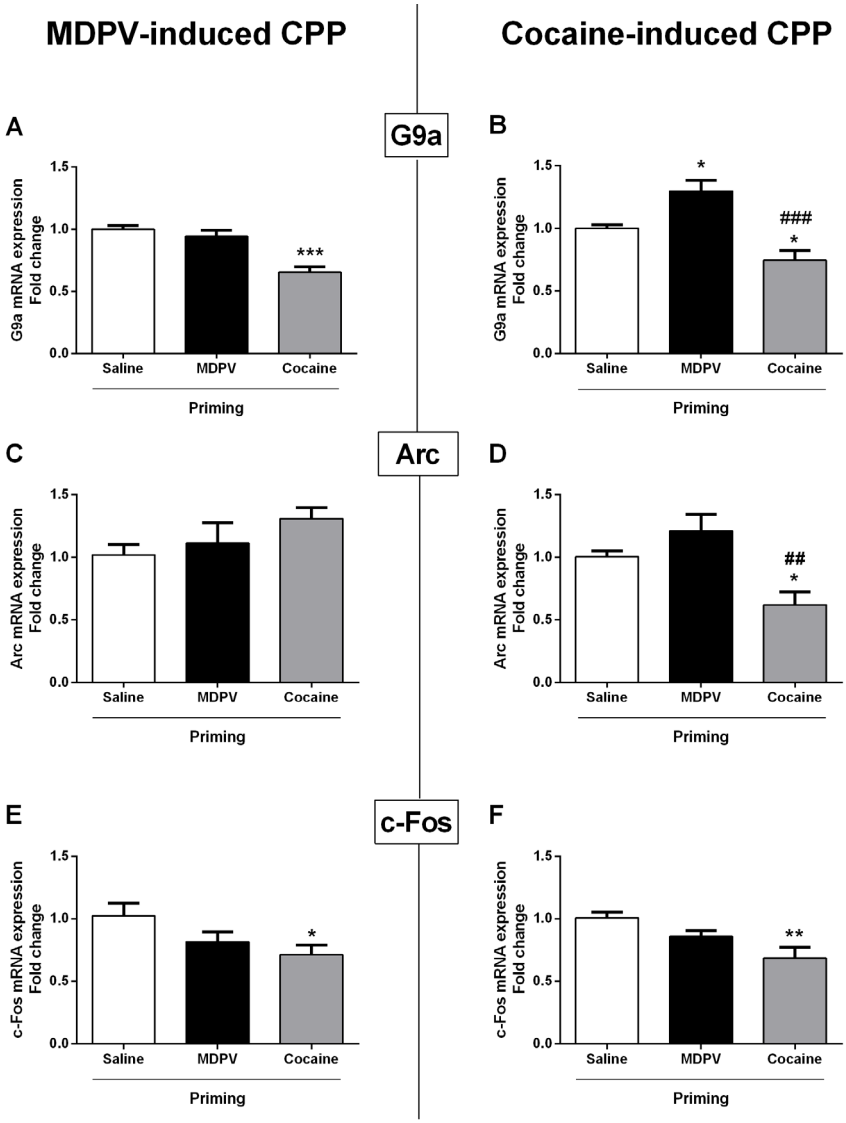

Figure 32. G9a (A, B), Arc (C, D), and c-Fos (E, F) gene expression in ventral striatum. Bars represent the fold change ± SEM in mRNA expression 2 h after the priming-dose (saline,

*MDPV or cocaine) given 12 days after the MDPV (A, C, E) or cocaine (B, D, F) -induced CPP. *P < 0.05; **P < 0.01; ***P < 0.001 significant difference vs priming with saline, ##P < 0.01, ###P < 0.001 significant difference vs priming with MDPV.*

Finally, when quantifying the mRNA encoding c-Fos (Figure 32E and 32F), a similar pattern of expression was observed after a priming with cocaine, regardless the conditioning treatment administered (cocaine conditioning: $F_{2,15} = 6.33$, $P < 0.05$; n=6/group); MDPV conditioning: $F_{2,14} = 4.853$, $P > 0.05$; n=5-6/group), respectively. In particular, an acute injection provoked a decrease of c-Fos expression ($P < 0.05$ in MDPV-conditioned mice and $P < 0.01$ in cocaine-conditioned mice), but not the acute MDPV administration.

3.3.5. DISCUSSION

Today, cocaine continues to be the most commonly illicit stimulant drug used in Europe, being even more prevalent in Southern countries as Spain (EMCDDA, 2018; EMCDDA, 2019). Moreover, in almost all European countries there has been an increase in the number of patients treated for the first time for cocaine addiction during the last 4 years. Prior to scheduling, MDPV was one of the most popular synthetic cathinones and was the principal substance detected in cathinone-poisoned users (Baumann et al. 2013). MDPV enhances dopamine neurotransmission, producing neurochemical effects similar to those of cocaine (Cameron et al., 2013; Cameron et al., 2013). MDPV users are effectively poly-drug users in almost all cases (Diestelmann et al., 2018). In fact, in a European case series of psychosis associated with acute recreational drug toxicity, cocaine was reported in 16.1 % of cases, but in half of these, more than one drug

was involved, including MDPV (Vallersnes et al., 2016). In this context, the present study investigates for the first time the ability of these two drugs to indistinctly restore drug-seeking behaviour and, thus, induce relapse in drug-taking.

Our results show that MDPV induces CPP in a range doses from 1 to 4 mg/kg, in line with previous reports in the literature (Gregg et al., 2016; Karlsson et al., 2014; King et al., 2015; Oliver et al., 2018). We observed that the preference induced by these doses was similar, with no dose–response relationship. The lack of dose–response effects usually observed in the CPP could be considered one of the weaknesses of this procedure (Aguilar et al., 2009). Nevertheless, we also confirmed that, after extinction, MDPV-induced CPP is robustly reinstated with doses up to 12.5 % (0.5 mg/kg) of the conditioning dose (Hicks et al., 2018). In this way, the present study corroborates the rewarding and reinstating ability of MDPV. In this context, the reinforcing effects of MDPV have also been demonstrated in the self-administration paradigm (Gannon et al., 2018; Geste et al., 2018; King et al., 2015).

Additionally, we aimed to explore if the ability of MDPV to reinstate drug-seeking behaviour is shared by cocaine, and if this occurs in the other direction, which would constitute cross-reinstatement. Cross-reinstatement is defined as the reinstatement of drug-seeking, following extinction, by a drug different to that administered during the acquisition of behaviour (in this case, CPP). Reinstatement of cocaine-induced CPP has been achieved with methamphetamine or methylphenidate (Achat-Mendes et al., 2003), and also by drugs belonging to other pharmacological classes, such as nicotine, ethanol, and morphine (Romieu et al., 2004). In addition, cocaine is capable of reinstating the CPP induced by morphine (Do Ribeiro Couto et al., 2005b).

For our experiments, the cocaine dose used was chosen based on previous studies showing a potent rewarding and reinstating effect of the conditioned preference (Maldonado et al., 2006; Ribeiro Do Couto et al., 2009; Rodríguez-Arias et al., 2009). Moreover, this cocaine dose (10 mg/kg) induced a similar preference as 2 mg/kg of MDPV. Nevertheless, although the CPP was equivalent with both drugs, the time needed to extinguish the preference was significantly longer after MDPV conditioning. It is well known that CPP is not a dose-dependent paradigm; however, we have previously observed that the maintenance of drug-induced CPP depends on the dose employed during conditioning, with higher doses requiring a greater number of extinction sessions (Ribeiro Do Couto et al., 2005a). It is important to highlight that our extinction procedure is natural and closely models real-life situations in which addicted individuals voluntarily stop taking the drug but continue to be exposed to the stimuli they associate with drug consumption. Therefore, our results suggest that, although the preference is equivalent, the neuroplasticity induced by MDPV is greater than that induced by cocaine, as the time needed to overcome MDPV preference is longer.

Furthermore, our results show for the first time that both drugs, cocaine and MDPV, can reinstate the CPP induced by the other drug. In this way, a cross-reinstatement between the two drugs became evident. As expected, we observed reinstatement of the preference with cocaine doses of 50% (5 mg/kg) and 25% (2.5 mg/kg) of the cocaine dose employed for conditioning (Blanco-Gandía et al., 2017; Reguilón et al., 2017). Similarly, preference was also reinstated in the MDPV-conditioned mice with 50% (1 mg/kg) and 25% (0.5 mg/kg) of the dose employed for conditioning. However, cross-reinstatement was obtained with 2 mg/kg and 10 mg/kg of MDPV and cocaine, respectively, but not with 1 mg/kg and 5 mg/kg.

In this sense, it is important to point out that such decreased sensitivity has been previously observed when cross-reinstatement was induced by cocaine in morphine-conditioned mice, but only with doses up to 10 mg/kg (Do Ribeiro Couto et al., 2005b). Moreover, there are some differences between the two drugs that could explain the decreased sensitivity observed in the cross-reinstatement. For instance, Wakabayashi et al. (2015) reported that MDPV, unlike cocaine, provokes cerebral vasoconstriction, leading to a poor supply of glucose to the nucleus accumbens. This implies that cocaine-users can be more susceptible to addiction than MDPV-users due to the presence of an interoceptive signal that results in earlier and more direct reward detection. That is to say, some differences underlying the two drugs' mechanism of action may be the reason why, despite their great similarity, they do not trigger the same responses, and so they are not fully interchangeable.

Cross-sensitization between the locomotor effects of MDPV and cocaine has previously been described in CD-1 mice at lower doses (Duart-Castells et al., 2019b; López-Arnau et al., 2017). However, in the present study, we aimed to determine the locomotor sensitization after a treatment schedule of drug administration similar to that employed in the CPP, evaluating the motor response to a drug challenge (saline, MDPV or cocaine) administered 12 days after the last drug administration. Behavioural sensitization is defined as an increased motor response after intermittent drug administration (Kalivas and Stewart, 1991; Steketee and Kalivas, 2011). Supporting an interaction in the conditioned rewarding effects of the two drugs, we also found that, following an experimental protocol that mimicked the CPP drug administration, exposure to either drug induced the development of sensitization, but only to the locomotor effects of cocaine. This result is in line with previous studies carried out in our and in other

laboratories using different schedules of cocaine administration (Duart-Castells et al., 2019b, Ferrer-Pérez et al., 2019, 2018). Nonetheless, this schedule of drug administration did not induce behavioural sensitization to MDPV. In this sense, we must take into consideration that the dose of MDPV used induced a higher increase in the locomotor response than the dose of cocaine employed. Thus, although other authors have reported sensitization to MDPV's locomotor effects, the pattern of drug administration in their studies was completely different (Berquist et al., 2016; Kohler et al., 2018; Watterson et al., 2016). For instance, the authors in question administered one dose of 0.5 or 1 mg/kg of MDPV to Sprague-Dawley rats, once daily, for 7 days, and obtained a significant increase in the motor response after the last drug administration on day 7. Similarly, we reported a locomotor sensitization to MDPV, but when administering lower doses to CD-1 mice (Buenrostro-Jáuregui et al., 2016; Duart-Castells et al., 2019b). Conversely, and in accordance with our present results, when higher doses of MDPV were administered in other studies, no locomotor sensitization response was obtained (Watterson et al., 2016). Furthermore, cross-sensitization between MDPV and cocaine or methamphetamine has also been described with low doses of MDPV (Kohler et al., 2018; Watterson et al., 2016). Considering all the evidence, the higher doses employed in our study could have induced an intense motor response that could have reached a ceiling effect.

To summarize, both cocaine and MDPV induce CPP, though memories associated with MDPV need more time to be extinguished. Moreover, both drugs have the ability to induce cross-reinstatement of drug-seeking, although at higher doses than those needed to reinstate their own CPP; thus, even though both drugs act on the dopaminergic system, there seem to be differences between them. In addition, these specific treatments of cocaine and MDPV sensitize to the locomotor effects of cocaine.

It has been suggested that drug priming induces reinstatement and cross-reinstatement of CPP through the activation of the mesocorticolimbic dopaminergic system, thereby influencing incentive motivation and appetitive goal-directed behaviour (Wang et al., 2000). Accordingly, in the present study, we sought to determine the expression of five different factors of the mesocorticolimbic dopaminergic system related with dopaminergic activation in drug addiction and neuroplasticity - G9a, ΔFosB, CB1 receptor, CDK5 and Arc - 24 h after the MDPV or cocaine CPP test. As ΔFosB is a long-lasting transcription factor, its expression was also evaluated 12 days later. G9a, Arc and c-Fos were also assessed after a priming dose of both drugs, also given 12 days after the CPP test.

Regarding G9a, a specific dimethyltransferase that acts as a negative regulator of gene expression, its effect is usually very fast. It is generated within 1 h and, sometimes, especially after MDPV administration, it vanishes 1 h later (Duart-Castells et al., 2019b). Thus, when we determined the expression of G9a 48 h after the last injection, no changes were observed. However, one of G9a's target genes, ΔFosB, was significantly increased after MDPV conditioning, and this increment persisted for 12 days. ΔFosB is the only member of the Fos family characterized by its high persistence over time (Carle et al., 2007; Ulery et al., 2006; Ulery-Reynolds et al., 2009). This stably-expressed truncated form of FosB plays a critical role in enhanced motivation, reward, drug reinforcement and long-term neural plasticity. In this sense, the long-lasting signal observed in the present study co-occurred with the longer time required for MDPV-seeking behaviour to be extinguished.

Under these conditions we did not find modifications in Arc, CB1 receptor or CDK5 protein expression in the same brain area. By contrast, in a

previous study using CD-1 mice, we described an increase of CDK5 induced by a chronic schedule of MDPV (1.5 mg/kg, twice daily, for 7 days). Thus, it is likely that the effect of MDPV on CDK5 expression is dose-dependent or mouse strain-dependent. It has been suggested that the induction of CDK5 leads to a decrease in the efficacy of the dopamine 1 receptor signal. Bib et al. (2001) described that the CDK5/DARPP-32 pathway performs a homeostatic negative feedback with respect to the behavioural effects of cocaine. In our case, the lack of changes in CDK5 expression suggest that the administration regime we used might not have been intense enough to generate such a homeostatic response. Alternatively, we cannot exclude the possibility that such differences were due to differences of mouse strain.

Arc is another protein involved in neural plasticity, although we did not find any alteration in its expression. Fumagalli et al. (2006) observed a long-lasting increase of this protein after a repeated cocaine regime over 14 days, but not after a shorter one (5 days). Accordingly, this could be the reason why we did not find any effect on Arc protein levels.

Finally, in relation to CB1 receptor expression, the effect of cocaine or MDPV consumption on the endocannabinoid transmission in reward-related areas of the brain is relatively under-researched. We did not observe any protein expression alteration when analysing the whole ventral striatum. In this sense, Bystrowska et al. (2018) observed a slight increase in CB1 receptor expression only in the ventral tegmental area and in the nucleus accumbens shell. Furthermore, recent publications suggest that the implication of the endocannabinoid transmission in cocaine reward and reinstatement might be mediated by other mechanisms, but not alterations in CB1 receptor protein levels in the ventral striatum (Freund et al., 2003; Hájos et al., 2001; Pistis et al., 2004).

The expression of some of these factors was also assessed 12 days after the CPP procedure, 2 h after a priming dose of saline, cocaine or MDPV. The aim was to determine early acute, and potential short-lasting effects of the priming drug. As mentioned above, it should be remembered that the extinction time varied from mouse to mouse and depended on the treatment received. Therefore, in many cases, this simulation would have mimicked early reinstatement and, more importantly, allowed us to perform a comparison at the same time point, although drug-seeking behaviour had not yet been extinguished.

As in the other set of experiments, we also assessed the levels of G9a mRNA and found out that, 2 h after a priming dose, different effects were observed between MDPV and cocaine. The effect of cocaine on the early expression of G9a was always a decrease, regardless the conditioning drug (MDPV or cocaine), whereas the priming with MDPV induced an increase in G9a mRNA only in cocaine-conditioned mice. Indeed, in a previous study, we already described the different effect of cocaine and MDPV on G9a expression and, thus, on BDNF generation (Duart-Castells et al., 2019b).

To explain the effects on Arc expression observed, it is important to bear in mind that MDPV-conditioned mice still had significantly increased levels of ΔFosB when a new drug was administered. When MDPV-treated mice received a cocaine challenge, they exhibited a lower G9a repressor signal and no alteration in Arc expression. Such decrease in G9a expression, along with the increase in ΔFosB levels, point to a significant activation of neuroplasticity (see Figure 33). This neuroplasticity observed after a priming dose of cocaine seemed to be higher in mice conditioned with MDPV than in those conditioned with cocaine, in which Arc decreased and ΔFosB remained unchanged.

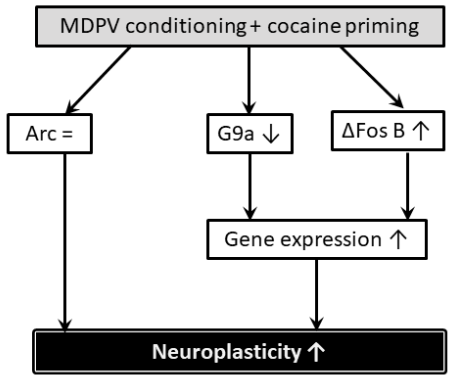

Figure 33. Schematic depiction of the biomolecular changes triggered in brain of mice after a cocaine injection given 12 days after MDPV-induced CPP.

Finally, as mentioned previously, in the present study we have observed locomotor sensitization only to cocaine, regardless the conditioning drug. In this sense, Todtenkopf et al. (2002) carried out an experiment relating repeated cocaine dosage with locomotor sensitization to this drug. They found that repeated cocaine administration (15 mg/kg twice daily, for 5 consecutive days) resulted in a robust sensitization that correlated with a significant decrease in the density of c-Fos in the nucleus accumbens only in animals challenged shortly after withdrawal. Indeed, the same pattern of c-Fos expression during withdrawal was described by Renthal et al (2008) after chronic treatment with amphetamine. In this sense, we also found that a cocaine priming decreased c-Fos, which was also correlated with a locomotor sensitization effect, regardless the conditioning drug used. When animals were challenged with MDPV, the same tendency to decline was observed, even though it did not reach statistical significance. In accordance, no locomotor sensitization to MDPV was observed.

To sum up, although MDPV 2 mg/kg displays a stronger psychostimulant effect than cocaine 10 mg/kg, both doses seem to be equivalent in their rewarding properties. However, memories associated with MDPV need more time to be extinguished. Importantly, our results show that MDPV and cocaine restore drug-seeking behaviour of both, though the relapse in drug-taking is always higher with the conditioning drug.

The fact that MDPV-treated mice show increased ΔFosB protein levels correlates with its longer extinction time with respect to cocaine and suggests an activation of neuroplasticity mechanisms that persists for at least 12 days. In addition, the neuroplasticity mechanisms that both drugs activate may notably differ, since MDPV- conditioned mice are more responsive to a new cocaine exposure, implying a high vulnerability to cocaine abuse.

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3.3.7. SUPPLEMENTARY MATERIAL

Table S2. Experimental design. Sets of animals used and experimental procedure followed in each experiment. *PND: post-natal day. Pre-C: pre-conditioning. Post-C: post-conditioning.*

Experiment 1 MDPV-induced reinstatement of the CPP

	PND	42-44	45-48	49	>50
n	Group	Pre-C	Conditioning	Post-C	Reinstatement
14	MDPV 1 mg/kg		MDPV 1 mg/kg		MDPV 0.5 mg/kg
14	MDPV 2 mg/kg		MDPV 2 mg/kg		MDPV 1 mg/kg; MDPV 0.5 mg/kg
14	MDPV 4 mg/kg		MDPV 4 mg/kg		MDPV 2 mg/kg; MDPV 1 mg/kg; MDPV 0.5 mg/kg

Experiment 2 Cross-reinstatement between MDPV and cocaine

	PND	42-44	45-48	49	>50
n	Group	Pre-C	Conditioning	Post-C	Reinstatement
14	MDPV – MDPV		MDPV 2 mg/kg		MDPV 1 mg/kg MDPV 0.5 mg/kg
15	MDPV – Cocaine		MDPV 2 mg/kg		Cocaine 10 mg/kg
14	Cocaine – Cocaine		Cocaine 10 mg/kg		Cocaine 5 mg/kg Cocaine 2.5 mg/kg
14	Cocaine – MDPV		Cocaine 10 mg/kg		MDPV 2 mg/kg

Experiment 3 Locomotor activity

	PND	45-48	61
n	Group	Drug administration	Challenge
8	Saline – MDPV	Saline	MDPV 2 mg/kg
8	MDPV – MDPV	MDPV 2 mg/kg	MDPV 2 mg/kg
8	Cocaine – MDPV	Cocaine 10 mg/kg	MDPV 2 mg/kg
8	Saline – Cocaine	Saline	Cocaine 10 mg/kg
8	MDPV – Cocaine	MDPV 2 mg/kg	Cocaine 10 mg/kg
8	Cocaine – Cocaine	Cocaine 10 mg/kg	Cocaine 10 mg/kg

Experiment 4 Biochemical analysis

	PND	45-48	50	61	
n	Group	Drug administration		Priming	2 h post-priming
12	Saline 24 h	Saline	Brain extraction		
6	Saline 12 days	Saline		Saline	Brain extraction
12	MDPV 24 h	MDPV 2 mg/kg	Brain extraction		
12	MDPV – Saline	MDPV 2 mg/kg		Saline	Brain extraction
6	MDPV - MDPV	MDPV 2 mg/kg		MDPV 2 mg/kg	Brain extraction
6	MDPV – Cocaine	MDPV 2 mg/kg		Cocaine 10 mg/kg	Brain extraction
12	Cocaine 24 h	Cocaine 10 mg/kg	Brain extraction		
12	Cocaine – Saline	Cocaine 10 mg/kg		Saline	Brain extraction
6	Cocaine – MDPV	Cocaine 10 mg/kg		MDPV 2 mg/kg	Brain extraction
6	Cocaine – Cocaine	Cocaine 10 mg/kg		Cocaine 10 mg/kg	Brain extraction

Part II

Second-generation synthetic cathinones

CHAPTER 4

3.4. Role of the amino terminal group in the pharmacological profile of novel synthetic cathinones structurally related to α-PVP and pentedrone

Comparative of their rewarding and psychostimulant effects

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** Chapter 4 is adapted from a paper pending to be submitted in near future. Therefore, it has been included and structured as a full article, but it may be modified as new results are obtained prior to being send for peer review. On date of its final edition, it constitutes the most updated version.*

3.4.1. ABSTRACT

The emergence of new psychoactive substances (NPS), including synthetic cathinones, continues to be a matter of health concern. Although a first-generation of synthetic cathinones has already been banned and illegal in most countries, a new second-generation of synthetic cathinones has recently broken into the drug market (i.e. α-pyrrolidinovalerophenone, α-PVP, and pentedrone). When one of these NPS falls under legislative control, the drug market responds by producing new different alternatives, usually structurally related. In this sense, the main goal of the present study was to characterize the pharmacological profile as well as the psychostimulant and rewarding properties of new second-generation synthetic cathinones, concretely, of different α-aminovalerophenone derivatives structurally related to α-PVP and pentedrone, which only differ in their amino-substituent.

All compounds tested are potent DA and NA uptake inhibitors, with weak effects on SERT. Accordingly, high DAT/SERT ratios (>300) were obtained, pointing out their high abuse potential. Moreover, a positive correlation between DA and NA uptake IC_{50} values was observed. Importantly, our data revealed that all the substances are more potent as DAT blockers than cocaine, and the potency as reuptake inhibitors increased when the amino-group expanded from a methyl to an ethyl and to a pyrrolidine or piperidine ring. In addition, all the derivatives inhibit hOCT-2 function.

Regarding QSAR studies, a positive correlation was observed between hDAT/hSERT ratio and the CLogP of the amino-substituent, pointing to a high abuse liability when increasing lipophilicity. The interaction of these compounds has been assessed at molecular level by means of molecular

docking, evincing the effect of the differential substitutions on the biological activity. A significant correlation was observed between predicted binding affinities and affinity constants (K_i) for hDAT. Additionally, and although these compounds are not potential SERT inhibitors, the potency at inhibiting 5-HT uptake appeared to be inversely proportional to lipophilicity, surface area and steric bulk of the amino substituent. Therefore, further research involving other synthetic cathinones with more serotoninergic activity are needed to corroborate these results.

Importantly, our study provides the first evidence that N-ethyl-pentedrone, N,N-diethyl-pentedrone and α-piperidinevalerophenone (α-PpVP) are able to induce potent psychostimulant and rewarding effects in mice. Therefore, our findings demonstrate the abuse liability of all the aminovalerophenone derivatives tested, especially N-ethyl-pentedrone, a NPS currently available in several Western countries.

3.4.2. INTRODUCTION

The emergence of New Psychoactive Substances (NPS) in the illicit drug market, including synthetic cathinones (*a.k.a.* "bath salts", "legal highs" or "research chemicals"), continues to be a matter of public health since their consumption is still associated with and has resulted in several deaths and acute intoxications (EMCDDA, 2019, UNODC, 2019). As of 2018, 119 countries and territories have reported 890 NPS to the United Nations Office on Drugs and Crime (UNODC) with more than 300 identified in the United States of America (USA). Although some of them are under law restrictions, others are easily available through online shops or the *darknet* (EMCDDA, 2019, UNODC, 2019).

When one of these NPS is banned and illegal, the drug market responds by producing different alternatives, usually related structurally and sometimes with minor chemical modifications. In this sense, when the "first-generation cathinones" (i.e., methylone, mephedrone and 3,4-methylenedioxypyrovalerone (MDPV)) was classified by the Drug Enforcement Administration (DEA) as Schedule I compounds (DEA, 2011), a "second-generation" emerged, which includes α-pyrrolidinovalerophenone (α-PVP) and pentedrone (DEA, 2014). For instance, α-PVP, also known as *flakka,* is structurally similar to MDPV. The simple removal of the 3,4-methylendixoy group from MDPV led to α-PVP structure which, at that time was not scheduled and was considered to largely emerged as a replacement to MDPV. However, many other synthetic cathinones analogues have not been yet classified by authorities since, among many other reasons, they are still pending scientific evidence of mode of action, toxicity and abuse potential.

Synthetic cathinones are known to interact with monoamine transporters and inhibit their function. It has been demonstrated that α-PVP acts as a potent blocker of the dopamine (DA) and noradrenaline (NA) transporters (DAT and NET, respectively) (Marusich et al., 2014; Meltzer et al., 2006). Preclinical studies have also described α-PVP to produce long-lasting increases in locomotor activity, to induce conditioned place preference and to fully substitute for discriminative stimulus effects of both cocaine and methamphetamine (Gatch et al., 2015a; Marusich et al., 2014). Moreover, α-PVP has also been shown to facilitate intracranial self-stimulation and maintain self-administration in rats (Watterson et al., 2014, Huskinson et al., 2017). Pentedrone is another "second-generation" cathinone closely related to α-PVP only differing in their amino-group substituent (see Figure 34). Similar to α-PVP, pentedrone also blocks DAT and NET and

exhibits psychostimulant, rewarding and reinforcing properties (Hwang et al., 2017; Gatch et al., 2015b; Javadi-Paydar et al., 2018; Simmler et al., 2014).

Numerous structure-activity relationship (SAR) studies on synthetic cathinones have shown to correlate molecular properties of different substituents with their pharmacological and toxicological profile (Eshleman et al., 2017; Kolanos et al., 2015; Walther et al., 2019; Niello et al 2019; for review see also Glennon and Dukat 2017; Baumann et al 2018). Saha and colleagues (2015 and 2019) demonstrated how modifications at both, α-carbon alkyl chain and the N-group of methcathinone, are able to generate the so-called "hybrid compound" which behaves as a blocker at DAT and as a releaser at SERT. In this study, we explore different structural modifications in the amino-terminal group by describing a set of five α-aminovalerophenone derivatives: pentedrone, α-ethylaminovalerophenone (N-ethyl-pentedrone), α-diethylaminovalerophenone (N,N-diethyl-pentedrone), α-PVP and α-piperidinevalerophenone (α-PpVP) (Figure 34). This may shine light on how structural modifications in the amino-terminal group may impact the psychostimulant and rewarding effects as well as the potency at inhibiting monoamine transporters.

Taken together, the aim of the present study was, firstly, to characterize the *in vitro* pharmacology of five aminovalerophenone derivatives. Thus, we determined their potency to inhibit DA and NA uptake in rat synaptosomes and monoamine uptake in HEK293 cells expressing human DAT, NET, SERT (uptake-1), organic cation transporter-2 and -3 (OCT-2 and OCT-3, respectively, uptake-2) (Mayer et al., 2018 and 2019), as well as their binding affinities for DAT, NET and SERT. Secondly, we studied the interaction mechanism of these compounds at molecular level by means of molecular docking, evincing the effect of the differential substitution on the biological

activity. Finally, we sought to assess their potential psychostimulant and rewarding effects at different doses, and establish a SAR between the different amino-substituents and their pharmacological profile. Altogether, this study may provide a molecular and behavioural explanation for abuse liability associated with synthetic cathinones.

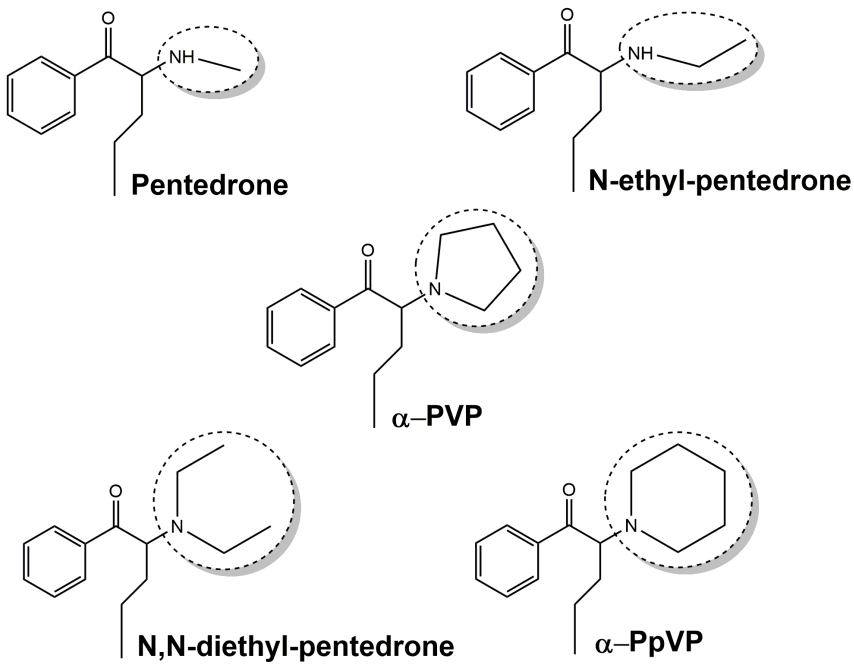

Figure 34. Chemical structure of the α-aminovalerophenone derivatives. *Compounds share a similar chemical structure, only differing by their amino-substituent: methylamino- (pentedrone), ethylamino- (N-ethyl-pentedrone), diethylamino- (N,N-diethyl-pentedrone), pyrrolidine-ring (α-PVP) or piperidine-ring (α-PpVP).*

3.4.3. MATERIALS AND METHODS

Subjects

Adult Swiss CD-1 mice (Charles River, Lyon, France) weighing 25–30 g (8 weeks-old) were used for the behavioural experiments. For the synaptosomal preparation, male Sprague-Dawley rats (Janvier, Le Genest, France) weighing 225–250 g (2-3 months-old) were used. The animals were housed in temperature-controlled conditions (22 ± 1°C) under a 12 h light/dark cycle and had free access to food and drinking water. All animal care and experimental protocols in this study complied with the guidelines of the European Community Council (2010/62/EU) and were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010).

Drugs and materials

α-Aminovalerophenone derivatives were synthetized in racemic form as hydrochloride salts as described in *Suplementary material section*. Solutions for injection were freshly prepared daily in isotonic saline solution (0.9% NaCl, pH 7.4). [^{3}H]DA, [^{3}H]NA [^{3}H]5-HT, [^{3}H]imipramine and [^{3}H]WIN35,428 were purchased from Perkin Elmer Inc. (Boston, MA, USA). [^{3}H]1-Methyl-4-phenylpyridinium ([^{3}H]MPP$^+$), was supplied by American Radiolabeled Chemicals (St. Louis, MO, USA). Cocaine was generously provided by the Spanish National Institute of Toxicology. All other reagents were of analytical grade and purchased from several commercial sources.

[³H]DA and [³H]NA uptake experiments in rat synaptosomes

Rat synaptosome preparation

Rat synaptosome suspensions were prepared as described by Pubill et al., (2005), with minor modifications. Briefly, in each experiment two rats were decapitated under isofluorane anaesthesia and the striatum or frontal cortex were dissected out, homogenized (5 mM Tris-HCl and 320 mM sucrose in Milli-Q water) and centrifuged at 1,000xg at 4°C for 10 min. After discarding the pellet, the supernatant was centrifuged at 13,000 x g for 30 minutes at 4 °C. Then the pellet 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 $MgCl_2 \cdot 6H_2O$, 0.41 $MgSO_4 \cdot 7H_2O$, 4.17 $NaHCO_3$, 0.34 $Na_2HPO_4 \cdot 7H_2O$, 5.5 glucose and 20 HEPES-Na) containing pargyline (20 mM) and ascorbic acid (1 mM).

Plasmalemmal [³H]DA and [³H]NA uptake experiments

Competitive blockade of [³H]DA and [³H]NA uptake was performed as described by López-Arnau et al., (2012), with some modifications, in synaptosomes from rat striatum or grontal cortex, respectively. Briefly, reaction tubes were composed of 0.125 mL of the drugs at different concentrations in HEPES-buffered solution containing pargyline and ascorbic acid. Moreover, 0.025 mL of [³H]DA or [³H]NA was also added (final concentration 5 nM or 20 nM, respectively). After addition of 0.1 mL of synaptosome suspension, incubation was carried out for 5 min. Then, the uptake reaction was terminated by rapid vacuum filtration through Whatman GF/B glass fibre filters (Whatman Intl Ltd., Maidstone, UK) presoaked 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 [^{3}H]DA or [^{3}H]NA uptake was determined at 4°C in parallel samples containing cocaine (300 μM) or desipramine (20 μM). respectively.

Uptake inhibition and transporter binding assays in HEK293 cells

Cell culture and membrane preparation

Human embryonic kidney (HEK293) cells were used for the uptake and binding experiments. The generation of stable, monoclonal cell lines expressing the human isoforms of SERT, NET, DAT, OCT-2 or OCT-3 has been described previously (Mayer et al., 2016a and 2016b). HEK293 were maintained in DMEM supplemented with heat-inactivated 10% FBS, 100 U/ml penicillin and 100 μg/ 100 ml streptomycin, and cultured to a subconfluential state in a humidified atmosphere (5% CO_2, 37°C). Geneticin (G418; 50 μg/ml) was added to maintain the selection process.

For membrane preparations, stably transfected HEK293 cells (hDAT and hSERT) were harvested from 15-cm dishes 80-90% confluent. Briefly, cells were washed twice with ice-cold phosphate buffered saline (PBS), mechanically detached from the dish with a plastic scraper in the same ice-cold PBS and pelleted by centrifugation (400 x g for 10 minutes at 4°C). The resulting pellet was resuspended in hypotonic HME buffer (20 mM HEPES NaOH, 2 mM $MgCl_2$, 1 mM EDTA; pH 7.4), followed by two freeze-thaw cycles in liquid nitrogen and homogenization through sonication at 4°C. Thereafter, membranes were collected by centrifugation (40,000 x g for 30 min at 4°C) and resuspended in an appropriate volume of HME buffer. The membrane preparations were kept at -80°C until use.

Protein concentration was determined using the Bio-Rad Protein Reagent (Bio Rad Laboratories, Hercules, CA).

Uptake inhibition assays

Before starting the uptake inhibition experiments, the media was removed and replaced with Krebs-HEPES-Buffer (KHB; 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2 \cdot 2\ H_2O$, 2mM $MgCl_2 \cdot 6\ H_2O$ supplemented with 20 mM D-glucose; pH 7.3). Afterwards, cells were preincubated with different concentrations of the test drugs diluted in KHB for 5 min in case of the uptake-1 inhibition assays (hDAT, hNET and hSERT) and 10 min for the uptake-2 assays (OCT-2 and OCT-3). Then, the tritiated substrates were added: 0.02 μM [^{3}H]MPP^+ for hDAT and hNET, 0.1 μM [^{3}H]5-HT for hSERT and 0.05 μM [^{3}H]MPP^+ for hOCT-2 and hOCT-3. The uptake incubation times were 3 min for hDAT and hNET, 1 min for hSERT and 10 min for uptake-2 experiments. The uptake was terminated by removing the tritiated substrate, washing the cells with ice-cold KHB and lysing them with sodium dodecyl sulfate (SDS) 1%. The lysate was added to scintillation fluid and the released radioactivity was quantified with a beta-scintillation counter (Perkin Elmer, Waltham, MA, USA). Non-specific uptake was determined in parallel samples containing cocaine 100 μM for hDAT and hNET, paroxetine 30 μM for hSERT and decynium-22 (D22) 100 μM for hOCT-2 and hOCT-3.

Transporter binding assays

Membrane preparations expressing the transporters hDAT and hSERT were incubated with radiolabelled selective ligands at concentrations equal or close to K_d. The drugs were diluted in binding buffer (120 mM NaCl,

3 mM KCl, 2 mM $MgCl_2$, 10 μM $ZnCl_2$ and 20 mM Tris pH 7.4 for hDAT; 120 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 20 mM Tris pH 7.4 for hSERT) and tested at increasing concentrations (0.1 nM – 300 μM) in duplicate. The binding reactions were performed in tubes containing 25 μl of [^{3}H]WIN35,428 (hDAT, final concentration 10 nM) or [^{3}H]imipramine (hSERT, final concentration 3 nM) diluted in the corresponding reaction buffer, 5 μg of membranes and 100 μl of the tested drug dilution. Non-specific binding was determined in the presence of cocaine 100 μM (for hDAT) and paroxetine 3 μM (for hSERT). Incubation was performed for 1 hour at 20 ºC. The binding reactions were terminated by rapid filtration as described previously and washed with ice-cold wash buffer containing 120 nM NaCl, 2 mM $MgCl_2$, 10 mM Tris, 100 μM $ZnCl_2$ for hDAT, and 120 nM NaCl, 2 mM $MgCl_2$ and 10 mM Tris, for hSERT). Afterwards, radioactivity was quantified as described above.

Horizontal locomotor activity (HLA)

HLA was measured as described by Duart-Castells et al., (2019), with minor modifications. Briefly, the animals were given their corresponding i.p. injection (saline (5 ml/kg), pentedrone (3, 10 or 30 mg/kg), N-ethyl-pentedrone (1, 3 or 10 mg/kg), N,N-diethyl-pentedrone (3.5, 12.5 or 35 mg/kg), α-PVP (1, 3 or 10 mg/kg) or α-PpVP (7.5, 25 or 75 mg/kg) and immediately placed in the open field arena. HLA was video-monitored for 60 min using a tracking software (Smart 3.0 Panlab, Barcelona, Spain) and their total travelled distance (in cm) was measured. On one hand, pentedrone and α-PVP doses were chosen following the results reported by Hwang et al., (2017) and Wojcieszak et al., (2018). On the other hand, there is no data available about the doses of N-ethyl-pentedrone, N,N-diethyl-pentedrone and α-PpVP used in animal research. Therefore, these doses were chosen

according to the psychostimulant effect induced by pentedrone or α-PVP at the medium doses tested in order to have, at least, one dose equally effective for all the substances. Moreover, all the highest doses used were always 10-fold higher than the lowest dose tested, a protocol also used by the aforementioned authors (Hwang et al., 2017 and Wojcieszak et al., 2018).

Conditioned place preference (CPP)

The potential of the five α-aminovalerophenone derivatives to induce rewarding effects was determined using a place conditioning paradigm (unbiased), as described by Duart-Castells et al., (2019). The drugs were administered at the same doses used in the HLA experiments.

Interaction mechanism of α-aminovalerophenone derivatives

All computational procedures were conducted using MOE 2019.01 softare [Molecular Operating Environment, Chemical Computing Group, Canada, 2019]. Structural model of hDAT protein was obtained by applying homology modeling on hDAT amino acid sequence (Uniprot ID: Q01959) and considering the crystal structure of *Drosophila* DAT complexed with methamphetamine as template (available in the Protein Data Bank, PDBID: 4XP6 (Wang et al., 2016). Although hDAT and dDAT proteins have moderate sequence similarity (56%), the active site is highly conserved (> 80% of homology). The obtained hDAT three-dimensional model (RMSD = 0.191 Å) complexed with methamphetamine was finally prepared by applying the QuickPrep protocol available in MOE.

Molecular docking was applied to predict the interaction mechanism of compounds under study, defining the triangle matcher as placement method

and considering both ligand and receptor flexible by means of induced fit approach. The GBVI/WSA ΔG score function was used for quantifying the free energy of binding of the 100 resulting conformations for each molecule. The drugs were built as (S)-enantiomers in protonated form and they were docked into the hDAT model using the previous protocol.

Data analysis

Competition curves were plotted and fitted by nonlinear regression. Data were best fitted to a sigmoidal dose-response curve and an IC_{50} or EC_{50} value was obtained. *Ki* (affinity) values were calculated using the Cheng-Prusoff equation: $Ki = IC_{50}/(1+[\text{radioligand concentration}/Kd])$. One-way ANOVA, and subsequent post hoc test (Tukey-Kramer), was used to determine overall α-aminovalerophenone derivatives effects on HLA, CPP as well as DA, NA and 5-HT uptake in both rat synaptosomes and HEK cells. Moreover, t-student test was also performed when studying the selectivity for each monoamine uptake inhibition. Pearson correlation analyses were also performed when needed. All analysis were carried out using GraphPad Prism (GraphPad software, USA). Molecular and physicochemical descriptors were calculated for the different amino-terminal groups using ChemBioOffice Ultra and Data Warrior software. Lipophilicity descriptors included calculated partition coefficient (CLogP). Molecular surface and steric bulk were also investigated using Total Surface Area (TSA) and calculated molar refractivity (CMR), respectively (Hevener et al., 2008).

3.4.4. RESULTS

Monoamine uptake inhibition

[³H]DA and [³H]NA uptake in rat brain synaptosomes

The potency of tested compounds to inhibit DA and NA uptake is depicted in Figure S1 and respective IC_{50} values for [^{3}H]DA and [^{3}H]NA uptake at DAT and NET are compiled in Table 2.

All compounds inhibited [^{3}H]DA and [^{3}H]NA uptake in rat synaptosomes. All drugs inhibited [^{3}H]NA uptake with a similar potency, with the exception of N,N-diethyl-pentedrone, which was considerably less potent compared to the others (> 4-fold less) and 70-fold compared to α-PVP (α-PVP ≈ N-ethyl-pentedrone ≈ α-PpVP ≥ pentedrone > N,N-diethyl-pentedrone; $F_{4,14}$=21.11, $P < 0.001$). Regarding [^{3}H]DA uptake inhibition, the order in terms of potency was as follows: α-PVP > N-ethyl-pentedrone ≈ α-PpVP > pentedrone >> N,N-diethyl-pentedrone ($F_{4,14}$=94.01, $P < 0.001$). As can be seen, the order of potency inhibiting both catecholamines is similar. This is exemplified in Figure S2A, wherein a significant correlation between lC_{50} values of [^{3}H]DA and [^{3}H]NA uptake was found (r^2= 0.9413, $P < 0.01$).

Monoamine uptake inhibition in transfected HEK293 cells

Concentration-response curves are depicted in Figure S3. Corresponding IC_{50} values and hDAT/hSERT inhibition ratios are presented in Table 2.

While assessing uptake-1 assays, all tested drugs exhibit preferentially uptake inhibition at hDAT and hNET with weak potencies at hSERT. Additionally, among the 5 compounds tested, a very similar order of potency for hDAT (α-PVP ≈ N-ethyl-pentedrone ≈ α-PpVP > pentedrone > N,N-diethyl-pentedrone; $F_{4,10}$=30.61, $P < 0.001$) and hNET inhibition (α-PVP ≈ N-ethyl-pentedrone > α-PpVP = pentedrone > N,N-diethyl-pentedrone; $F_{4,10}$=568.7, $P < 0.001$) was observed. Consequently, a significant correlation between IC_{50} values at blocking hDAT and hNET was found (r^2= 0.8097, $P < 0.05$) (Figure S2B).

The order of potency for hSERT was as follows: pentedrone ≈ N-ethyl-pentedrone >> α-PVP > N,N-diethyl-pentedrone ≈ α-PpVP; $F_{4,10}$=179.1, $P <0.001$). It is important to point out that when studying [^{3}H]5-HT uptake inhibition at hSERT, the very low potencies of N,N-diethyl-pentedrone, α-PVP and α-PpVP were not sufficient to allow for correct fitting of the data. However, an estimation of the IC_{50} values was performed in order to obtain a hDAT/hSERT ratio.

Molecular and physicochemical descriptors were also calculated for the different amino-substituents, correlating them with some QSAR parameters such as CLogP, total surface area, volume and CMR. As shown in Table S3, hSERT, but not hDAT and hNET IC_{50} values, significantly correlated with CLogP, total surface area, volume and CMR of the amino-substituents. In addition, the hDAT/hSERT ratios also correlated with CLogP.

With regards to uptake-2 inhibition experiments (see Figure S3 and Table 2), α-aminovalerophenone derivatives blocked hOCT-2 within the IC_{50} value range of 10 – 60 μM. No considerable effect was observed on hOCT-3. Hence, inhibition potency values were estimated in all cases > 1 mM.

Table 2. Affinity and potency of substituted cathinones at monoamine transporters. *Monoamine uptake-1 and uptake-2 inhibition: values are IC_{50} given as μM (mean and 95% confidence intervals (CI). Transporter binding affinities: values are given as μM (mean ± SD). All determinations were performed per triplicate and at least three-times (n=3-4).*

	Monoamine uptake inhibition								**Transporter binding affinities**	
	Rat brain synaptosomes		Transfected HEK293 cells						Transfected HEK293 cells	
	Uptake-1		Uptake-1				Uptake-2			
Compound	[^{3}H]DA	[^{3}H]NA	[^{3}H]MPP$^+$ uptake at hDAT	[^{3}H]MPP$^+$ uptake at hNET	[^{3}H]5-HT uptake at hSERT	hDAT/hSERT ratio	[^{3}H]MPP$^+$ uptake at hOCT-2	[^{3}H]MPP$^+$ uptake at hOCT-3	hDAT	hSERT
Pentedrone	1.742 (1.371 – 2.112)	0.369 (0.267 – 0.471)	0.215 (0.163 – 0.267)	0.594 (0.540 – 0.643)	78.03 (69.270 – 86.790)	363	26.95 (23.996 – 29.90)	> 1000	0.146 ± 0.03	11.42 ± 1.15
N-ethyl-pentedrone	0.824 (0.718 – 0.930)	0.108 (0.097 – 0.119)	0.076 (0.041 – 0.111)	0.168 (0.160 –0.176)	78.47 (77.997 – 78.94)	1029	57.58 (46.97 – 68.19)	> 1000	0.049 ± 0.02	17.13 ± 0.9
N,N-diethyl-pentedrone	3.535 (3.175 – 3.895)	1.672 (1.075 – 2.269)	0.445 (0.325 – 0.565)	3.260 (3.025 –3.495)	> 100	2356	47.81 (46.745 – 48.87)	> 1000	0.398 ± 0.05	75.9 ± 36.81
α-PVP	0.139 (0.104 – 0.174)	0.024 (0.023 – 0.025)	0.032 (0.027 – 0.037)	0.067 (0.069 – 0.075)	> 100	21801	17.49 (13.498 – 21.48)	> 1000	0.011 ± 0.004	52.2 ± 5.06
α-PpVP	1.055 (0.917 – 1.193)	0.320 (0.260 – 0.38)	0.058 (0.046 – 0.070)	0.519 (0.488 – 0.550)	> 100	19518	12.88 (12.15 – 13.61)	> 1000	0.078 ± 0.03	> 100

hDAT/hSERT ratio = 1/DAT IC_{50} : 1/SERT IC_{50}

Transporter binding affinities

The binding affinity constants (*Ki)* of the α-aminovalerophenone derivatives for hDAT and hSERT are summarized in Table 2.

All drugs exhibited binding affinity for hDAT in the medium-low nanomolar range (< 400 nM). For instance, N-ethyl-pentedrone, α-PVP and α-PpVP were more potent than cocaine in binding to hDAT. Conversely, all drugs presented substantially lower affinity to hSERT, with pentedrone showing the highest affinity (*Ki*). α-PVP and the compound with the bulkier amino-substituent (α-PpVP) displayed poor binding affinity to hSERT.

Effect of α-aminovalerophenone derivatives on spontaneous locomotor activity

As described in *Material and Methods section*, the locomotor activity induced by three different doses of the drugs was determined (Figure 35) One-way ANOVA of HLA results revealed a significant effect of the variable Dose for all the compounds tested ([pentedrone], ($F_{3,34}$=22.7; P < 0.001); [N-ethyl-pentedrone], ($F_{3.36}$=98.42; P < 0.001); [N,N-diethyl-pentedrone], ($F_{3,36}$=52.89; P < 0.001); [α-PVP], ($F_{3,40}$=72.09; P < 0.001); [α-PpVP], ($F_{3,36}$=12.01; P < 0.001)). Overall, all compounds increased locomotor activity in a dose-dependent manner in mice.

When analysing the locomotor activity induced by the medium doses tested of each drug, one-way ANOVA yielded no significant effect of the variable Compound ($F_{4,46}$=0.8724; P > 0.05), which means that the medium doses used in this study were equally effective. Consequently, the rank order of potency for eliciting hyperlocomotion was α-PVP = N-ethyl-pentedrone > pentedrone > N,N-diethyl-pentedrone > α-PpVP.

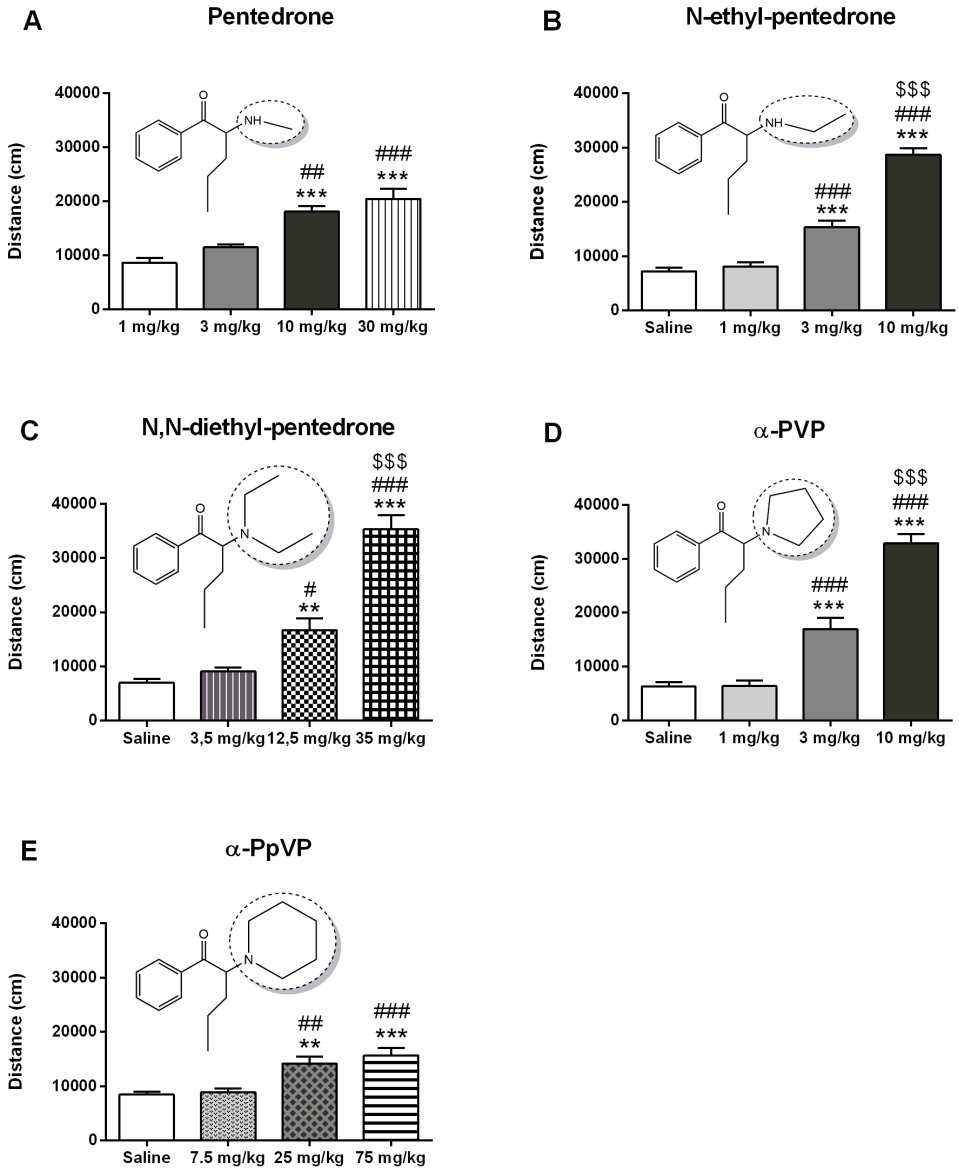

Figure 35. Effects of α-aminovalerophenone derivatives on cumulative locomotor activity in CD-1 mice. *Bars represent mean ± SEM of the distance travelled in 60 min. (n=9-12/group). **P<0.01 and ***P<0.001 vs saline; #P<0.05, ##P<0.01 and ###P<0.001 vs the lower drug-dose; $$$P<0.001 vs the medium drug-dose.*

Effect of α-aminovalerophenone derivatives on CPP

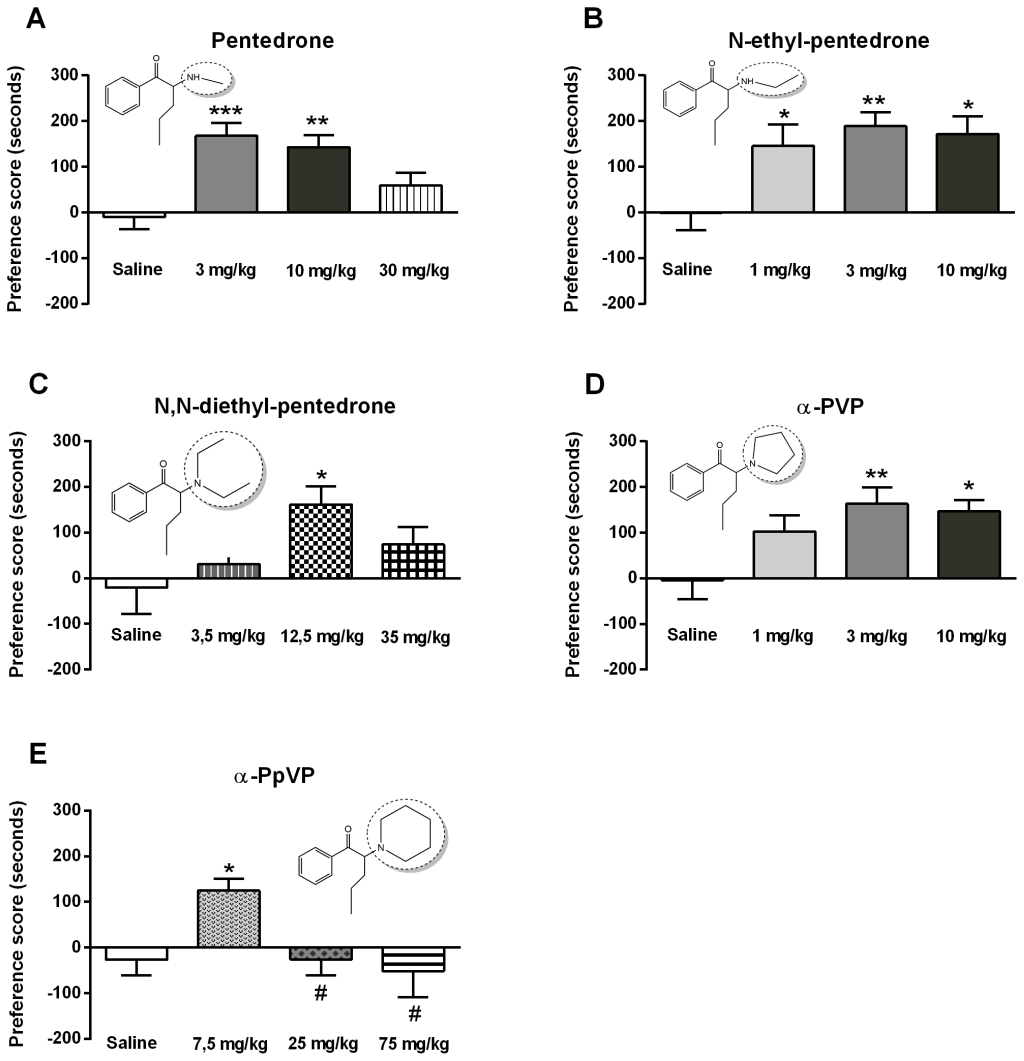

Figure 36. Effects of α-aminovalerophenone derivatives on conditioned place preference (CPP) test in CD-1 mice. *Bars represent mean ± SEM of the preference score (difference between the time (in seconds) spent in the drug-paired compartment on the test day and the pre-conditioning day). (n=12-15/group). *P<0.05, **P<0.01 and ***P<0.001 vs saline; #P<0.05 vs the lower drug-dose.*

As shown in Figure 36, on the test day, one-way ANOVA revealed a significant effect of Dose for all the compounds tested ([pentedrone], ($F_{3.51}$=8.819; P < 0.001); [N-ethyl-pentedrone], ($F_{3.49}$=5.044; P < 0.01); [N,N-diethyl-pentedrone], ($F_{3.49}$=2.802; P < 0.05); [α-PVP], ($F_{3.52}$=4.555, P < 0.01); [α-PpVP], ($F_{3.52}$=4.179; P = 0.01)).

Molecular docking with α-aminovalerophenone derivatives

The most favourable binding mechanism for all the considered compounds was assessed by molecular docking. According to the literature, the binding pocket of DAT can be divided into three subsites (A-C, Figure 37A) (Andersen et al., 2010; Cheng et al., 2015). As expected, the phenyl ring is mainly located in subsite B (Saha et al., 2015), which corresponds to an amphiphilic site where the phenyl group can interact with Phe 76 of TM1 helix through π-interaction. However, results suggest a more stable configuration for α-PpVP, in which the phenyl group was oriented towards the hydrophobic subsite C.

Interestingly, the pyrrolidine group of α-PVP is subtly directed to TM6, renouncing to interact with Asp 79, an amino acid that interacts with methamphetamine indeed (Figure 37B). This is in contrast to N-ethyl-pentedrone, pentedrone and N,N-diethyl-pentedrone, which show similar binding mechanisms stabilising an hydrogen bond between the amino group and Asp 79. The addition of an extra carbon atom in pentedrone seems not to affect the binding mechanism (Figure 37C-D), but the high hydrophobicity of N,N-diethyl-pentedrone contrasts with the high polarity of subsite A and modifies its interaction mechanism (Figure 37E). Nevertheless, the major change is observed in α-PpVP, where the phenyl and propyl groups exchange their orientations in comparison to α-PVP. It may be due to the steric

hindrance found by the piperidine substructure when attempting to fit subsite A and the hydrophobic complementarity obtained when the phenyl group is located within subsite C (Figure 37F).

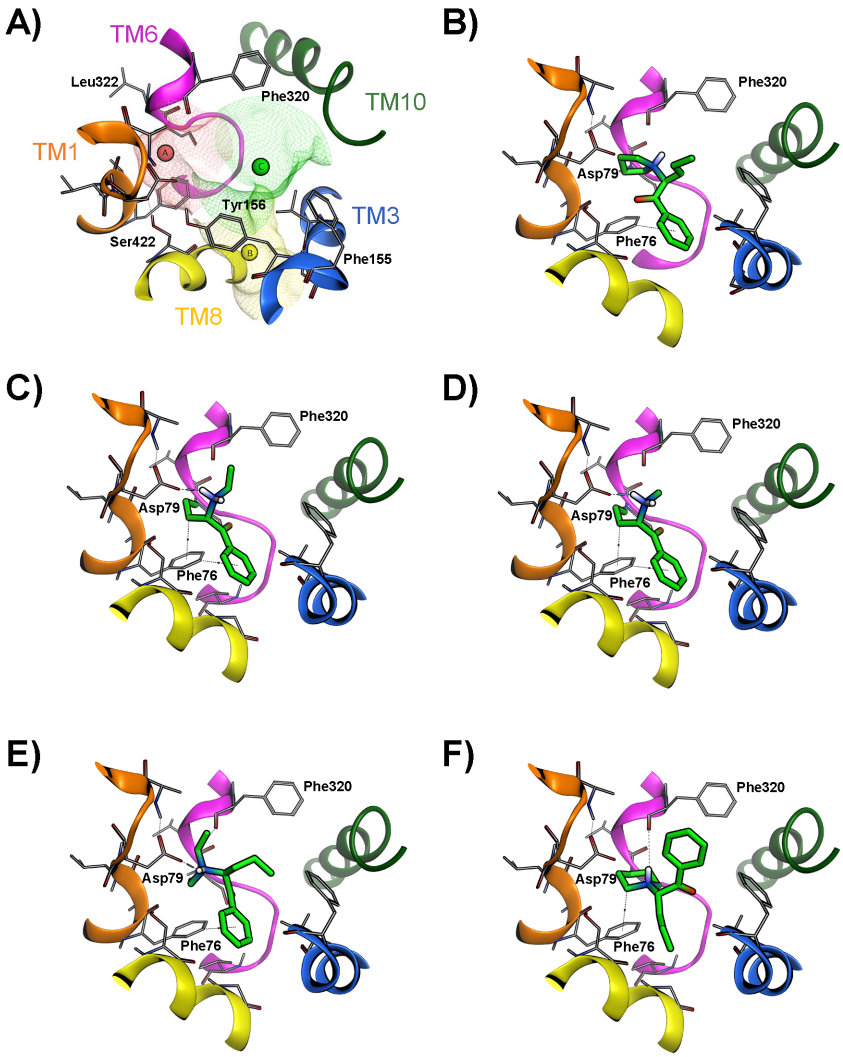

Figure 37. Molecular representation of DAT binding site identifying the three different subsites A to C by colored surfaces (A). Binding mechanism predicted by molecular

docking for α-PVP (B), N-ethyl-pentedrone (C), pentedrone (D), N,N-diethyl-pentedrone (E) and α-PpVP (F).

Predicted binding affinities (approximated as the docking score value) agree with *Ki* experimental data and follow the same order.

3.4.5. DISCUSSION

The emergence of NPS have become a global phenomenon often linked to socioeconomic and health burdens with increased incidences of acute toxicities and fatalities (Baumann and Volkow, 2016; Logan et al., 2017). The main goal of the present study is to characterize the pharmacological profile as well as the psychostimulant and rewarding properties of five different α-aminovalerophenone derivatives, which structurally differ only in their amino substituents (Figure 34). It must be pointed out that the present work highlights pharmacological and behavioural effects of N-ethyl-pentedrone, a novel NPS currently available on the Internet. Moreover, we provide insights into other substances structurally related to pentedrone and α-PVP, which may appear as next generation NPS in the foreseeable future.

A first evidence of the direct blockade of DA and NA uptake was obtained in rat brain synaptosomes. All of the five synthetic cathinones tested in this study are potent DA and NA uptake inhibitors. As with other synthetic cathinones, a positive correlation between DA and NA uptake inhibition (IC_{50} values) was observed. Thereafter, pharmacological profiling of the five drugs from synaptosomal preparations were further corroborated by assays in heterologous systems. The expression of cloned transporters in heterologous systems (in this case HEK293 cells) enabled us to investigate

direct interactions of the synthetic cathinones to a single transporter type in absence of other machinery normally present in synaptosomes. Accordingly, the effect of α-aminovalerophenone derivatives on uptake-1 and uptake-2 inhibition (hDAT, hNET, hSERT and hOCT-2 and hOCT-3, respectively) were evaluated. Similarly to synaptosomal data, uptake inhibition in HEK293 cells demonstrated that all five compounds tested potently inhibit DA and NA uptake but with weak 5-HT uptake inhibition. In spite of that, the IC_{50} values were estimated to allow us to calculate the hDAT/hSERT ratio. Drug selectivity at DAT vs SERT is a key determinant of drug abuse potential for monoamine transporter substrates and inhibitors since a high DAT/SERT ratio is associated with high abuse liability (Negus and Miller, 2014; Negus and Banks 2017). High hDAT/hSERT ratios (> 300) were obtained, indicating that all the α-aminovalerophenone derivatives tested possess a high abuse potential, posing a threat to public health. Many studies have reported similar observations when comparing synaptosomal preparations and heterologous cell line data in order to elucidate the mechanism of action (i.e. identifying blockers or releasers) of different compounds. However, frequent discrepancies in absolute potency estimations (i.e. IC_{50} values) have been found (Baumann et al. 2014; Saha et al. 2015; Sandtner et al. 2016; Mayer et al. 2016b).

We extracted binding affinities of the α-aminovalerophenone compounds to monoamine transporters from membranes extracted from HEK293 cells expressing SERT or DAT. As expected, submicromolar affinity interactions with hDAT were also observed. Importantly, a significant correlation was observed between predicted binding affinities (docking analysis) and *Ki* results for hDAT.

SAR and QSAR studies attempt to explain how a functional group (in our study the substitution at the N-terminal) of a molecule influences

its action at its target. Correlational analysis allows to identify if that action might be related to a specific physicochemical or molecular property of the substituent (Glennon and Dukat 2017). Kolanos et al., (2013) performed a SAR study which "deconstructed" MDPV (3,4-methylenedioxy analogue of α-PVP) and examined which structural moieties account for potent DA uptake inhibition. Regarding the amino-substituent, they conclude that a tertiary amine is the major contributor to the potent effect of MDPV as a DAT blocker, compared to analogues with a secondary and a primary amine. These results agree with the computational simulations obtained by molecular docking in which the molecular geometry defined by the amine induces a differential binding mechanism. Similarly, Glennon and Young (2016) describe that abbreviation of the pyrrolidine ring of MDPV to its simplest tertiary, secondary and primary amine analogues resulted in a progressive decrease in potency at inhibiting DAT. Additionally, Kolanos et al., (2015) reported that when the pyrrolidine ring of α-PVP is expanded to a piperidine ring, there is a several-fold reduction in potency as DAT reuptake inhibitor. The five compounds tested in the present study only differ in their amino-substituent, including secondary (pentedrone, N-ethyl-pentedrone) and tertiary amine analogues (N,N-diethyl-pentedrone, α-PVP and α-PpVP). In our experiments, α-PVP and N-ethyl-pentedrone appeared to be the most potent drugs at inhibiting DAT and NET, followed by α-PpVP. Conversely, N,N-diethyl-pentedrone was the weakest. Taken together, the present *in vitro* data extend previous findings to reveal that the potency of these compounds to block reuptake at hDAT and hNET increases when the amino group expands from a methyl to an ethyl but decreases from a pyrrolidine to a piperidine ring. It is important to highlight the results obtained for N,N-diethyl-pentedrone, which show the lowest affinities and potencies to block uptake in hDAT and hNET. Therefore,

we suggest that possibly being a primary, secondary or tertiary amine may not be the sole contributing factor that may account for potency at DA and NA uptake inhibition. No positive correlation was observed between IC_{50} values of uptake inhibition of the 5 compounds tested at hDAT or hNET with molecular and physicochemical parameters related with lipophilicity (CLogP), molecular surface (Total surface Area and Volume) or steric bulk (CMR). However, a positive correlation was observed between hDAT/hSERT ratio and the CLogP of the N-terminal group; the higher the lipophilicity of the substituent, the higher is its selectivity for hDAT vs hSERT, and therefore, a higher abuse liability. Additionally, a significant correlation was also observed between IC_{50} values of uptake inhibition for the five compounds tested at hSERT and CLogP, Total Surface Area, Volume and CMR. Thus, it seems that the potency inhibiting 5-HT uptake improves with decreasing bulk and lipophilicity of the amino substituent. Accordingly, pentedrone and N-ethyl-pentedrone, which are the substances with the least bulk in the amino-substitution, are more potent in inhibiting hSERT mediated uptake, even though the effect remains being weak. These correlations on hSERT inhibition are extremely useful information and must be corroborated in further research involving other synthetic cathinones with more serotoninergic activity.

In addition, uptake-2 inhibition assays were also performed in order to evaluate the possible effects of such compounds on other monoamine transporters known to be of low-affinity/high-capacity: hOCT-2 and hOCT-3 (Koepsell, 2013; Engel and Wang 2005). OCT-2 participates in reabsorption of endogenous compounds such as choline or monoamine neurotransmitters, and in the reabsorption of ultrafiltrated drugs (Koepsell et al, 1999). OCT-3, also known as extraneuronal monoamine transporter (EMT), even though it is expressed in neurons (Vialou et al., 2008; Gasser et

al., 2017; Mayer et al., 2018), has an special role in neurotransmitter uptake in the brain (Jonker and Schrinkel, 2004; Koepsell et al., 1999; Koepsell and Endou, 2004). The null involvement of hOCT-1-3 in the cocaine transport *in vivo* and *in vitro* has also been described (Chapy et al., 2014). However, a significant inhibition of OCTs by D-amphetamine and MDMA was reported by Amphoux et al., (2006). Meanwhile only hOCT-1 and hOCT-2 were sensitive to cocaine. In addition, amphetamine is known to promote non-exocytotic release of substrates in the presence of cocaine in an OCT-3-dependent manner (Mayer et al., 2018). Recently, Mayer et al., (2019) described that the combination of MDPV and mephedrone interrupts the function of hDAT, hNET and hSERT but promotes release via OCT-3, which result in drastically elevated monoamine levels that may lead to deleterious side effects (Mayer et al., 2019).

Our findings demonstrated that all the five compounds tested significantly inhibit hOCT-2 function with similar potencies. By contrast, and like cocaine (Amphoux et al., 2006), all the compounds tested did not produce any effect on hOCT-3 function.

To assess the psychostimulant and rewarding properties of the five compounds, we used a motor performance and CPP test, respectively, which are standard paradigms in assessing the abuse potential of drugs in animals (FDA/CDER, 2010). As DA and NA uptake inhibitors, all the compounds stimulate locomotion. When comparing the medium doses used for reaching the same locomotor effect, we can conclude that N-ethyl-pentedrone and α-PVP are the substances that needed less dose (3 mg/kg), followed by pentedrone (10 mg/kg), N,N-diethyl-pentedrone (12.5 mg/kg), and finally, α-PpVP (25 mg/kg). The present study also confirms previous reports about psychostimulant effects of α-PVP and pentedrone (Gatch et al., 2015a;

Giannotti et al., 2017; Marusich et al., 2014; Marusich et al., 2016; Wojciesak et al., 2018; Javadi-Paydar et al., 2018; Gatch et al., 2015b; Hwang et al., 2017), and extends this observation to other α-aminovalerophenone derivatives.

As expected, the rank order of *in vivo* potencies seems to go in parallel with their potencies to inhibit hDAT function. The substances that needed less dose to produce the same behavioural effects (N-ethyl-pentedrone and α-PVP), are the ones with highest affinities and potencies at inhibiting hDAT. However, it is important to highlight the result obtained for α-PpVP; despite being one of the most potent substances inhibiting hDAT, a high dose is required to induce the same locomotor effect in relation to other compounds. Considering these results, further studies are needed to fully elucidate the discrepancies observed between the *in vitro* and the *in vivo* behavioural studies for α-PpVP. At this point, we can only hypothesize that some pharmacokinetic and/or metabolic effects such as absorption and/or crossing blood-brain-barrier may be involved in such difference in *in vitro* vs *in vivo* effects.

Finally, we also demonstrated that all the compounds tested rewarding effects. In fact, our results for pentedrone and α-PVP are consistent with previous studies (Hwang et al., 2017; Gatch et al., 2015a). As described by studies where CPP test was used, no dose-response relationship was observed for any drug. However, it is noteworthy to mention that N-ethyl-pentedrone was able to induce conditioning at all the doses tested. Once again, these results demonstrate the high abuse liability of this synthetic cathinone currently available on the drug market. Moreover, the results obtained for α-PpVP are of interest since only the lowest dose induced place-conditioning in spite of not producing hyperlocomotion. The other two doses tested

(25 mg/kg and 75 mg/kg) produce an increase in the locomotor activity but do not exert rewarding properties.

In summary, all of the α-aminovalerophenone compounds studied act as potent DA and NA uptake inhibitors, with weak activity on SERT. Regarding the amino-group, the potency at inhibiting DAT and NET increased from a methyl to an ethyl group and decreased from a pyrrolidine to a piperidine ring. However, we cannot conclude that tertiary amines are more potent DA and NA uptake inhibitors since N,N-diethyl-pentedrone (a tertiary amine) was the weakest compound inhibiting monoamine uptakes. A positive correlation between the hDAT/hSERT ratio and the CLogP of the amino-substituent exists, pointing to a high abuse liability with increased lipophilicity of these substituents. In addition, although these compounds are not potential SERT inhibitors, the potency at inhibiting 5-HT uptake appeared to be inversely proportional to some physicochemical and molecular parameters such as lipophilicity, surface and bulk of the N-substituent. Further research, characterizing other synthetic cathinones with preferential SERT activity is needed in order to corroborate these results. Finally, our study also provides the first evidence that N-ethyl-pentedrone, N,N-diethyl-pentedrone and α-PpVP are able to induce psychostimulant and rewarding effects in mice.

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3.4.7. SUPPLEMENTARY MATERIAL

Synthesis of aminovalerophenone derivatives

The synthesis and characterization of the five cathinones was carried out through three steps, following the procedure formerly described (Meltzer et al., 2006). First of all, the ketone intermediate was prepared by alkylation of the nitrile compound (Step 1), followed by acidic hydrolysis. The n-BuMgCl was added in a dropwise manner to a solution of benzonitrile in toluene in anhydrous conditions. After three hours at room temperature, the reaction was complete. A solution of H_2SO_4 (4%) was added into the reaction mixture previously cooled. The organic layer was extracted with Et_2O, dried ($MgSO_4$), filtered and reduced in vacuo to an oil. The α-bromination reaction was carried out by adding bromine in a dropwise manner to a solution of the ketone intermediate in Et2O in presence of $AlCl_3$ in catalytic amounts (Step 2). The excess of bromine is neutralized with a solution of $Na_2S_2O_3$. The organic layer is separated, dried ($MgSO_4$), filtrated and reduced in vacuo to an oil. The αbromoketone was dissolved in Et_2O, and the corresponding amine (methylamine, ethylamine, diethylamine, pyrrolidine and piperidine), was added all at once (Step 3). After 24-48 h the reactions were completed. The reaction mixtures were extracted with HCl 1 N and then back-extracted into Et_2O by basification to pH 10 with NaOH 1 M. The organic layers were dried ($MgSO_4$), filtered and reduced in vacuo to an oil. Each product was dissolved in EtOH and a mixture of Et_2O with HCl (3 M) in CPME was added in a dropwise manner in order to obtain the hydrochloride salt. Solids were collected by filtration. The identification of the five compounds was assessed by proton nuclear magnetic resonance (^{1}H NMR) ($CDCl_3$) yielding the following results:

α-methylaminovalerophenone hydrochloride (pentedrone): δ 10.55 (s, 1H), 9.16 (s, 1H), 7.96 (dd, J = 8.4, 1.2 Hz, 2H), 7.70 – 7.64 (m, 1H), 7.53 (t, J = 7.8 Hz, 2H), 4.94 (s, 1H), 2.81 (s, 3H), 2.30 – 2.16 (m, 1H), 2.16 – 2.02 (m, 1H), 1.61 – 1.46 (m, 1H), 1.45 – 1.29 (m, 1H), 0.89 (t, J = 7.3 Hz, 3H); α-ethylaminovalerophenone hydrochloride (N-ethyl-pentedrone): δ 7.99 – 7.98 (d, J = 7.4 Hz, 2H), 7.72 – 7.68 (t, J = 7.4 Hz, 1H), 7.58 – 7.54 (t, J = 7.6 Hz, 2H), 6.99 (s, 0H), 4.97 (s, 1H), 3.24 (s, 1H), 3.04 (s, 1H), 2.33 – 2.29 (d, J = 12.4 Hz, 1H), 2.21 – 2.14 (m, 1H), 1.56 – 1.53 (t, J = 7.2 Hz, 3H), 1.48 – 1.32 (m, 2H), 0.90 – 0.86 (t, J = 7.2 Hz, 3H); α-diethylaminovalerophenone hydrochloride (N,N-diethyl-pentedrone): 8.18 - 8.10 (m, 2H), 7.75 (ddt, J = 7.9, 7.0, 1.2 Hz, 1H), 7.66 - 7.57 (m, 2H), 5.34 (dd, J = 6.3, 4.9 Hz, 1H), 3.48 (dq, J = 14.6, 7.3 Hz, 1H), 3.36 - 3.25 (m, 3H), 3.11 (dq, J = 14.3, 7.3 Hz, 1H), 2.06 - 1.92 (m, 2H), 1.41 (t, J = 7.3 Hz, 3H), 1.33 (t, J = 7.3 Hz, 3H), 1.30 - 1.12 (m, 3H), 0.86 (t, J = 7.3 Hz, 3H); α pyrrolidinovalerophenone hydrochloride (α-PVP): δ : 12.42 (s, 1H), 8.02-7.96 (m, 2H), 7.72-7.67 (m, 1H), 7.58-7.54 (m, 2H), 5.25 (dt, J = 8.0, 5.1 Hz, 1H), 3.82 (m, 2H), 3.62 (m, 1H), 2.95 (dq, J = 10.5, 7.7 Hz, 1H), 2.03 (m, 2H), 2.19 (m, 3H), 1.35 (m, 1H), 1.47 (m, 1H), 0.89 (t, J = 7.3 Hz, 3H); α- piperidinevalerophenone hydrochloride (α-PpVP): δ : 12.40 (s, 1H), 8.02 – 7.97 (m, 2H), 7.73 – 7.67 (ddt, J = 7.4 Hz, 2H), 7.58 – 7.52 (m, 1H), 5.03 (dt, J = 10.3, 3.8 Hz, 1H), 3.67 (d, J = 12.0 Hz, 1H), 3.51 – 3.45 (m, 1H), 3.42 – 3.37 (d, J = 21.1 Hz, 1H), 2.75 – 2.64 (m, 1H), 2.53 – 2.45 (m, 1H), 2.41 – 2.33 (m, 1H), 2.30 – 2.17 (m, 1H), 2.03 – 1.97 (m, 2H), 1.96 – 1.87 (m, 2H), 1.75 – 1.63 (d, J = 12.5 Hz, 1H), 1.47 – 1.30 (m, 2H), 0.91 (t, 3H);

Chemical purity of the obtained compounds was also assessed by thin layer chromatography ^{1}H NMR, ^{13}C NMR and mass spectrometry. All analytical data were consistent with the assigned structure with over 98% purity for the cathinone derivative.

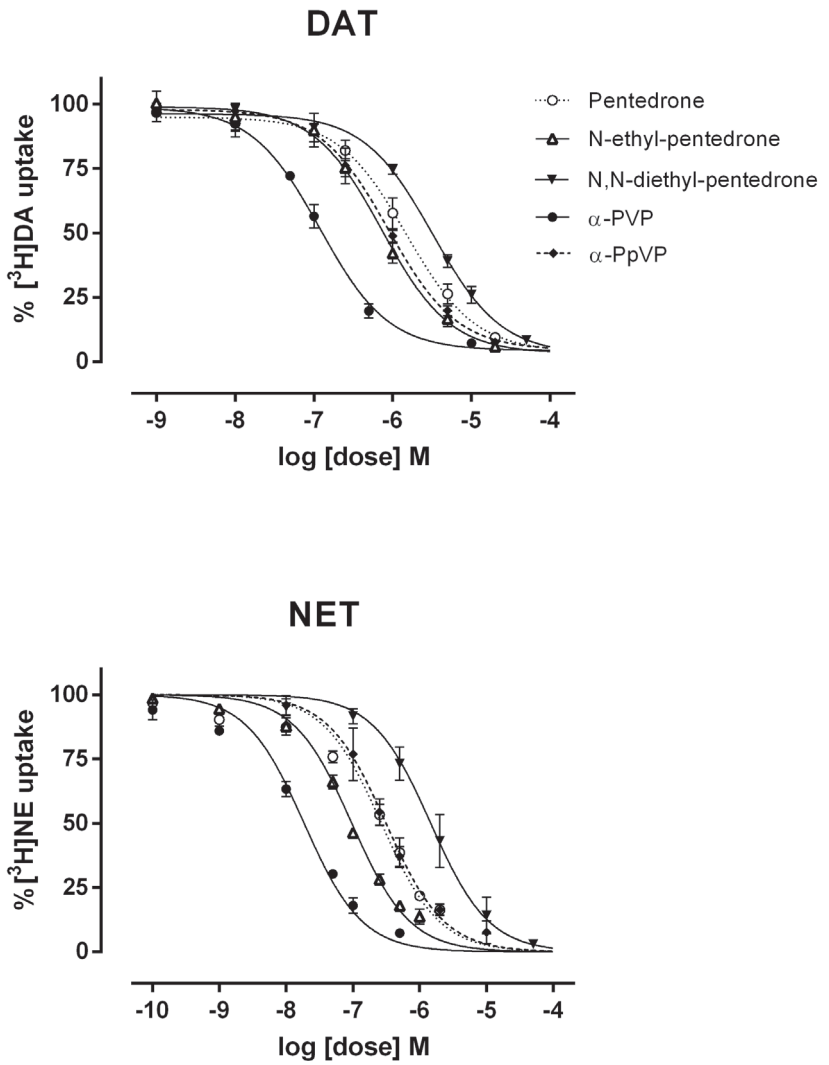

Figure S1. Effects of α-aminovalerophenone derivatives on [³H]DA and [³H]NA uptake inhibition in rat brain synaptosomes. *Data are expressed as a percentage of control uptake (mean ± SD) of 3-4 independent experiments performed in duplicate.*

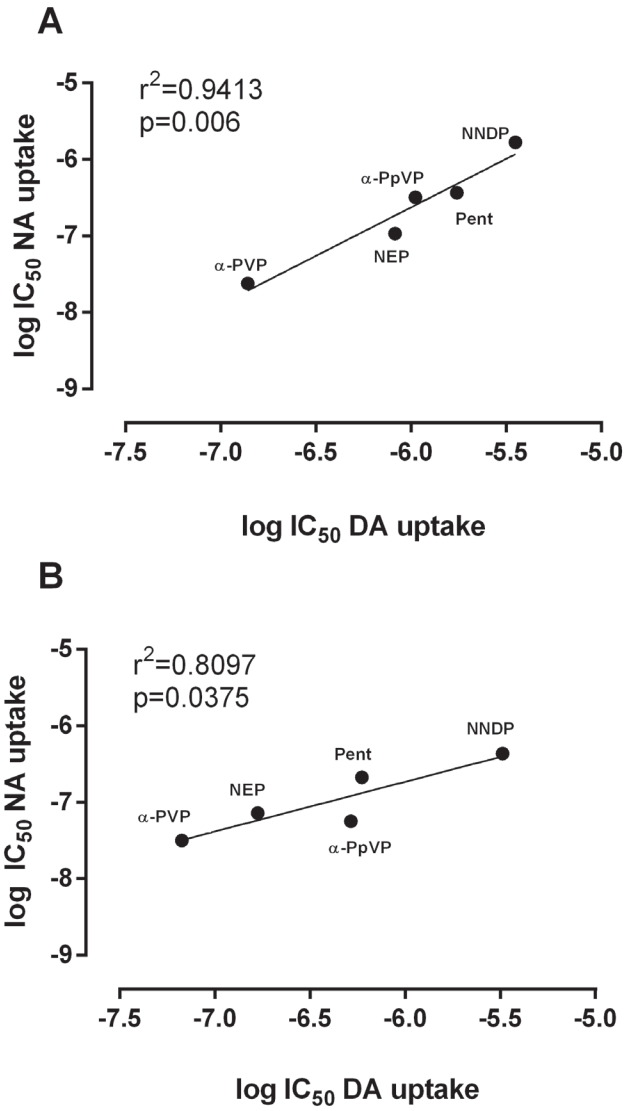

Figure S2. Correlations between logIC$_{50}$ values at dopamine (DA) and noradrenaline (NA) transporters by α-aminovalerophenone derivatives, assessed either in rat brain synaptosomes (A) or transfected HEK293 cells (B). *Pent: Pentedrone; NEP: N-ethyl-pentedrone; NNDP: N,N-diethyl-pentedrone.*

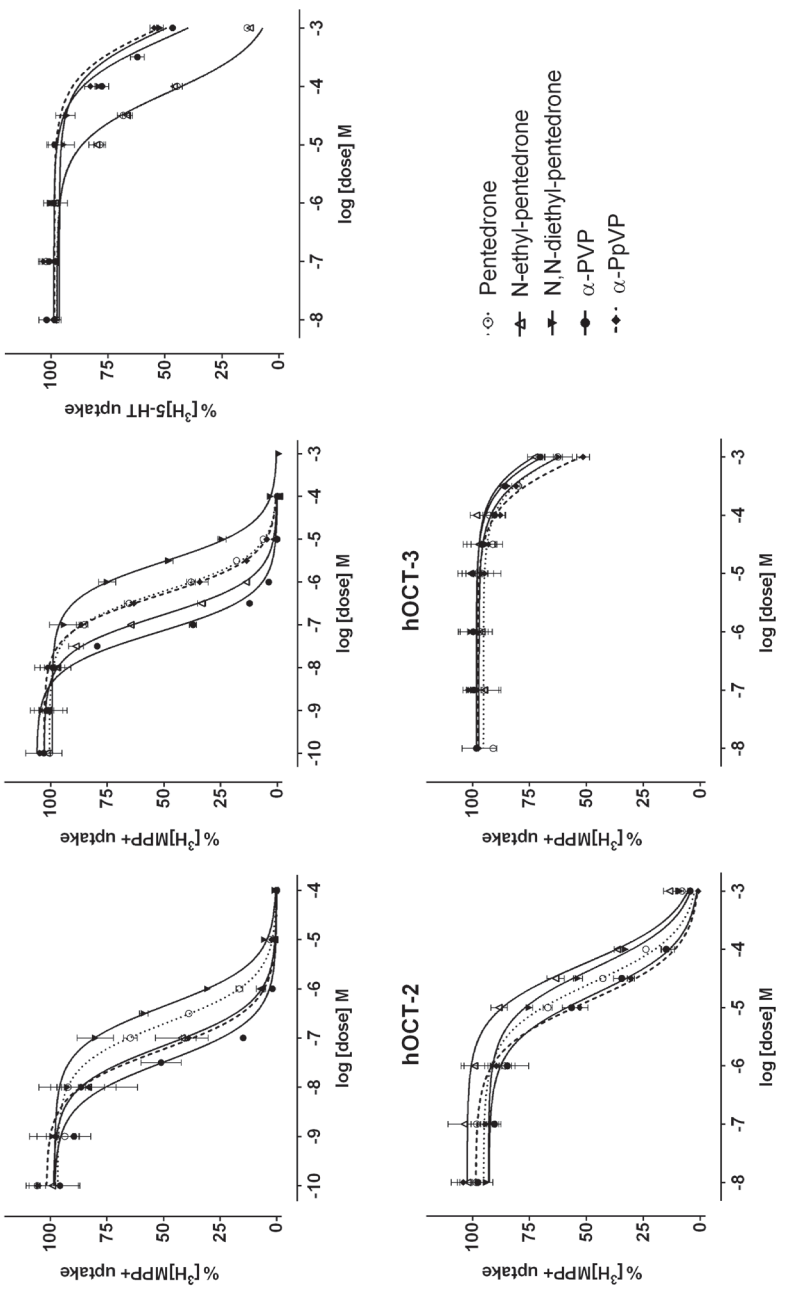

Figure S3. Effects of α-aminovalerophenone derivatives on [³H]MPP⁺ uptake inhibition at hDAT, hNET, hOCT-2 and hOCT-3, and [³H]5-HT uptake inhibition at hSERT in HEK293 cells. *Data are expressed as a percentage of control uptake (mean ± SD) of 3 independent experiments performed in triplicate.*

Table S3. Correlation analysis between molecular or physicochemical descriptors and log IC_{50} or hDAT/hSERT ratio values of monoamine uptake in transfected HEK293 cells.

	Log IC_{50} hDAT		Log IC_{50} hNET		Log IC_{50} hSERT		Log hDAT/hSERT ratio	
	r^2	p	r^2	p	r^2	p	r^2	p
CLogP	0.082	P > 0.05	0.008	P > 0.05	**0.917**	**P < 0.05**	**0.806**	**P < 0.05**
Total Surface Area (Å^2)	0.013	P > 0.05	0.066	P > 0.05	**0.922**	**P < 0.01**	0.639	P > 0.05
Volume (Å^3)	0.024	P > 0.05	0.050	P > 0.05	**0.925**	**P < 0.01**	0.677	P > 0.05
CMR	0.045	P > 0.05	0.029	P > 0.05	**0.926**	**P < 0.01**	0.734	P > 0.05

CLogP; partition coefficient.

CMR; calculated molar refractivity

4 DISCUSSION

4.1. Part I: MDPV

The tidal wave of new emerging psychoactive substances has completely modified the drug scene and the current landscape of addiction. Currently, hundreds of new designer drugs are being consumed by millions of people worldwide regardless most of them are barely known. Safety data on toxicity and carcinogenic potential of many NPS are not available or very limited, and information on long-term adverse effects or risks derived from their consumption are still largely unexplored. Furthermore, purity and composition of products containing NPS are often unknown, which places users at a high risk as evidenced by hospital emergency admissions and deaths, sometimes associated with polydrug use (UNODC, 2019a). To date, not only the authorities and health professionals but also the general population need to be clearly informed and aware of the dangers derived from NPS spread and use. In this regard, this doctoral dissertation aims to contribute to scientific knowledge by providing new insights about MDPV, that is, about the neuroadaptive changes underlying its abuse, addictive properties, behavioural effects, as well as about its relationship with cocaine, the most consumed psychostimulant worldwide.

4.1.1. Behavioural sensitization to MDPV and cocaine

By way of reminder, behavioural sensitization to psychostimulants is the phenomenon whereby repeated intermittent exposure to drugs results in a progressive and enduring increase in the motor stimulant response to the drug (Kalivas et al., 1993; Kuczenski & Segal, 1969; Pierce & Kalivas, 1997; Robinson & Berridge, 1993; Steketee & Kalivas, 2011). It has been largely associated with dynamic changes in neural processes induced by repeated drug exposure (Nestler, 2001b; Robinson & Berridge, 1993). The fact that different addictive drugs produce the same physiological effects suggests that they may exert some of their effects through the same neural mechanisms. Indeed, cross-sensitization between addictive drugs has already been described (Lessov & Phillips, 2003; Vezina & Stewart, 1989). In this context, since cocaine and MDPV share the same mechanism of action, a cross-sensitization between them could be expected.

A first evidence of the sensitizing properties of MDPV was obtained in a preliminary research of our group (Buenrostro-Jáuregui et al., 2016). In this study we found out that repeated exposure to a low dose of MDPV (0.3 mg/kg, once daily for 5 days) during adolescence, induced behavioural sensitization not only to itself but also to cocaine, providing thus a first evidence for a relationship between MDPV and cocaine. In a second study, furthermore, we reported that repeated exposure to a moderate dose of MDPV (1.5 mg/kg, twice daily, for 7 days) during adolescence could effectively potentiate cocaine abuse liability in adulthood by sensitizing the neural circuitry underlying associations between cocaine and its related stimuli (López-Arnau et al., 2017).

The present thesis (Chapter 2) not only corroborates the sensitizing properties of an intermediate regime of MDPV (1.5 mg/kg, once daily for

5 days), but also provides a first evidence of the existence of a cross-sensitization between cocaine and MDPV. Therefore, repeated administration of MDPV or cocaine sensitizes to cocaine and MDPV locomotor effects. This finding is on chief significance since the sensitization phenomenon has been frequently proposed to influence susceptibility to drug-craving and drug-relapse by increasing the reinforcing value of an acute drug administration, even after long withdrawal periods (Camí & Farré, 2003; Robinson & Berridge, 1993). Hence, we have demonstrated that repeated use of one of these psychostimulants may favour relapse when a new dose is taken after withdrawal, but it also may favour the onset of an addiction to the other drug when taken for the first time. Altogether, from a clinical point of view, this feature represents a basic step to provide new knowledge about factors involved in the vulnerability to cocaine and MDPV addiction.

Furthermore, it is well known that MDPV is from 10 to 50-fold more potent as a DAT blocker than cocaine (Baumann et al., 2013; Simmler et al., 2013), and therefore, that it exerts more powerful behavioural responses. In our study we corroborate this statement since we could observe that a dose of MPDV of 1.5 mg/kg induced a higher locomotor activity than a dose of cocaine 10 times higher, 15 mg/kg.

The process of behavioural sensitization includes two distinct phases: initiation and expression. The initiation phase refers to the period during which the increased behavioural response following daily drug administration is associated with an increase in extracellular DA concentration. Behavioural sensitization continues to increase after the cessation of drug administration, and this procedure produces long-lasting sensitization, known as the expression of sensitization (Baik, 2013). If we look at the expression of sensitization (challenge day, 10 days after drug

cessation), we could say that repeated administration of cocaine 15 mg/kg or MDPV 1.5 mg/kg induce equivalent sensitization, thus, in this regard, those doses can be considered equieffective.

Finally, it is important to point out that, in Chapter 3, when following a treatment schedule which mimics that regularly used for CPP experiments (MDPV 2 mg/kg or cocaine 10 mg/kg, once daily for 4 days), we observed behavioural sensitization to the motor effects of cocaine but not to MDPV. One the one hand, regarding cocaine-induced sensitization, our result is consistent with previous studies (Chapter 2, Ferrer-Pérez et al., 2019, 2018). However, on the other hand, we must take into account that although sensitization to MDPV locomotor effects has already been reported to date by our group (Chapter 2, Buenrostro-Jáuregui et al., 2016; López-Arnau et al., 2017) and others (Berquist et al., 2016; Kohler et al., 2018; Watterson et al., 2016), the drug administration regime that we used in our last study (Chapter 3) was completely different. For instance, the mentioned authors obtained a significant increase in the motor response to MDPV when administering doses of 0.5 or 1 mg/kg to Sprague-Dawley rats, once daily for 7 days. Similarly, as mentioned above, we reported locomotor sensitization when administering also lower doses (0.3 and 1.5 mg/kg). Conversely, and in accordance with our results from Chapter 3, when higher doses of MDPV were used in other studies, drug sensitization was no longer observed (Watterson et al., 2016). Furthermore, cross-sensitization between MDPV and cocaine or methamphetamine has also been described but with lower doses of MDPV (Berquist et al., 2016; Kohler et al., 2018; Watterson et al., 2016). Overall, it seems reasonable to hypothesize that the higher dose employed in our latest study could have induced an intense motor response that could have reached a ceiling effect, being the reason why we did not observe sensitization to MDPV. In addition, this lack of drug sensitization

may be a result of increasing levels of stereotypic behaviour at high doses, which has already been reported (Aarde et al., 2013; Fantegrossi et al., 2013).

4.1.2. Behavioural effects after a sensitization regime of MDPV

The drug administration protocol followed in Chapter 1 (MDPV 1.5 mg/kg, twice daily, for 7 days), besides inducing behavioural sensitization to itself and to cocaine, caused, additionally, other behavioural abnormalities.

Firstly, mice treated repeatedly with MDPV showed increased anxiety-like responses in the EPM that were apparent mainly long after treatment, when animals had already become adults. Such anxiety-like behaviour encouraged us to test other behavioural abnormalities in the OF, a test that provides an initial screen for locomotion, anxiety and emotional-related behaviours (Bailey & Crawley, 2009). After MDPV treatment, mice displayed a different behaviour: they ventured more frequently into the central area of the OF, where they stayed longer. This conduct is considered atypical because it involves leaving a defensive zone to enter in a more exposed one, so it opposes to the rodents' natural aversion to brightly lit open areas. Moreover, it reflects a non-anxious-related behaviour. However, considering the results obtained in the EPM paradigm, we could not attribute this atypical behaviour to an anxiolytic effect of MDPV, but to an increased willingness to explore. Additionally, repeated exposure to the OF is a method for assessing habituation to an increasingly familiar environment. Accordingly, our results can be interpreted as a faster habituation to a new environment induced by MDPV, favouring a risky behaviour (Clément et al., 1995). In fact, increased exploration in the

centre along with a major number of visits to the same area by the rodents is interpreted as a tendency for novelty-seeking and risk-taking behaviour, which, in turn, has been associated with predisposition to rewarding and addictive behaviours (Valvassori et al., 2017). Consistent with our results, the same pattern has also been observed with other drugs, for example, short after a prolonged treatment with MDMA (Abad et al., 2014), which, in addition, also induced a lasting anxiety-like response (Rodríguez-Arias et al., 2011).

Finally, case studies have reported that MDPV, as other synthetic cathinones, induces an aggressive behaviour in humans (James et al., 2011; Murray et al., 2012; Penders et al., 2012). In this sense, our study is, to our knowledge, the first that really proves that MDPV exposure can increase aggressiveness in mice, as we evidenced that animals were more aggressive shortly after MDPV withdrawal, albeit such greater aggressiveness faded over time. Afterwards, our results have been corroborated by other research groups (De-Giorgio et al., 2019).

4.1.3. Neuroadaptive changes underlying MDPV abuse

Repeated exposure to a drug causes repeated perturbation of the intracellular pathways, which first initiates and then maintains the long-lasting molecular and cellular adaptations that underlie addiction. Hence, the process of addiction can be considered as a form of drug-induced neural plasticity (Nestler, 2001a; Nestler et al., 1993). In this regard, we aimed to elucidate the neuroadaptive changes, that is changes in the expression of specific neural biomarkers, underlying such sensitization observed to

cocaine and MDPV, as well as the related abnormal behaviours. For that, we focused especially on the DR1 – cAMP and the BDNF – TrkB intracellular signalling pathways. In this sense, the present thesis aims to describe the molecular changes occurred in reward-related brain areas (especially in the striatum and the cortex) at two different moments during forced abstinence: shortly after exposure to MDPV (1, 2 or 24 h after the last dose) or later after (10, 12 or 21 days after treatment).

The discussion collects and jointly discusses all the results obtained for the same biomarker throughout all the chapters. Importantly, although MDPV and cocaine share their mechanism of action – both drugs are mainly DAT blockers – and are similar enough to produce cross-sensitization, some differences in the intracellular responses that they trigger must be highlighted and thus will be explained in detail all along the discussion.

DR2:DR1 ratio

Drugs of abuse elevate brain DA levels, and *in vivo*, chronic drug use is generally accompanied by a selective decrease in striatal DR2 availability in the brain (Nader et al., 2006; Volkow et al., 1991). Accordingly, the ratio of DR2:DR1 signalling alters towards DR1. However, such reductions on DR2 availability recover during abstinence. In this sense, DR2 availability is predictive of future seeking behaviour (Nader et al., 2006). Furthermore, changes in the ratio DR2:DR1 towards DR1, either by downregulation of DR2s or upregulation of DR1s, are considered to contribute to cocaine-induced behavioural sensitization (Thompson et al., 2010). Similarly, in Chapter 1, repeated exposure to a sensitizing regime of MDPV induced an initial increase of DR1 population and a DR2 reduction (López-Arnau et al., 2017). Hence, the DR2:DR1 ratio altered towards DR1. Nevertheless,

after three weeks of abstinence, DR1 population decreased substantially and DR2 availability returned to normal.

Addiction-related changes may reflect increased motivation for drugs but reduced pharmacological impact on reward processes (tolerance), leading to compensatory increases in drug intake. In this context, Edwards et al., (2007) suggested that such changes could result in enhancing DR2 and reducing DR1 responsiveness, respectively. Furthermore, the emergence of these features from early to late withdrawal, as it occurred in our study with MDPV, parallels time-dependent increases in drug-seeking behaviours that have been shown to persist from weeks to months following chronic cocaine self-administration. Thus, reduced DR1 function along with increased DR2 function, seem to exacerbate relapse to cocaine use despite efforts to abstain. As we obtained similar results in the pattern of expression of DR1 and DR2 populations in early and late withdrawal, we could hypothesize the same regarding the role of such receptors in MDPV-related behaviours.

Furthermore, Chen et al., (2005) observed a positive association of reduced DR2 and aggressive and pathological violent behaviours. Therefore, we cannot rule out the possibility such decrease in DR2 population evidenced early after MDPV exposure could contribute, in addition, to the aggressiveness observed at the same time. Moreover, this behavioural effect faded during withdrawal, when DR2 availability returned to normal.

ΔFosB

ΔFosB is one of the most studied transcription factors in drug addiction. It is known to play a role in drug-induced hyperlocomotor response, the rewarding effects and relatively prolonged sensitization to

drugs. For instance, there is compelling evidence that ΔFosB is involved in close to one quarter of all the genes influenced by chronic cocaine exposure in the NAcc (McClung & Nestler, 2003), thus its expression is involved in the increased locomotor and rewarding responses to cocaine and causes increased cocaine-seeking behaviour and self-administration (Whisler et al., 1999).

Repeated exposure to MDPV also provokes an enduring accumulation of ΔFosB in ventral striatum, which, in addition, persisted for a long period of time (López-Arnau et al., 2017). Moreover, such striatal accumulation of ΔFosB, along with an increase in CREB expression, has been associated to MDPV-induced behavioural sensitization (Buenrostro-Jáuregui et al., 2016; López-Arnau et al., 2017).

Nevertheless, in Chapter 1, following the same drug administration protocol as López-Arnau et al., (2017) (MDPV 1.5 mg/kg, twice daily for 7 days), we also observed an increase of ΔFosB protein levels within the OFC, which, in turn, was even higher than that observed in VS. Moreover, the abnormalities observed after a repeated MDPV exposure co-occurred with such increase of ΔFosB protein levels within the OFC. Importantly, this feature has already been related not only to increased risk-taking behaviours, but also to locomotor sensitization to cocaine (Winstanley et al., 2009a, 2009b). Therefore, we cannot rule out the possibility that such increase of ΔFosB levels in the OFC is involved in both behavioural abnormalities observed after MDPV exposure: behavioural sensitization and increased risk-taking behaviour. Hence, we extend knowledge suggesting that behavioural sensitization to MDPV might be associated not only to the accumulation of ΔFosB in VS, but also within the OFC.

Consistent with our previous findings, in Chapter 3, we also observed a long-lasting accumulation of ΔFosB in VS after repeated MDPV exposure (2 mg/kg, once daily for 4 days) which, in addition, persisted for at least

12 days after drug withdrawal. Such overexpression, furthermore, correlates with the long time needed to extinguish memories associated with MDPV after CPP conditioning, suggesting an active role of ΔFosB underlying the rewarding effects of MDPV. Conversely, following our treatment schedule, cocaine exposure (10 mg/kg) did not induce ΔFosB accumulation, which correlates with the shorter time needed to extinguish the preference in comparison to MDPV-conditioned animals.

ΔFosB primarily functions as a transcriptional activator, while it acts as a repressor for a small subset of genes (McClung & Nestler, 2003). Interestingly, this differential activity of ΔFosB is a function of the duration and the degree of its expression (Nestler, 2008). From our studies, a different pattern of expression of ΔFosB target genes and other related biomarkers has been observed in comparison to those induced by cocaine, strengthening the belief that MDPV and cocaine are siblings rather than identical twins, so they trigger different intracellular responses.

c-Fos

One of ΔFosB target genes is *c-fos*. Induction of c-Fos is considered an early marker of neural activation, and it is also essential for the behavioural responses to cocaine (Zhang et al., 2006). Renthal et al., (2008) demonstrated that ΔFosB mediates an epigenetic desensitization to the *c-fos* gene after chronic exposure to a psychostimulant, which helps to create the molecular switch that enables its own accumulation. In accordance, in López-Arnau et al., (2017) we corroborated such negative association between both factors in a way that, although a challenge of cocaine significantly increased c-Fos expression as an undoubtedly reflection of neuronal activation by the drug, such increase was less pronounced in MDPV-pretreated animals, which

presented high ΔFosB protein levels. Hence, our results are in line with previous findings demonstrating that repeated injections of cocaine cause blunted responses to acute cocaine-induced increases in the expression of immediate early genes, including *c-fos* (for a review see McCoy et al., 2011).

In Chapter 3, however, we found that after a cocaine injection given during the extinction of previously drug-conditioned animals, the transcription of the *c-fos* gene modestly decreased. Conversely, when animals were challenged with MDPV, no changes in c-Fos mRNA were observed.

Altogether, at this point, some controversy regarding c-Fos expression must be noted since our results from Chapter 3 seem inconsistent with what we found previosly in López-Arnau et al., 2017. The main differences between our two studies were: the drug administration protocol followed, the mouse strain used, the brain area and cellular compartment examined and the biomolecule quantified. Therefore, some of these parameters must be affecting differently the expression of c-Fos. Although both treatments induced the accumulation of ΔFosB within the VS, an increment that, in fact, was higher after the treatment followed in Chapter 1, consistent with the higher exposition to MDPV; a new drug exposure of sensitized animals after a withdrawal period, triggered an opposite response on c-Fos expression. Concretely, a cocaine injection increased c-Fos protein levels in dorsal striatum of MDPV-pretreated animals (Chapter 1), while it decreased c-Fos mRNA in ventral striatum of MDPV- and cocaine-pretreated mice in Chapter 3, both observations performed at the same time-point: 2 h after the acute drug injection.

Under basal conditions, c-Fos mRNA and protein are expressed at very low, barely detectable, levels in most brain regions. Following induction,

c-Fos protein primarily accumulates in the nucleus. However, the time course of c-Fos mRNA and protein expression runs differently: mRNA levels peak between 30 and 60 min whereas protein levels peak between 1 and 3 h following an acute challenge, and this expression returns to basal levels within 4-6 h. Moreover, the half-life of c-Fos mRNA is approximately 15 min, therefore, mRNA encoding c-Fos should return to normal levels when examining at a time-point optimal for observing peak protein levels. Therefore, at a same time-point, nuclear c-Fos protein expression would correspond to earlier exposure to an stimulus, while total mRNA expression would correspond to later exposure to a different stimulus. Nevertheless, some exceptions to this time course must be noted, including delayed or prolonged patterns of *c-fos* activation. Consequently, the presence or absence of changes in c-Fos can depend on many factors, including whether mRNA or protein levels are being examined. Traditionally, it is assumed that changes in mRNA will always lead to changes in protein expression. However, there are multiple additional factors that can influence protein expression, including post-transcriptional modifications, inefficient translation, rapid mRNA decay or protein degradation and therefore changes in *c-fos* are not always consistent with its protein levels. Furthermore, c-Fos activation only occurs upon robust stimulation, thus, sometimes to capture subtle changes in response to weaker stimulus might be difficult. Even though qPCR and western blotting are quantitative approaches that allow an accurate assessment of mRNA and protein expression changes, these approaches lack the spatial resolution as they require bulk tissue dissection. Thus, subtle differences can go unnoticed when analysing larger samples (i.e. the ventral striatum instead of the NAcc or the whole cellular lysate instead of the nuclear). Taken together, a failure of an experiment to influence c-Fos expression may be a consequence of several interacting factors, so it is

necessary to be cautious when interpreting null findings (see McReynolds et al., 2018 for more in-depth discussion). Alternatively, we cannot exclude the possibility that such differences might be due to the mouse strain used: Swiss CD-1 (Chapter 1) or OF1 (Chapter 3) mice. In this context, further studies need to be performed to understand why and how *c-fos* expression tends to decrease after an acute administration of psychostimulants given during the CPP extinction phase in OF1 -conditioned mice.

CDK5

Cdk5 is another widely recognized target gene for ΔFosB, whose activation, however, is not only under positive control of ΔFosB, but it is also regulated by the extracellular signal-regulated kinase (ERK), the phosphorylation of which is increased in the NAcc by drugs of abuse through a DR1-dependent mechanism (Valjent et al., 2004). *Cdk5* is an example of a gene that is induced by chronic, but not acute, cocaine administration (Bibb et al., 2001).

Unlike other cyclin-dependent kinases, CDK5 has its own activation factors: p35 and p39. However, it can be also activated by p25 and p29, the calpain-mediated cleavage products of p35 and p39. It is thought that when CDK5 is activated by p35 or p39, it takes part in physiological processes, while CDK5/p25 and CDK5/p29 are related with neurotoxic and neurodegenerative processes, that is an aberrant and pathological activity of the protein (Angelo et al., 2006; Cheung & Ip, 2007; Hawasli & Bibb, 2007). For instance, CDK5/p25 seems to hyperphosphorylate Tau, mainly at Thr 205 and Ser 202, which aggregates as tangles in the neurons of Alzheimer's disease patients, impairing learning and memory (Patrick et al., 1999).

In Chapter 1 we observed that CDK5 is induced by chronic MDPV administration (1.5 mg/kg, twice daily for 7 days), as occurs with cocaine, and, apparently, such increment was not accompanied by an aberrant and pathological activation of the protein nor by an alteration of the levels of phospho-Tau at Thr 205. Moreover, it is noteworthy that unlike ΔFosB, whose levels declined over time, the expression of CDK5 remained stable during the whole withdrawal, that is for at least 3 weeks after drug cessation. Therefore, we can suggest that the hyperdopaminergic state observed in mice after repeated exposure to MDPV might activate the mitogen-activated protein kinase pathway (MAPK/ERK) which would contribute to the stable induction and expression of CDK5 besides ΔFosB.

Additionally, in Chapter 3, we also measured CDK5 protein levels in ventral striatum after repeated exposure to MDPV (2 mg/kg, once daily for 4 days), and, under these conditions, we did not find any modification in its expression. Hence, it is likely that the induction of CDK5 by drugs may depend on the drug administration protocol and the dose. It is known that CDK5 phosphorylates DARPP-32 at Thr 75, which results in a decreased DR1/cAMP signalling, considered to be as an adaptative change to drug addiction, concretely, an homeostatic negative feedback against the behavioural effects of drugs (Bibb et al., 2001; Nishi et al., 2011). In this context, we can hypothesize that the administration regime used in Chapter 3 may have not been intense enough to generate such a homeostatic response, thus inducing changes in CDK5 expression, while the regime used in Chapter 1 was.

Regarding cocaine (10 mg/kg, once daily for 4 days), no changes in CDK5 expression were observed, albeit no ΔFosB accumulation was evidenced either.

GluA2

ΔFosB induces GluA2 expression in the NAcc (Kelz et al., 1999). GluA2 is an AMPA receptor subunit that reduces permeability to Ca^{2+} and thus, decreases its overall current (Song & Huganir, 2002).

Growing evidence indicates that cocaine indirectly influences glutamate transmission producing persistent maladaptive forms of neuroplasticity that lead to cocaine-seeking behaviour (for a review see Schmidt & Pierce, 2010). It is known that cocaine increases extraneuronal levels of glutamate in the NAcc shortly after its administration (Smith et al., 1995), which decreased along withdrawal (Baker et al., 2003). Consistent with these results, several studies have shown that AMPA receptors contribute to the reinstatement of cocaine-seeking in such a way that the administration of AMPA receptor agonists directly into the NAcc promote reinstatement of cocaine seeking, while an antagonist completely blocks it (for a review see Schmidt & Pierce, 2010).

Nonetheless, studies examining glutamate receptor subunit mRNA and protein expression in the NAcc of animals receiving non-contingent injections of cocaine and in animals self-administering intravenous injections of cocaine report inconsistent findings. For instance, although some studies have demonstrated no changes in the expression of GluA2 subunits in the NAcc after withdrawal from either cocaine-self administration (Conrad et al., 2008; Ghasemzadeh et al., 1999) and non-contingent cocaine repeated exposure (Churchill et al., 1999), others have reported increased GluA2 levels after cocaine self-administration (Lu et al., 2003).

Additionally, cocaine withdrawal in cocaine-sensitized animals has been associated with a slowly developing redistribution and subsequent

increased expression of AMPA receptors in the surface of the NAcc neurons (Boudreau et al., 2007; Boudreau & Wolf, 2005; Conrad et al., 2008), which, in turn, internalize after a cocaine challenge (Boudreau et al., 2007). Some controversy must be noted since Boudreau et al., reported an increased surface/intracellular ratio for GluA2 subunit 14 and 21 days after non-contingent repeated cocaine exposure but not on day 1, whereas Conrad et al., showed a small increase of such ratio only on withdrawal day 1 but not on day 45 after cocaine self-administration. Instead, Conrad et al., (2008) reported that after prolonged withdrawal from cocaine, the normal complement of GluA2-containing AMPA receptors is supplemented by the addition of GluA2-lacking receptors (GluR1/3 and/or homomeric GluR1).

Collectively, these studies indicate that repeated exposure to cocaine followed by a period of forced abstinence, when extracellular glutamate levels are decreased in the NAcc, is associated with increased numbers of synaptic AMPA receptors combined with the higher conductance of GluA2-lacking AMPA receptors, which may increase reactivity of neurons to cocaine-related cues, resulting in intensification of drug craving and relapse.

In our study (Chapter 1), we did not observe any effect on GluA2 subunit expression shortly after repeated non-contingent administration of MDPV, as it has been described for cocaine (Churchill et al., 1999). However, and by contrast to cocaine, GluA2 subunit decreased substantially over withdrawal (day 21) and an apparent internalization of the subunit was suspected, even though it did not reach statistical significance. Therefore, although the neuroadaptations produced during cocaine and MPDV withdrawal appear to be different, the final net result does not seem to differ as much: there is a lower presence of GluA2-containing AMPA receptors on the cell surface, and thus, a higher conductance.

Accordingly, we may speculate that this adaptation of the GluA2 AMPA-subunit is the result of the likely decreased glutamatergic neurotransmission present after withdrawal from repeated MDPV exposure, as it has been observed after repeated cocaine exposure (Baker et al., 2003). Likewise, the glutamate system has been associated with the reinforcing and psychostimulant properties of MDPV (Gregg et al., 2016).

NFkB

Nfκb is another induced -target gene of ΔFosB (Ang et al., 2001). There are a number of subunits than can comprise a transcription factor dimer of the NFκB family, including p65, RelB, c-Rel, p50 and p52, being p65 one of the most common subunits which generally heterodimerize with p50 and thus, trigger active transcription (Nennig & Schank, 2017).

It has been reported that chronic, but not acute, cocaine exposure increases NFκB p65 subunit in the NAcc by means of a ΔFosB -mediated mechanism (Ang et al., 2001; Russo et al., 2009b). By contrast, even though ΔFosB accumulates after repeated exposure to MDPV (López-Arnau et al., 2017), in Chapter 1 we did not observe any change in NFκB protein levels in VS neither 2 h nor 24 h (data not shown) after treatment, suggesting a different modulation of NFκB expression by cocaine and MDPV.

G9a

G9a is a histone 3 lysine 9 -specific dimethyltransferase that acts as a negative regulator of several genes including *fosB, arc* and *bdnf* (Walker et al., 2013; Zhang et al., 2014). It has already been reported the essential role of G9a in cocaine-induced plasticity and related behaviours, specifically,

through a ΔFosB-dependent mechanism (Maze et al., 2010). Although many cocaine-induced alterations in gene expression appear transiently after drug exposure, numerous genes display enhanced transcriptional inducibility following drug administration (i.e. sensitization). Accordingly, this phenomenon can be largely dependent on G9a activity in the NAcc. An initial proposed hypothesis was that in response to acute cocaine exposure, G9a acts to maintain homeostatic levels of gene expression in the NAcc by repressing genes known to enhance synaptic plasticity in the same brain area. Nevertheless, following chronic exposure, G9a recruitment to target gene promoters is diminished, leading to the enhanced transcriptional expression of associated transcripts. These data suggest that, as G9a is repressed by chronic, but not acute, cocaine administration, G9a target genes become more sensitive (i.e. more likely to be induced) by future exposures to the drug (Maze et al., 2010; Maze & Nestler, 2011). In this sense, Maze et al., (2010) proved that localized knockout of G9a in NAcc neurons increased cocaine-conditioned place preference in mice, whereas G9a overexpression diminished it. However, more recently, Anderson et al., (2019) have demonstrated disparate results, so they found that an RNAi-mediated knockdown of G9a in NAcc shell neurons exerts powerful reductions in cocaine self-administration, cocaine-seeking and basal anxiety-related behavioural responses. Moreover, deletion of G9a in DR1 striatal neurons reduces cocaine-place preference, and so, unrepressed genes in these neurons may also reduce cocaine self-administration and relapse behaviours (Maze et al., 2014). Consistent with this, G9a overexpression enhances cocaine self-administration, stress-induced reinstatement and anxiety during forced abstinence (Anderson et al., 2018). Altogether, these new evidences suggest that G9a reductions observed after chronic drug exposure (i.e. cocaine and morphine) may represent a common adaptive response of neurons to

compensate for the negative effects of chronic drug use on the brain. In this sense, there would seem to be numerous genes normally suppressed by G9a that act to counter the concomitant increase in addiction-promoting gene expression that also are unmasked by overall G9a reductions, for instance, ΔFosB. At this point, therefore, some controversy regarding the role of G9a in drug addiction can be noted: while previous studies proposed that repeated cocaine exposure decreases G9a in the NAcc and thereby enhances expression of certain addiction-promoting genes, recent studies suggest the opposite, that countering this cocaine-induced decrease in G9a activity actually exacerbates addiction and sensitivity to relapse.

In the present doctoral thesis, G9a acquired a special relevance, thus mRNA levels encoding G9a were determined in all chapters devoted to MDPV study (Part I).

In Chapter 1, unexpectedly, G9a was overexpressed in VS (contains NAcc) 24 h after repeated exposure to MDPV (1.5 mg/kg, twice daily for 7 days), evincing, thus, a key difference between the mechanisms underlying cocaine and MDPV addiction. Furthermore, as MDPV-treated animals showed increased ΔFosB protein levels in the same brain area, alternative mechanisms must modulate G9a expression, aside from ΔFosB. In this sense, we could suggest that most probably the overexpression of genes known to enhance synaptic plasticity, which are at the same time G9a target genes (i.e. *arc, fosB, cdk5* or *nfκb*) might have been limited or even blocked by such G9a overexpression, a feature that could explain, at least partly, the aforementioned differences observed in gene expression induced by repeated exposure to cocaine or to MDPV.

In Chapter 2, we determined G9a expression in VS after either an acute or repeated exposure to MDPV or cocaine. Consistent with what was described by

Maze et al., (2010), G9a was overexpressed 1 h after acute cocaine administration, which returned to normal within 2 h. Surprisingly, the same pattern of expression was observed after a single exposure to MDPV. Nevertheless, following repeated exposure to drugs, some discrepancy with Maze et al., (2010) must be highlighted. Regarding cocaine, in our study we observed an initial overexpression of G9a, as occurred after an acute administration, which may reflect the acute effect of the drug. However, it reverted to normal within 24 h. Accordingly, we did not find any G9a repression as Maze et al., (2010) described, although we cannot rule out the possibility that such repression could have been observed shortly after 24 h. Conversely, regarding MDPV, we did not observe any change in G9a expression 2 h after the repeated drug exposure, as occurred after an acute administration, but a tendency to decline after 24 h, like cocaine. In this context, therefore, our findings suggest that G9a expression after MDPV- intake may be time and dose-dependent, since we observed different modulation of G9a in Chapter 1 and 2, which only differ in the drug administration protocol followed. Furthermore, it seems that G9a regulation runs temporarily different for cocaine than for MDPV.

Importantly, G9a expression in mPFC was not altered neither by acute nor repeated doses of the drugs, suggesting that G9a expression appears to be differentially modulated as a function not only of the treatment schedule, the dose used and the time of the determination, but also of the brain area analysed. Accordingly, changes in G9a expression induced by cocaine and MDPV seem to occur in the ventral striatum, where they probably play a relevant role in the regulation of behavioural responses to the drugs (Maze et al., 2014), rather than in the mPFC.

Finally, in Chapter 3, following a repeated exposure to MDPV or cocaine, no changes in G9a expression were observed 48 h after the last drug

injection. In this sense, our results are in agreement with what we reported previously: the effects of the drugs might have faded (returned to normal) at that time. In relation to MDPV-treated animals, as occurred in Chapter 1, the increased ΔFosB protein levels observed in VS did not repress G9a, thus alternative mechanism must certainly modulate G9a expression. In addition, the pattern of administration might not have been strong enough to produce lasting changes in G9a expression, while it was in Chapter 1. In any case, there are no reports concerning G9a expression after more than 24 h after the last drug injection.

Furthermore, G9a expression was also determined 12 days after the CPP paradigm, 2 h after an acute dose of saline, cocaine 10 mg/kg or MDPV 2 mg/kg given to drug-conditioned animals (Chapter 3). Under these conditions, we found out, once more, different effects triggered by MDPV and cocaine. On one hand, the effect of an acute cocaine injection on the early expression of G9a was always a repression, regardless of the conditioning drug. Such repression might reflect the adaptative response of neurons to a new drug administration after withdrawal. On the other hand, we did not see any change in G9a expression 2 h after the MDPV acute injection in MDPV-conditioned animals, consistent with what we found in Chapter 2.

Finally, it is important to point out that, although several putative G9a target genes in the NAcc have been identified using chromatin immunoprecipitation (ChIP) with an anti-G9a antibody, when determining the expression of these genes after the non-contingent administration of drugs to mice, we did not see any relationship between the levels of mRNA G9a and the mRNA levels encoding its target genes Arc (Chapter 1 and 3), FosB, CDK5, NFκB (Chapter 1) or BDNF (Chapter 2), suggesting additional

regulatory mechanisms of their expression. Furthermore, although in some conditions we did not see changes in G9a mRNA, we cannot discard possible changes in the enzyme protein levels or in its activity, as well as in H3K9m2 levels.

Overall, the huge complexity and variability of the effects of drugs of abuse on G9a expression becomes evident. That is, many genes, including *c-fos* and *g9a*, are differentially regulated depending on several factors like the region of the mesolimbic pathway being analysed, the moment within the withdrawal period, as well as the drug administration protocol followed, among other parameters. Accordingly, further studies are needed to completely elucidate and understand the role of G9a in drug addiction.

Arc

Arc is an immediate early gene considered a reliable index of activity-dependent synaptic modifications (Fumagalli et al., 2006; Salery et al., 2017). Therefore, its activation by psychomotor stimulants has been interpreted primarily as a key step influencing long-term plasticity in neurons (Nestler, 2001b). For this reason, the effects of different drugs of abuse on Arc expression have been largely studied after both acute and chronic expositions (Fumagalli et al., 2006; Kodama et al., 1998; Schiltz et al.,2005). For example, Fumagalli et al., 2006 have described the corticostriatal regulation of Arc expression after cocaine exposure, evidencing an overexpression of the protein in striatum after both acute and chronic exposures. In fact, compelling evidence suggests that Arc contributes to the long-term effects of drugs such as cocaine, probably by alteration of the morphology of dendrites and spines observed in different brain regions after drug exposure (Norrholm et al., 2003; Robinson & Kolb, 1999, 2004). Nevertheless, it is

important to highlight that Arc expression, like c-Fos, G9a and many other factors, is differentially modulated, even by the same drug, as a function of the treatment schedule, the dose used, and the brain area analysed. Moreover, a discrepancy between transcriptional (mRNA levels) and translational (protein levels) processes has also been observed, in such a way that an increased mRNA does not translate in increased protein levels in the same brain area (Fumagalli et al., 2006).

As described for cocaine, Arc expression is up-regulated 24 h after repeated MDPV exposure in ventral striatum (Chapter 1). However, such overexpression dissipated over withdrawal until reaching protein levels substantially lower than those of the control group, on day 21. At this point, as an attempt to elucidate the reason for such decline, we measured the mRNA levels encoding Arc and we observed that, interestingly, they were significantly increased. Therefore, a discrepancy between Arc mRNA and protein levels at the same time become evident. As mentioned above, such inconsistency has also been described after subchronic and chronic cocaine exposures (Fumagalli et al., 2006). Therefore, as Fumagalli et al., (2006) did, we can reasonably attribute this controversy to the fact that repeated administration of MDPV might prolong Arc mRNA half-life, for example by reducing Arc mRNA turnover or inhibiting protein synthesis. This possibility, in addition, is in agreement with Ichikawa et al., (2003), who elegantly showed that, after *in vitro* stimulation of neuronal activity, Arc mRNA accumulates because of protein synthesis inhibition.

All considered, the increase in Arc expression observed shortly after MDPV repeated exposure herein reported may alter the morphology of dendrites and spines in striatum, thereby setting the stage for drug addiction.

In Chapter 3, Arc protein levels were measured after a weaker drug administration protocol in comparison to that used in Chapter 1 (MDPV 2 mg/kg, once daily for 4 days vs MDPV 1.5 mg/kg, twice daily for 7 days, respectively). In this study, we did not observe any variation in Arc protein levels in ventral striatum, 48 h after the last drug injection. Overall, our results are consistent with what has already been observed after a similar cocaine exposure, for which an Arc overexpression was only detected 2 h after the last drug-injection, but not later (Fumagalli et al., 2006). Accordingly, Arc modulation depends on the pattern of administration of the drug, which was different in Chapter 1 and 3, and thus, that may be the reason why we obtained distinct results.

Additionally, in the same Chapter 3, mRNA levels encoding Arc were determined 12 days after the CPP paradigm, 2 h after an acute dose of saline, cocaine 10 mg/kg or MDPV 2 mg/kg. In this context, a single exposure to MDPV did not alter Arc transcription. By contrast, after a cocaine administration, no effect was observed in MDPV-conditioned animals but a decrease in cocaine-conditioned ones.

BDNF

BDNF is a neurotrophic factor that has been widely studied. Research done over the last decades point BDNF to be involved in the long-term neuronal adaptations leading to functional modifications in the synapses associated with cocaine abuse and related behaviours, such as behavioural sensitization and drug-conditioning (Ghitza et al., 2010; Horger et al., 1999; Le Foll et al., 2005; McGinty et al., 2010; Russo et al., 2009a). Nevertheless, the *bdnf* gene is differentially regulated in regions of the mesolimbic pathway during the abstinence period following repeated cocaine administration

(McCarthy et al., 2012). Indeed, as happens with c-Fos, G9a and Arc, the regional selectivity and timing of cocaine effects can vary widely depending on the experimental conditions, reason why the literature regarding the effects of cocaine on BDNF expression is confusing with many contradictory observations (for a review see Li & Wolf, 2015).

In Chapter 2, we found out that either an acute or repeated exposure to MDPV upregulate BDNF mRNA levels in mPFC, the brain area where is mainly produced. Indeed, BDNF protein is chiefly supplied to NAcc by anterograde transport from cortical pyramidal neurons in frontal cortex, rather than by local production. Importantly, BDNF is initially synthesized as its precursor (proBDNF) and subsequently cleaved into mature BDNF (mBDNF) (Lessmann et al., 2003). Nevertheless, such early increment in *bdnf* transcription induced by MDPV was not accompanied by any alteration in proBDNF protein levels in NAcc. Furthermore, despite the discrete up-regulation of mRNA levels in mPFC and the null effects on proBDNF, mBDNF protein levels were reduced in the NAcc shortly after repeated exposure to the drug, albeit they returned to normal levels within 10 days. The decoupling herein observed between transcriptional and translational processes has already been observed by other authors (Fumagalli et al., 2007), and could be attributed to the complex regulation of BDNF synthesis and transport. Alternatively, upregulation of mRNA levels might be a compensatory response to the primary loss of mBDNF protein.

Very importantly, such decline observed in mBDNF levels induced by repeated exposure to MDPV appears to be involved in the development of behavioural sensitization to the drug. Hence, pretreatment with 7,8-dihydroxyflavone, a selective TrkB agonist, during the induction period, completely blocked the development of sensitization to the cathinone

derivative. In addition, such finding was corroborated by the fact that, the previous administration of ANA-12, a potent and selective TrkB antagonist, prevented the effect of the flavone. However, when assessing the rewarding properties of MDPV in the CPP paradigm, 7,8-DHF did not modify CPP acquisition, suggesting that the BDNF – TrkB pathway has a specific effect on motor sensitization to MDPV, but not to its rewarding effects.

By contrast, no effect on *bfnf* mRNA transcription, proBDNF nor mBDNF protein levels were evidenced by cocaine treatment at any time until 10 days of withdrawal. Accordingly, no effect of the 7,8-DHF on the development of behavioural sensitization to the drug was observed. Therefore, additional signalling pathways may be involved in the development of behavioural sensitization to cocaine, highlighting again the different intracellular responses that it triggers despite the great similarity of its mechanism of action with that of MDPV.

It is noteworthy that, regarding mBDNF levels, the differences observed between cocaine and MDPV in the *in vitro* determinations agree with the results of the *in vivo* experiments, so that 7,8-DHF only prevented the behavioural sensitization induced by MDPV. Taken together, our results suggest that the decrease in mBDNF levels induced by MDPV, leading to a decreased receptor stimulation, is involved in the sensitization developed to the drug. In fact, the same effect of 7,8-DHF on the development of behavioural sensitization after repeated administration of methamphetamine was described by Ren et al., (2014). Hence, even though MDPV and methamphetamine have substantial differences regarding their mechanism of action, a decrease in BDNF protein levels has been found during early withdrawal of both substances (Chen et al., 2014). Therefore, this suggests that METH and MDPV abusers may suffer from a severe disfunction on the

BDNF – TrkB signalling, which is importantly involved in the development of behavioural sensitization, pointing TrkB modulation as a target to prevent MDPV and METH sensitization. In fact, models with reduced BDNF expression exhibit a variety of alterations in the DA system (Apawu et al., 2015), which indicates that BDNF has some direct influence on this system.

DR3

It is thought that the induction of DR3 in the striatum is triggered by a DR1/DR5 receptor stimulation-dependent elevation of BDNF in cortico-striatal neurons (Guillin et al., 2004; Le Foll et al., 2005). Specifically, BDNF, synthesized in either VTA neurons or neurons originating from the cortex, controls DR3 expression, which is selective of the NAcc (Guillin et al., 2004). Accordingly, DR3 expression is elevated in drug addiction (Staley & Mash, 1996) and it is known to be critically involved in reactivity to drug-associated stimuli. In fact, DR3 modulation controls behavioural sensitization and BDNF increases the behavioural response and conditioned reward to cocaine (Horger et al., 1999) and mephedrone (Ciudad-Roberts et al., 2015).

In our study (Chapter 2), even though exposure to MDPV induced an increase of cortical BDNF transcription, we did not observe any change in accumbal mRNA encoding DR3, neither after acute nor repeated exposure. Furthermore, we did not find any change after cocaine acute or repeated exposure, for which no changes in BDNF cortical levels were observed either at early withdrawal.

TH

Tyrosine hydroxylase catalyses the rate-limiting step in the biosynthesis of the catecholamines DA, NA and adrenaline. Repeated cocaine exposure is known to induce short-term changes such as increased TH expression in the NAcc (Rodriguez-Espinosa & Fernandez-Espejo, 2015). Similarly, in Chapter 1, repeated exposure to MDPV induced an increase of TH protein levels that was apparent not only shortly after treatment, but also after 21 days of abstinence. Therefore, our findings suggest a long-lasting adaptative change in *th* gene expression induced by MDPV that can be mediated by several mechanisms (Kumer & Vrana, 1996).

DAT

DAT is the target of MDPV and cocaine, so both psychostimulants act mainly blocking DAT by directly binding to it and reducing its uptake transport rate. Repeated exposure to cocaine in rodents can alter levels of DAT mRNA and protein, although the nature of these changes has been highly inconsistent and seems to be influenced by many factors, including route of administration, dose, and withdrawal period (Kuhar & Pilotte, 1996). Hence, increases, decreases or no changes in DAT expression following cocaine exposure have been reported. In relation to MDPV, Colon-Perez et al., (2018) reported DAT downregulation 24 h after a single administration of MDPV to rats and suggested that potent binding of MDPV to DAT may trigger internalization and prolonged alteration in homeostatic regulation of DA and functional brain network reorganization.

In Chapter 1 we did not observe changes 24 h after the treatment, but a decrease in total DAT protein levels 21 days after the last drug injection.

Moreover, such decrease in DAT co-occurred with increased TH levels and an altered DR2:DR1 ratio towards DR1, leading to a hyperdopaminergic status. As increased TH expression could lead to increased DA levels in the synaptic cleft, DAT reduction could be explained, at least partly, as a mechanism to guarantee a resilience response to avoid terminal injury by oxygen radicals derived from high DA intracellular levels, since increased DA uptake through its specific transporter can result in oxidative damage via the cytosolic oxidation of the neurotransmitter (Masoud et al., 2015). Alternatively, DAT downregulation may be a homeostatic response once MDPV disappears from the brain and leaves an increased population of free transporters that, otherwise, would dramatically remove DA from the synapses. In this sense, our results were later backed up (see López-Arnau et al., 2019 in the *Annex IV* section for more in-depth discussion).

CB1 receptor

The effect of cocaine or MDPV consumption on the endocannabinoid transmission in reward-related areas of the brain is relatively under-researched. Bystrowska et al., (2018) have recently reported a significant decrease in CB1 receptor expression in the prefrontal cortex, dorsal striatum, and the basolateral and basomedial amygdala of rats following cocaine self-administration, and an increase in CB1 receptor expression in the ventral tegmental area and, in a very discrete way, in the NAcc shell. Besides this study, most of the studies concerning the role of the endocannabinoid transmission in cocaine addiction include the use of exogenous modulators of the system, for instance, CB1 receptor antagonists such as SR141716. In this way, while Chaperon et al., (1998) described that the coadministration of SR141716 with cocaine in the conditioning phase abolishes the acquisition of

CPP to cocaine in rats, Martin et al., (2000) demonstrated cocaine-induced CPP in CB1 receptor knockout mice. Considering this controversy, many different explanations have surged. However, a more recent evidence indicates that SR141716 might antagonize, not only CB1, but also a still uncharacterized cannabinoid receptor which can be solely responsible for cocaine-induced CPP (Pistis et al., 2004).

Altogether, our findings from Chapter 3 are in agreement with this, since we did not observe any change in CB1 protein expression when analysing the whole ventral striatum after MDPV and cocaine-induced CPP. Hence, we suggest that the implication of the endocannabinoid transmission on cocaine or MDPV reward and reinstatement might be mediated by other mechanisms but not alterations in CB1 receptor protein levels in the ventral striatum.

4.1.4. Neurotoxicity

There are nearly no reports on MDPV-induced neurotoxicity. In fact, a limited neurotoxic potential for MDPV could be expected due to its unique nature as a catecholamine transporter blocker, rather than a releaser, which would block extracellular dopamine from being internalized in the terminal, where its subsequent oxidation would generate toxic reactive oxygen species (Masoud et al., 2015). However, Valente et al., (2017) reported that oxidative stress and mitochondrial dysfunction play a role in MDPV-induced neuronal damage *in vitro*, ultimately leading to cell death by apoptosis. By contrast, Adám et al., (2014) demonstrated that MDPV does not affect apoptosis in the adult mice brain. Moreover, MDPV seems to

prevent methamphetamine-induced signs of neurotoxicity, by impeding its uptake via DAT (Anneken et al., 2015).

Nevertheless, given the hyperdopaminergic status induced after repeated MDPV exposure (Chapter 1) and because increased DA uptake through its specific transporter can result in oxidative damage via the cytosolic oxidation of the neurotransmitter, we aimed to investigate a putative neurotoxic effect of the cathinone derivative.

Shortly after repeated drug exposure we found out that 4-HNE, a robust marker of oxidative stress (Perluigi et al., 2012) tripled its levels, suggesting an important oxidative effect generated by DA or possibly by the reactive quinones generated from the MDPV metabolism (Baumann et al., 2017). Nevertheless, and consistent with the available literature (Angoa-Pérez et al., 2017; Anneken et al., 2015), such oxidative stress did not seem to be severe enough to cause glial activation. Furthermore, it was transient, so it disappeared over time without leaving an apparent trace of further relevant consequences.

4.1.5. Rewarding properties of MDPV

In Chapter 3, our first findings corroborate the results garnered from other studies (Gannon et al., 2017; Gregg et al., 2016; Karlsson et al., 2014; Watterson et al., 2014; Watterson & Olive, 2014), since we proved that MDPV induces CPP in a range of doses from 1 to 4 mg/kg. Moreover, we observed that the preference induced by these doses was similar, with no dose–response relationship. We also confirmed that, after extinction, MDPV-induced CPP is robustly reinstated with doses up to 12.5 %

(0.5 mg/kg) of the conditioning dose (Hicks et al., 2018). In this way, the present study evidences the rewarding and reinstating ability of MDPV.

Additionally, considering the locomotor cross-sensitization observed between cocaine and MDPV in Chapter 2, we aimed to explore if the ability of MDPV to reinstate drug-seeking behaviour is shared by cocaine, and if this occurs in the other direction, which would constitute cross-reinstatement. In this context, a dose of 10 mg/kg of cocaine induced a similar preference as 2 mg/kg of MDPV. Nevertheless, although the CPP was equivalent with both drugs, the time needed to extinguish the preference was significantly longer after MDPV conditioning. Therefore, our results suggest that, although the preference is equivalent, the neuroplasticity induced by MDPV may be greater than that induced by cocaine, as the time needed to overcome MDPV preference was longer. In addition, our study provides, to our knowledge, the first evidence that both drugs, cocaine and MDPV, can reinstate the CPP induced by the other drug. In this way, a cross-reinstatement between the two drugs became evident. As in previous studies, we observed reinstatement of the preference with cocaine doses of 50% (5 mg/kg) and 25% (2.5 mg/kg) of the cocaine dose employed for conditioning (Blanco-Gandía et al., 2017; Reguilón et al., 2017). Similarly, preference was also reinstated in MDPV-conditioned mice with 50% (1 mg/kg) and 25% (0.5 mg/kg) of the dose employed for conditioning. However, cross-reinstatement was obtained with 2 mg/kg and 10 mg/kg of MDPV and cocaine, respectively, but not with 1 mg/kg and 5 mg/kg. In this sense, it is important to point out that such decreased sensitivity has previously been observed when cross-reinstatement was induced by cocaine in morphine-conditioned mice, but only with doses up to 10 mg/kg (Ribeiro Do Couto et al., 2005). Hence, some differences underlying the mechanism of action of the two drugs may be the reason

why, despite their great similarity, they do not trigger the same responses, and so they are not fully interchangeable.

Finally, it is important to bear in mind that, as discussed above, and by contrast to cocaine (for a review see Li & Wolf, 2015) and other cathinones as mephedrone (Ciudad-Roberts et al., 2015), our results point that BDNF is not directly involved in the rewarding properties of MDPV, thus, modulation of the BDNF – TrkB pathway with either TrkB agonists or antagonists (data not shown) do not modify CPP acquisition.

4.2. Part II: Second-generation synthetic cathinones

The overwhelming apparition of synthetic cathinones on the illicit drug market has promoted the action of many governments which have started to pass laws to render these drugs illegal. In this context, the first-generation of synthetic cathinones, including MDPV, has been listed in the 1971 Convention (UNODC, 2017). Nevertheless, the problem remains: when one of these drugs is banned and illegal, the drug market suppliers respond by producing new unregulated alternatives, and they do so by taking advantage of a legal loophole whereby, by performing slight chemical modifications on the already banned drugs, new compounds with similar psychoactive properties are obtained, with no explicit legislation regarding their sale, purchase or use. Consequently, these new synthetic cathinones become easy to obtain, especially through online shops or the *darknet*. In this sense, a second-generation of synthetic cathinones including α-PVP and pentedrone has recently appeared in the market (Brandt et al., 2010; Shanks et al., 2012). As α-PVP is the 3,4-methylendioxy-lacking counterpart of MDPV, it has been considered to emerge as a replacement to MDPV.

In this context, while the first part of the present doctoral dissertation focuses on studying MDPV, the second one is devoted to characterizing the *in vitro* pharmacology as well as the psychostimulant and rewarding properties of novel synthetic cathinones structurally related to α-PVP and pentedrone, and thus, ultimately, to MDPV. More concretely, it tries to elucidate the role of the amino terminal group of these molecules in their interaction with monoaminergic transporters.

α-PVP and pentedrone, like MDPV, have proved to be potent uptake blockers at DAT and NET, with much weaker effects at SERT (Eshleman et al., 2017; Kolanos et al., 2015; Meltzer et al., 2006; Simmler et al., 2014), and consequently, they exert potent psychostimulant and rewarding effects (Gatch et al., 2015a; Gatch et al., 2015b; Giannotti et al., 2017; Hwang et al., 2017; Javadi-Paydar et al., 2018a, 2018b; Marusich et al., 2014), being α-PVP and MDPV the most potent and effective. In this sense, Kolanos et al., (2013) found that the bulky pyrrolidine ring and the flexible α-carbon chain are critical attributes for potent uptake inhibition at DAT, whereas the 3,4-methylenedioxy ring moiety is of little consequence in this regard. Conversely, the 3,4-methylenedioxy ring is known to increase the affinity and thus, potency at inhibiting SERT (Eshleman et al., 2017). Therefore, MDPV is the most potent SERT inhibitor *in vitro,* albeit none of them can be considered to produce real effects on serotoninergic activity *in vivo* at the doses usually consumed.

In Chapter 4 we extended the study to other novel cathinones structurally related to α-PVP and pentedrone, which only differ in their amino substituent: N-ethyl-pentedrone, N,N-diethyl-pentedrone and α-piperidinevalerophenone (α-PpVP). In this regard, we found out that all compounds studied act as potent DA and NA uptake inhibitors, with weak

activity on SERT. Regarding the amino group, the potency at inhibiting DAT and NET increased from a methyl to an ethyl group and to a pyrrolidine or piperidine ring. However, we cannot conclude that tertiary amines are more potent DA and NA uptake inhibitors since N,N-diethyl-pentedrone (a tertiary amine) was the weakest compound inhibiting monoamine uptakes. Furthermore, a positive correlation exists between the hDAT/hSERT ratio and the CLogP of the amino-substituent, pointing to a high abuse liability when increasing lipophilicity of the substituent. In addition, and although these compounds are not potential SERT inhibitors, the potency at inhibiting 5-HT uptake appeared to be inversely proportional to some physicochemical and molecular parameters such as lipophilicity, surface and bulk of the N-substituent. Accordingly, further research using other synthetic cathinones with more SERT activity are needed in order to corroborate these results. Importantly, our study also provides the first evidence that N-ethyl-pentedrone, N,N-diethyl-pentedrone and α-PpVP are able to induce psychostimulant and rewarding effects in mice. Therefore, our findings demonstrate the abuse liability of all the amino-valerophenone derivatives tested, especially N-ethyl-pentedrone, a NPS currently available in several Western countries.

5 CONCLUSIONS

The present doctoral dissertation has contributed to increasing scientific knowledge regarding novel psychoactive substances, especially about MDPV, but also about the new second-generation synthetic cathinones. Overall, the results obtained lead to the following conclusions:

Part I: MDPV

Chapter 1: Neuroadaptive changes and behavioural effects after a sensitization regime of MDPV.

1.1 Repeated exposure to MDPV induces an enduring anxiety-related behaviour, increases aggressiveness shortly after drug withdrawal and promotes a quick habituation to new environments, interpreted as a tendency for novelty-seeking and risk-taking behaviour.

1.2 Repeated exposure to MDPV induces an overexpression of plasticity-related factors (Arc, CDK5) and TH, which, along with the decrease of DR1 and DAT populations, points to a dopamine imbalance, concretely, to a hyperdopaminergic status. Furthermore, MDPV increases ΔFosB not only in ventral striatum, but also within the orbitofrontal cortex. Altogether, these alterations might be involved in the neuroadaptive changes underlying drug sensitization and behavioural-related abnormalities.

1.3 Despite the great similarities between MDPV and cocaine regarding their mechanism of action, the intracellular responses that they trigger in reward-related brain areas notably differ. For instance, they modulate the expression of G9a, NFκB and GluA2 differently.

Chapter 2: Role of the BDNF – TrkB signalling pathway in the development of behavioural sensitization to MDPV and cocaine.

1.1 Repeated administration of MDPV and cocaine cross-sensitizes to their locomotor effects.

1.2 MDPV and cocaine modulate the expression of BDNF distinctively: repeated exposure to MDPV induces an early increase of cortical mRNA levels encoding BDNF but a decrease in accumbal mBDNF protein. By contrast, no changes in BDNF expression induced by cocaine are evident at early withdrawal.

1.3 Pre-treatment with TrkB-agonists such as 7,8-DHF, completely blocks the development of behavioural sensitization to MDPV, but not to cocaine, without affecting CPP acquisition. Hence, the BDNF – TrkB signalling pathway plays a key role in the development of behavioural sensitization to MDPV, pointing TrkB modulation as a potential target to prevent sensitization to MDPV.

Chapter 3: Characterization of the rewarding properties elicited by MDPV and its interaction with cocaine.

1.1 MDPV induces CPP at doses ranging from 1 to 4 mg/kg with similar preference values. Furthermore, after extinction, MDPV-induced CPP can be robustly reinstated with doses up to 12.5% of the conditioning dose.

1.2 MDPV and cocaine reinstate the CPP induced by the other drug (cross-re-instatement), even though relapse in drug-seeking is always higher with the conditioning drug.

1.3 Memories associated with MDPV-conditioning need more time to be extinguished, a feature that correlates with the lasting accumulation of ΔFosB in the ventral striatum of these animals. Hence, the neuroplasticity induced by repeated MDPV exposure may be greater than that induced by cocaine.

1.4 MDPV-conditioned mice are more responsive to a new cocaine exposure, implying a high vulnerability to cocaine abuse.

Part II: Second-generation synthetic cathinones

Chapter 4: Role of the amino terminal group in the pharmacological profile of novel synthetic cathinones structurally related to α-PVP and pentedrone. Comparative of their rewarding and psychostimulant effects.

1.1 All the compounds tested are potent DA and NA uptake inhibitors, with weak effects at SERT. Regarding the amino group, the potency at inhibiting DAT and NET increases from a methyl to an ethyl group and to a pyrrolidine or piperidine ring.

1.2 A positive correlation exists between the DAT/SERT ratio and the CLogP of the amino substituent, pointing to a higher abuse liability when increasing lipophilicity of the substituent. Furthermore, potency at inhibiting SERT appears to be inversely proportional to lipophilicity, surface and bulk of the N-substituent.

1.3 All compounds tested induce psychostimulant and rewarding effects in mice, a fact that evidences their abuse liability.

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7 ANNEX

7.1. Annex I

The combination of ethanol with mephedrone increases the signs of neurotoxicity and impairs the neurogenesis and learning in adolescent CD-1 mice

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Toxicology and Applied Pharmacology 2016; 293:10-20

Toxicology and Applied Pharmacology 293 (2016) 10–20

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

The combination of ethanol with mephedrone increases the signs of neurotoxicity and impairs neurogenesis and learning in adolescent CD-1 mice

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ARTICLE INFO

Article history:
Received 30 July 2015
Revised 17 December 2015
Accepted 21 December 2015
Available online 30 December 2015

Keywords:
Adolescence
Alcohol
Cathinones
Mephedrone
Neurogenesis
Neurotoxicity

ABSTRACT

A new family of psychostimulants, under the name of cathinones, has broken into the market in the last decade. In light of the fact that around 95% of cathinone consumers have been reported to combine them with alcoholic drinks, we sought to study the consequences of the concomitant administration of ethanol on mephedrone -induced neurotoxicity. Adolescent male Swiss-CD1 mice were administered four times in one day, every 2 h, with saline, mephedrone (25 mg/kg), ethanol (2; 1.5; 1.5; 1 g/kg) and their combination at a room temperature of 26 ± 2 °C. The combination with ethanol impaired mephedrone-induced decreases in dopamine transporter and tyrosine hydroxylase in the frontal cortex; and in serotonin transporter and tryptophan hydroxylase in the hippocampus by approximately 2-fold, 7 days post-treatment. Furthermore, these decreases correlated with a 2-fold increase in lipid peroxidation, measured as concentration of malondialdehyde (MDA), 24 h post-treatment, and were accompanied by changes in oxidative stress-related enzymes. Ethanol also notably potentiated mephedrone-induced negative effects on learning and memory, as well as hippocampal neurogenesis, measured through the Morris water maze (MWM) and 5-bromo-2′-deoxyuridine staining, respectively. These results are of special significance, since alcohol is widely co-abused with amphetamine derivatives such as mephedrone, especially during adolescence, a crucial stage in brain maturation. Given that the hippocampus is greatly involved in learning and memory processes, normal brain development in young adults could be affected with permanent behavioral consequences after this type of drug co-abuse.

Introduction

Drug abuse is a matter of concern at all life stages but its occurrence at earlier ages, such as adolescence, is especially worrisome, as it can determine the social outcome of an individual. While adolescence is a crucial stage in brain maturation, experimentation with alcohol and other drugs during this stage is common; teenagers are not aware of the risks they are taking, as the regions of the brain that control impulses are still immature. Substance use during adolescence has been associated with alterations in brain structure, function, and neurocognition (reviewed by Squeglia et al., 2009). Moreover, it has been reported in studies with humans that drug consumption during adolescence increases the likelihood of drug abuse in adulthood (Izenwasser, 2005). Specifically, transformations in the prefrontal regions and limbic systems are thought to contribute to increased risk-taking and novelty/sensation seeking behaviors (Casey et al., 2008; Chambers et al., 2003; Spear, 2000).

Currently, most drug use during adolescence is attributable to recreational purposes and occurs in leisure environments, such as dance clubs and parties (Schifano et al., 2011). Alcohol is omnipresent due to its legal drug status (Winstock et al., 2011) while other drugs such as cannabis, cocaine and amphetamine derivatives are often associated with it (Elliott and Evans, 2014).

Recently, a new family of amphetamine derivatives generically referred to as cathinones (β-keto-amphetamines), broke into the drug market. They were initially sold through legal channels, mainly websites and smart shops, taking advantage of an existing legal loophole concerning their chemical structures. Due to this status, they were also called "legal highs", together with other designer drugs such as synthetic cannabinoids. The drug enforcement organization of many countries have made efforts to ban these substances, but the pace at which new compounds appear in the market exceeds the speed at which the necessary legal machinery for their illegalization is established (EMCDDA report, 2014).

Abbreviations: 5-HT, serotonin; BrdU, bromo-deoxyuridine; CAT, catalase; DA, dopamine; DAT, dopamine transporter; DG, dentate gyrus; EtOH, ethanol; Gpx, glutathione peroxidase; MDA, malondialdehyde; MDMA, 3,4-methylenedioxy-methamphetamine; Meph, mephedrone; NAc, nucleus accumbens; SERT, serotonin transporter; SOD, superoxide dismutase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

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http://dx.doi.org/10.1016/j.taap.2015.12.019
0041-008X/

Among these new drugs, mephedrone (4-methylmethcathinone) has become very popular, mainly due to its affordability, purity and initial legal high status (it is currently banned in several countries). It is known to have similar effects to other psychostimulant drugs such as 3,4-methylenedioxy-methamphetamine (MDMA, Brunt et al., 2012; Varner et al., 2013), or even superior (Winstock et al., 2010; Vardakou et al., 2011). Moreover, the abuse potential of cathinone derivatives is comparable to that of cocaine or MDMA (McElrath and O'Neill, 2011). Mephedrone users evidence a desire to re-dose (Winstock et al., 2011), increasing the risk of overdoses (Maskell et al., 2011; Wood et al., 2011).

Preclinical studies have shown that mephedrone stimulates the release of dopamine (DA), serotonin (5-HT) and norepinephrine and inhibits their re-uptake in the CNS (Kehr et al., 2011; Baumann et al., 2012; Lopez-Arnau et al., 2012; Martinez-Clemente et al., 2012). These mechanisms are similar to those of MDMA (reviewed by Green et al., 2003), which also induces species-dependent neurotoxicity when administered following a binge-dosing schedule in a hot environment (Sanchez et al., 2004). Neurotoxicity of amphetamine derivatives is a matter of concern and has been subject of a great amount of research. This led to undertake studies exploring a possible neurotoxic effect of mephedrone in rodents. Reported research evidences the need to perform neurotoxicity assays under different administration schedules and controlled room temperature. For example, Angoa-Perez et al. (2012) and den Hollander et al. (2013) reported no damage by mephedrone to DA or 5-HT systems when administered to mice, while our group more recently reported neurotoxicity using a dosing schedule which better agreed with mephedrone pharmacokinetics and exploring cerebral areas others than the striatum (Martinez-Clemente et al., 2013, 2014). Thus, using a two-day consumption pattern in mice, mephedrone induced a dopamine and serotonin transporter loss that was accompanied by a decrease in tyrosine hydroxylase and tryptophan hydroxylase 2 expressions one week after administration (Martinez-Clemente et al., 2014). Moreover, similar results have recently been reported in rats (Lopez-Arnau et al., 2015).

In the UK, around 95% of cathinone consumers combine them with alcohol (Winstock et al., 2011), thus studying the consequences of these combinations in adolescent subjects seems of the essence, since a potentiation of their effects may increase their abuse liability (Ciudad-Roberts et al., 2015) and neurotoxicity. Moreover, adolescents are less sensitive than adults to the depressant effects of ethanol, as well as to the subsequent hangover (reviewed by Witt, 2010), which facilitates the intake of higher amounts. Numerous studies report neurotoxic effects of ethanol itself in consumption models using adolescent rodents (reviewed by Guerri and Pascual, 2010), mainly leading to impairment in memory and visual and verbal tasks (Harper, 2007). Excitotoxicity and neuroinflammation seem to be involved in such deleterious effects (Pascual et al., 2007). Also, neurogenesis from the granular layer of the dentate gyrus of the hippocampus is impaired following treatment with ethanol (Morris et al., 2010; McClain et al., 2011; Ehlers et al., 2013) and adolescents are more sensitive than adults to such effects. (Crews et al., 2006).

To date, there are no available studies on the neurotoxic effects of the combination of cathinones and ethanol, although there have been reports on the effects of the combination of MDMA plus ethanol. Hernandez-Rabaza et al. (2010) described that this drug combination produces cognitive impairment in adolescent rats at doses that do not when administered alone. This impairment is accompanied by a decrease in survival of neuronal precursor cells as well as a decrease in the presence of mature cells in the dentate gyrus (DG) of the hippocampus. Furthermore, Izco et al. (2007) found that ethanol potentiates MDMA neurotoxicity through the production of hydroxyl radicals.

These antecedents and our recent works (Martinez-Clemente et al., 2014; Lopez-Arnau et al., 2015) led us to hypothesize that the combination of mephedrone with ethanol could also result in increased damage and cognitive impairment. Therefore the aim of the present work is to investigate the effects of this combination on several neurochemical and cognitive markers of neurotoxicity, as well as on hippocampal neurogenesis.

Materials and methods

Animals

All animal care and experimental protocols in this study complied with the guidelines of the European Community Council (86/609/ECC) and ARRIVE, and were approved by the Animal Ethics Committee of the University of Barcelona. Male adolescent Swiss CD-1 mice (Charles River, Lyon, France) of ages between PND 35-42 (20–32 g), were used for all experiments. The animals were housed 5–6 per cage at 22 ± 1 °C under a 12 h light/dark cycle with free access to standard diet and drinking water.

Drugs and reagents

Pure racemic mephedrone was synthetized and characterized in house as described previously (López-Arnau et al., 2012). Absolute ethanol was purchased from Scharlau (Barcelona, Spain) and diluted in saline at different concentrations, never exceeding 20% (*w*/*v*) to avoid tissue irritation. Mephedrone solutions for injection were prepared in saline or ethanol/saline solutions immediately before subcutaneous administration at a volume of 10 ml/kg. [^{3}H]WIN 35428 and [^{3}H]paroxetine were purchased from Perkin Elmer (Boston, MA, USA). Bromo-deoxy-Uridine and protease inhibitors were from Sigma-Aldrich. Bromo-deoxy-uridine was dissolved in saline containing 0.007 M NaOH. The rest of reagents were of analytical grade and purchased from several commercial sources.

In-vivo treatment

In a previous work (Martinez-Clemente et al., 2014), we assessed the neurotoxic effects of mephedrone in mice at three different treatment regimens: four doses of 25 mg/kg in one day; four doses of 50 mg/kg in one day; three doses of 25 mg/kg during two consecutive days. We found that the latter two elicited clear dopaminergic and serotonergic impairment in several areas of the brain. Given that the aim of this work was to determine whether ethanol is capable of increasing the neurotoxic effects of a mephedrone treatment regimen with little neurotoxic effects, we used four doses of 25 mg/kg (s.c.) in one day, every two hours, as a reference treatment for all experiments in this work.

In humans, the typical amount of mephedrone consumed over an evening/night is about 0.5–1 g, usually taken in doses of 100–200 mg every hour or two hours (Kelly, 2011). Following the body surface area normalization method (Reagan-Shaw et al., 2008), we calculated an equivalent dose in mice of 25 mg/kg, which corresponds to 2 mg/kg in a human. The interval of 2 h between doses was chosen according the mephedrone half-life in rats ($t_{1/2\beta} = 0.55$ h, Martinez-Clemente et al., 2013). Furthermore, during the whole duration of the treatment, room temperature was set at 26 ± 2 °C, at which this drug has been reported to induce signs of neurotoxicity (Martinez-Clemente et al., 2014), in order to reproduce the common hot conditions found in crowded dance clubs.

Given that we wanted to emulate recreational ethanol intake, we sought to find a regimen that caused blood ethanol concentration to level around 1.5 g/l during the whole duration of the treatment. Due to clearly different kinetics between mephedrone and ethanol (Bejanian et al., 1990; Martinez-Clemente et al., 2013), we administered changing doses of ethanol throughout the treatment schedule, which was given subcutaneously mixed in the same injection with mephedrone, which allowed constant ethanol plasma concentration, diminished distress to the animals and simplified treatment execution. No

signs of pain or discomfort were observed when the animals received ethanol by this route and at the concentrations used.

To set up ethanol dose combinations, we performed test experiments extracting blood samples from animals 1 h after each administration. Around 50 μl were extracted through jugular punction and placed in tubes coated with ethylenediaminetetraacetic acid (EDTA) to avoid coagulation. After centrifugation, ethanol concentration was immediately determined in serum through gas chromatography, using methanol as an internal standard (Macchia et al., 1995).

After testing several combinations, we chose decreasing doses of ethanol every two hours as follows: 2, 1.5, 1.5, 1 g/kg which rendered uniform blood concentrations ranging between 1 and 1.5 g/l. The time-course of ethanol blood concentrations after this treatment schedule is provided as supplementary material.

The treatment was repeated three times, differing in the time of sacrifice which allowed performing different studies. Thus, in Study 1, the mice were killed 7 days after the last injection, whereas in Study 2 and Study 3 they were killed 24 h and 28 days after, respectively. Treatment 1 and 3 were performed with 6 animals per group, while treatment 2 was performed with 6–8 animals, as sample pooling was required for lipid peroxidation assays.

Tissue sample preparation

Crude membrane preparation (collecting both synaptosomal and endosomal fraction) was prepared as described (Escubedo et al., 2005) with minor modifications. Mice from Study 1 were killed by cervical dislocation 7 days after treatment to perform radioligand binding to DAT and SERT and Western blotting of TH and TPH-2. Hippocampus, striatum and frontal cortex were quickly dissected out and stored at −80 °C until use. When required, tissue samples were thawed and homogenized through sonication at 4 °C 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 assays. 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 and then 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 (Bio-Rad Labs., Inc., Hercules, CA, USA), according to the manufacturer's instructions.

For all oxidative stress assays, animals from Study 2 were killed 24 h after treatment. For measuring the malondialdehyde (MDA) production, tissue samples were homogenized on ice in 30 volumes of the MDA lysis buffer (see below). The homogenates were centrifuged at 13,000×*g* for 10 min to remove insoluble material. Aliquots of the supernatant were used for lipid peroxidation assay. Samples for Western blot were prepared as described above.

DA and 5-HT transporter density

The density of the DA transporter in striatal or frontal cortex membranes was measured by [^{3}H]WIN 35428 binding assays. These were performed in tubes containing 5 nM [^{3}H]WIN 35428 in 0.1/0.32 M sodium phosphate/sucrose-buffer (pH 7.9) and 50 (striatum) or 100 μg (cortex) of membranes. Incubation was done for 2 h at 4 °C. Non-specific binding was determined in the presence of 30 μM bupropion.

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

All incubations were finished by rapid filtration under vacuum through Whatman GF/B glass fiber filters pre-soaked in 0.5% polyethyleneimine. Tubes and filters were washed rapidly twice with 4 ml of ice-cold buffer, and the radioactivity trapped in the filters was measured by liquid scintillation spectrometry.

Lipid peroxidation

Lipid peroxidation was assessed using a colorimetric assay kit (Lipid peroxidation assay kit, Sigma-Aldrich) following the manufacturer's instructions. Briefly, we measured the accumulation of thiobarbituric acid-reactive substances (TBARS) in homogenates from the frontal cortex and hippocampus, expressed in terms of malondialdehyde (MDA) content. Samples were incubated with thiobarbituric acid 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.

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) and catalase (CAT) in mice receiving the treatments. Due to limitations in the amount of remaining processed tissue after TBARS assays, a representative number of samples from each treatment group was used for WB studies. For each sample, 20 μg of protein was mixed with sample buffer, boiled, and loaded onto a 10% acrylamide gel. Proteins were electrophoresed and subsequently transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P; Millipore, USA). PVDF membranes were blocked overnight and incubated for 2 h 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 anti-Gpx (ab108427, Abcam, Cambridge, UK) dil. 1:1000; rabbit polyclonal anti-CAT (EMD Chemicals, La Jolla, CA, USA) dil 1:2500. 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 (Dako Cytomation, Denmark).

Immunoreactive protein was visualized using a chemiluminescence-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 (1:2500 mouse monoclonal antibody, Healthcare) or GAPDH (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.

Morris water maze

Spatial learning and memory were assessed in a Morris water maze one week after treatment of mice from Study 3 (see below for details). Animals were trained in the water maze, which consisted of a circular pool (100 cm diameter and 45 cm high) that was filled with water (22 ± 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 suppress room cues (Vorhees and Williams, 2006). 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. Four extra-maze distal cues were located equidistantly around the pool, labeling the N, S, E and W locations. These cues consisted of a black circle, triangle, square and

diamond shape drawn on a blank hard surface. 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 mouse and the escape latency (the time needed by each mouse 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). The area within 10 cm from the edge of the pool was defined as the 'border zone'. The platform was always located in the NE quadrant. Throughout six days of training, the mice received one training session per day, consisting of five trials, by using a semi-random set of start locations that were not equidistant from the goal, creating short and long paths to the platform (a total of 30 trials per animal were to reach asymptotic performance). 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 none of the start positions was repeated the same day. Therefore, the only way to perform well was to learn the relative location of the platform with respect to the distal cues. A trial was started by placing the mouse in the desired start position of the pool, facing the tank wall. The mice were allowed to swim to the hidden platform, and the escape latency was determined. If an animal did not escape within 60 s, it was gently placed on the platform or guided to it. The mice were allowed to rest for 30 s (inter-trial interval) on the platform (even those that failed to locate it). 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. In the probe trial, animals were assigned a start location which had not been used in any of the learning trials, to ensure that their spatial preference was a reflection of the memory of the goal location rather than for a specific swim path. Different parameters of each mouse's performance were analyzed: the total time and distance spent swimming in each quadrant, entries in each quadrant and time elapsed (latency) until the mouse first reached the target zone (absent platform).

Administration of BrdU and tissue preparations for neurogenesis assessment

Animals from Study 3 also received two injections of Bromodeoxyuridine (BrdU, 100 mg/kg, i.p.) (Burns and Kuan, 2005). The first injection was done 2 h after the last mephedrone injection and the second one was given 12 h later. BrdU is a thymidine analog that is incorporated into cells in place of a thymine base pair as the cell undergoes DNA replication during the S phase of the mitotic cell cycle, and as such is a measure of cell proliferation. 28 days after the first BrdU injection, animals were anesthetized with sodium pentobarbital and killed by transcardial perfusion, firstly with 30 ml of PBS and then with 60 to 100 ml of 4% paraformaldehyde (PFA). Their brains were removed, postfixed overnight in 4% PFA and equilibrated in a 30% sucrose solution in PBS. 30 μm coronal sections were collected on a freezing cryostat and stored free-floating in a cryoprotective solution (30% sucrose, 30% polyethyleneglycol in PBS) at −20 °C until used for immunohistochemical analysis.

Immunohistochemistry and its quantification

For BrdU detection, sections of interest were selected and washed with PBS, incubated in 2 N HCl at 37 °C for 30 min, washed in PBS, incubated in 0.1 M boric acid at 37 °C, washed in PBS and blocked for 1 h in a blocking solution (PBS containing 0.2 M glycine, 10% fetal bovine serum, 0.3% Triton X-100 and 0.2% gelatin) The tissue sections were stained overnight with specified combinations of the following primary antibodies: mouse anti-BrdU (1:250) and rabbit monoclonal anti-NeuN (1:500; Millipore). Secondary antibodies used for both primary antibodies were Alexa-Fluor 488 donkey anti-mouse IgG (1:500; Life Technologies) and Alexa-Fluor 594 goat anti-rabbit IgG (1:500; Life Technologies). Slices were finally washed and mounted on StarFrost (Knittel, Germany) coded slides using Fluoromount-G solution (Electron Microscopy Sciences).

Neurogenesis was evaluated by counting the cells that were double labeled with BrdU and NeuN (using fluorescent microscope Leitz DMIRB magnification X400). We counted the number of labeled cells in six coronal sections per mouse brain (180 μm apart), that were stained and mounted on coded slides, though the rostrocaudal extent of the granule cell layer (blind to the observer). The total number of cells counted in the selected coronal sections from each brain was multiplied by the volume index (the ratio between the volume of the DG and the total combined volume of the selected sections). Cellular co-labeling of BrdU and NeuN was confirmed by confocal microscopy (TCS SP2 Leica confocal microscope).

Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were compared using one-way or two-way ANOVA; Student's *t* test for paired data was used to assess differences in latency between days 1 and 6 of a same group. 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 GraphPAD Prism 4 (one-way) and SPSS (two-way ANOVA) software.

Results

Effect of the combination of mephedrone and ethanol on different markers of DA and 5-HT neurotoxicity

In Study 1, statistical analysis of the results from [^{3}H]WIN35428 binding showed an overall significant effect of treatment (ANOVA: $F_{3,20} = 7.08$, $P < 0.01$). 7 days post-treatment, mephedrone induced a loss in DA reuptake sites ([^{3}H]WIN35428 specific binding) in the frontal cortex by 25% (Fig. 1A). Ethanol significantly increased this effect ($P < 0.01$) to the level of duplicating it, bringing DAT levels down to 48% of basal values ($P < 0.001$). Ethanol alone did not significantly affect transporter density. DAT was not affected in the striatum by any of the drug treatments (Fig. 1B).

In the hippocampus (Fig. 1C), after treatments with mephedrone or ethanol, 5-HT reuptake sites, measured as specific [^{3}H]paroxetine binding, were decreased by 25% ($P < 0.05$) and 33% ($P < 0.01$) respectively ($F_{3,20} = 26.69$, $P < 0.001$). Their combination caused a significant increase in receptor density loss compared to mephedrone alone, reaching a 66% decrease over baseline levels ($P < 0.001$). Conversely, SERT levels were unaffected in the frontal cortex by any of the drug treatments (Fig. 1D).

In light of these results, we investigated the expression of the TH in the frontal cortex and TPH-2 in the hippocampus. There was a good relationship between the decrease in the [^{3}H]WIN35428 specific binding and the decrease in enzyme expression in the frontal cortex, where ethanol significantly increased TH depletion ($F_{3,20} = 11.46$, $P < 0.001$). Similar results were found for TPH-2, where the combination with ethanol caused a significant reduction over the group treated only with mephedrone ($F_{3,20} = 17.04$, $P < 0.001$).

Assessment of oxidative stress

Due to the robust changes in DA and 5-HT markers, we repeated the treatment (Study 2), euthanizing the animals 24 h after the first dose. The aim of this experiment was to elucidate whether this phenomenon could be explained by changes in oxidative stress markers. We used two approaches: determination of lipid peroxidation and oxidative stress-related enzymes.

Lipid peroxidation was measured as a raise in the MDA levels, a general indicator of the decomposition of polyunsaturated fatty acids. One

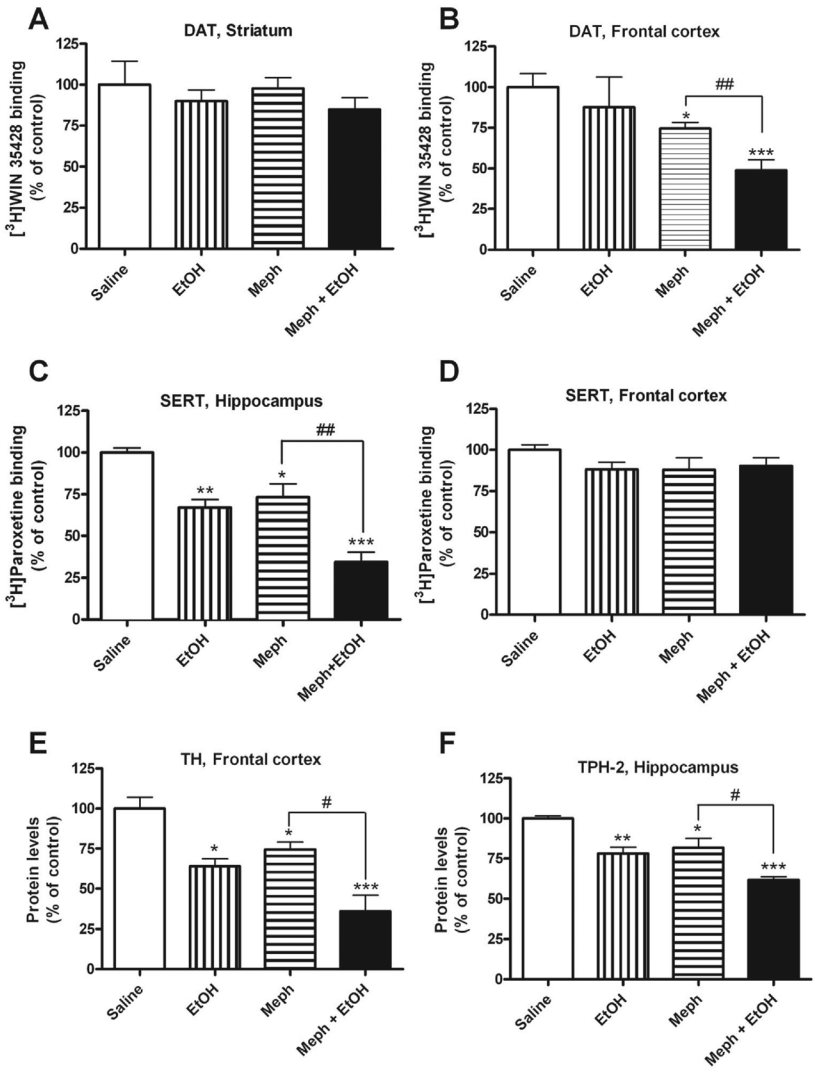

Fig. 1. Levels of dopamine (DAT, panels A and B) and serotonin (SERT, panels C and D) transporters in specific brain areas of adolescent CD-1 mice 7 days after being treated with either saline, ethanol, mephedrone or their combination, following the schedule described in the Materials and methods section. DAT and SERT were measured as specific binding of [^{3}H]WIN 35428 and [^{3}H]paroxetine, respectively. Panels E and F show the quantification of Western blots for tyrosine hydroxylase (TH) in the frontal cortex and tryptophan hydroxylase 2 (TPH-2) in the hippocampus. Values represent means ± SEM of values coming from 6 animals per group, normalized with respect to the saline-treated values. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline; #P < 0.05, ##P < 0.01 between the indicated groups.

way ANOVA revealed an overall effect of treatment both in the frontal cortex ($F_{3,16} = 8.08$, $P < 0.01$) and in the hippocampus (Fig. 2A) ($F_{3,11} = 8.10$, $P < 0.01$). Mephedrone alone only significantly increased MDA levels in the hippocampus. By contrast, the combination of mephedrone and ethanol caused substantial increases in the levels of MDA; these levels were significantly higher than those found for the mephedrone group in both assessed brain areas (Fig. 2A, B).

In order to support the hypothesis that these high amounts of MDA were produced by an increase in reactive oxygen species, we assessed the effect of ethanol on mephedrone-induced oxidative stress by measuring the levels of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx) in the remainder of the tissue used for MDA experiments. Due to the amount of tissue required for MDA determinations, quantification of the antioxidant enzymes was only feasible in the frontal cortex.

The treatment had statistically significant effects on Gpx ($F_{3,17} = 4.19$, $P < 0.05$) and CAT levels ($F_{3,17} = 16.89$, $P < 0.001$), which were significantly and similarly overexpressed in both mephedrone-treated groups (Fig. 3A, B).

Regarding SOD expression, although the overall ANOVA did not reach statistic significance due to the higher deviations, there is a clear tendency towards increase (around 85%) in both mephedrone-treated groups (Fig. 3C).

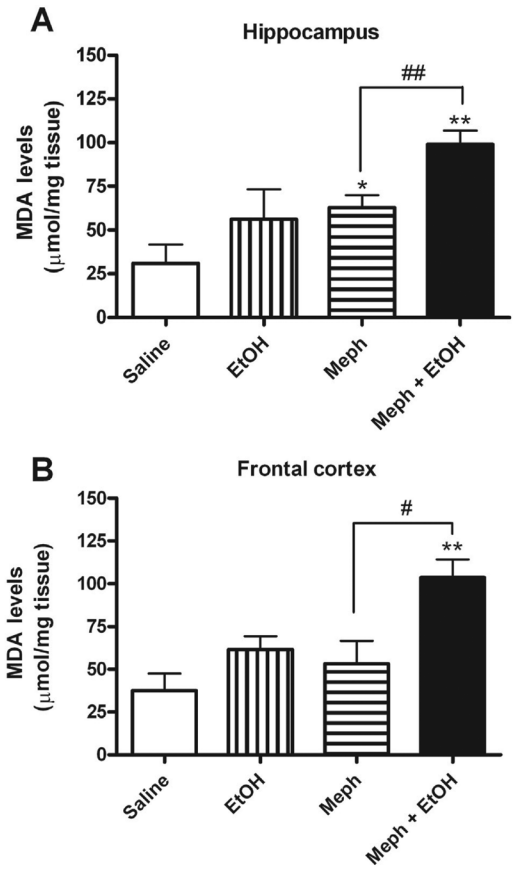

Fig. 2. Assessment of lipid peroxidation measured as levels of malondialdehyde (MDA) in hippocampus (A) and frontal cortex (B) from adolescent CD-1 mice, 24 h after being treated with either saline, ethanol, mephedrone or their combination, following the schedule described in the Materials and methods section. Values represent means ± SEM of the μmol of MDA per mg of tissue coming from 6 to 8 animals per group. *P < 0.05, **P < 0.01 vs. saline; #P < 0.05, ##P < 0.01 between the indicated groups.

Effects on the Morris water maze test

With the mice from Study 3, we investigated the effect of mephedrone, ethanol and their combination 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 ($F_{3,20} = 0.53$, $P > 0.05$; saline: 22.49 ± 1.26 cm/s; ethanol: 22.91 ± 0.91; mephedrone: 22.5 ± 1.36; mephedrone + ethanol: 21.13 ± 0.51 cm/s). Therefore, latency was taken to quantify the performance in the water maze. Overall, there was an appropriate learning of the task in all groups, as escape latency diminished over time. In the acquisition phase, two-way repeated measures ANOVA showed a significant effect of treatment and training days: variable treatment ($F_{3,20} = 11.93$, $P < 0.001$); variable days of acquisition ($F_{5,22} = 7.510$, $P < 0.001$). ANOVA also showed that the interaction between the variables treatment × day was almost significant ($F_{15,666} = 1.56$, $P = 0.07$). All the above reflects differential learning across the various groups, as can be seen in Fig. 4A.

Post-hoc analysis revealed inter-group differences in learning after day 3. On day 4, mephedrone + ethanol was significantly different to saline; on day 5, mephedrone and mephedrone + ethanol were

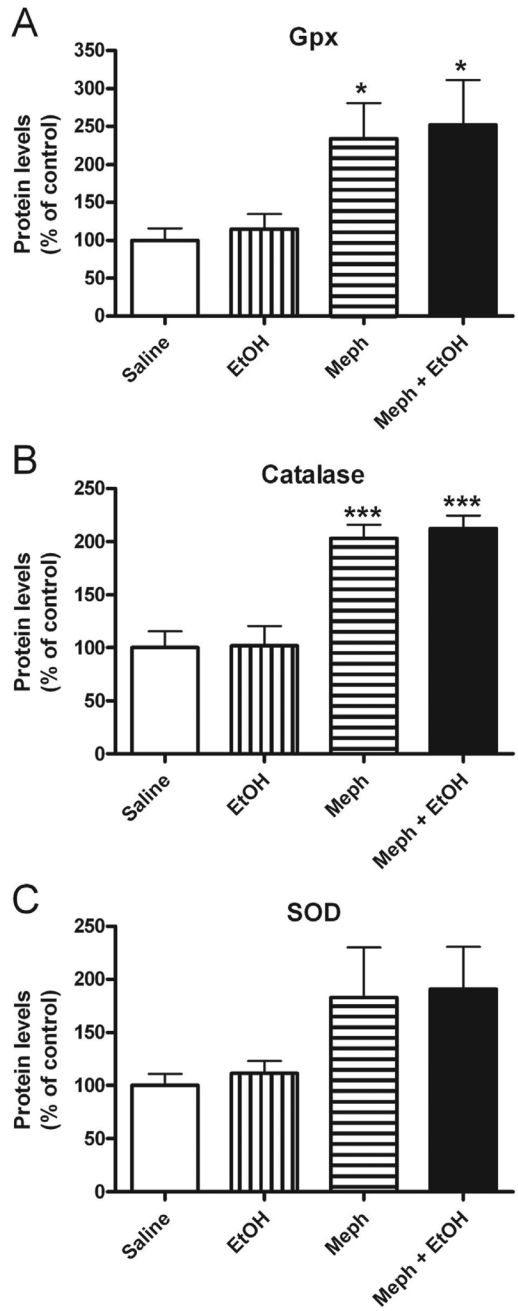

Fig. 3. Levels of enzymes related with antioxidant response in frontal cortex from adolescent CD-1 mice, 24 h after being treated with either saline, ethanol, mephedrone or their combination, following the schedule described in the Materials and methods section. Gpx1 (panel A), catalase (panel B) and SOD (panel C) levels were determined through Western blot and the quantifications by densitometry are depicted as bar graphs. Values represent means ± SEM of normalized values coming from 6 to 8 animals per group. *P < 0.05, ***P < 0.001 vs. saline.

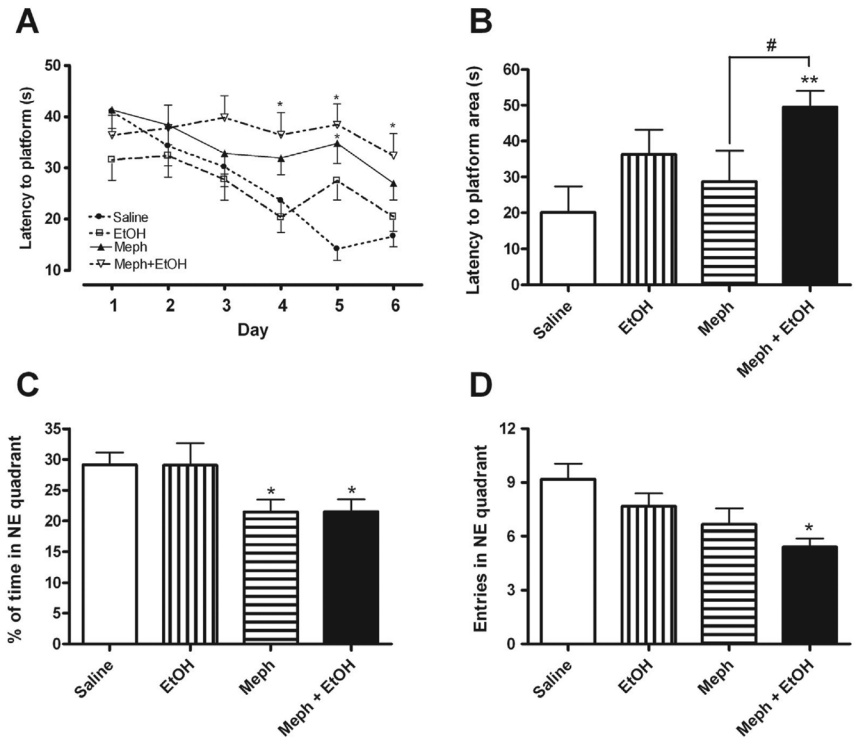

Fig. 4. Effects of treatment with either saline, ethanol, mephedrone or their combination on spatial learning and memory. Adolescent mice were treated as described in Materials and Methods and seven days after they were submitted to the Morris water maze paradigm, consisting of 6 days of training and 1 day of trial. Panel A shows the mean latency of differently treated mice to find the hidden platform, throughout the 6 days of training. Panel B represents, on the probe test day (day 7), the latency to first reach the area where the platform had been located during the training period. Finally, panels C and D depict the percentage of time spent and the number of entries of every group in the target (NE) quadrant, respectively, on the test day. Values represent means ± SEM coming from 6 animals per group. One-way or two-way ANOVA were performed where appropriate.*$P < 0.05$, **$P < 0.01$vs. saline; #$P < 0.05$ between the indicated groups.

significantly different to saline. On day 6, mephedrone + ethanol was, as on day 4, the only significantly different group to saline.

Comparison was performed between latency on day 1 and day 6 for each group individually in order to confirm learning (paired t-test). All groups showed significantly lower values on day 6, except for mephedrone + ethanol.

Twenty-four hours after the last training day acquisition, the probe trial demonstrated significant differences in several parameters:

Latency to reach target platform location. We measured the time employed by each animal before entering the area where the platform had been located during the learning phase. ANOVA revealed an overall effect of treatment ($F_{3,20} = 3.00$, $P < 0.05$). The mice treated with mephedrone + ethanol took longer than those of the other groups to reach that area (Fig. 4D). Post-hoc analysis showed significant difference between mephedrone + ethanol and saline, as well as between mephedrone and mephedrone + ethanol.

Time in each quadrant after 60 s. Two-way ANOVA revealed a significant effect of the interaction between the "quadrant" and "treatment" variables ($F_{9,76} = 2.53$, $P = 0.01$). Post-hoc analysis showed that time in the platform quadrant (NE quadrant) was different in the mephedrone ($P < 0.05$) and mephedrone + ethanol groups ($P < 0.05$) with respect to saline (Fig. 4E). In these groups, animals spent approximately 25% of the time in the NE quadrant, which can be attributable to chance, whereas in the saline and ethanol groups, this percentage was significantly higher, thus reflecting better memory. There were no inter-group differences in none of the other three quadrants.

Entries in the target quadrant. ANOVA revealed an overall effect of treatment over the number of entries in the platform quadrant ($F_{3,20} = 3.88$, $P < 0.05$) (Fig. 4F). Post-hoc analysis showed that only animals treated with mephedrone + ethanol attained significantly lower entry values than control animals ($P < 0.05$).

Effects on neurogenesis. Continuing the Study 3, 28 days after receiving the drug treatment (14 days post-MWM test) the animals were sacrificed and their sectioned brains were stained for BrdU and NeuN (Fig. 5 A-D). A one-way ANOVA of BrdU+ cells*volume index in the DG showed a main group effect ($F_{3,20} = 9.373$; $P < 0.001$). *Post hoc* test showed a significant decrease in newly formed cells in the DG of mice administered with mephedrone (75 ± 10%, $P < 0.05$) and mephedrone + ethanol (46 ± 2.75%, $P < 0.001$) with respect to saline. Furthermore, a significant difference was found between both mephedrone-treated groups ($P < 0.05$), indicating an increased deleterious effect of the combination. BrdU count in animals treated with ethanol alone was unaffected with respect to saline (Fig. 5D).

Discussion

Alcoholic drinks are frequently combined with the new psychostimulant substances (Elliott and Evans, 2014). It has been reported

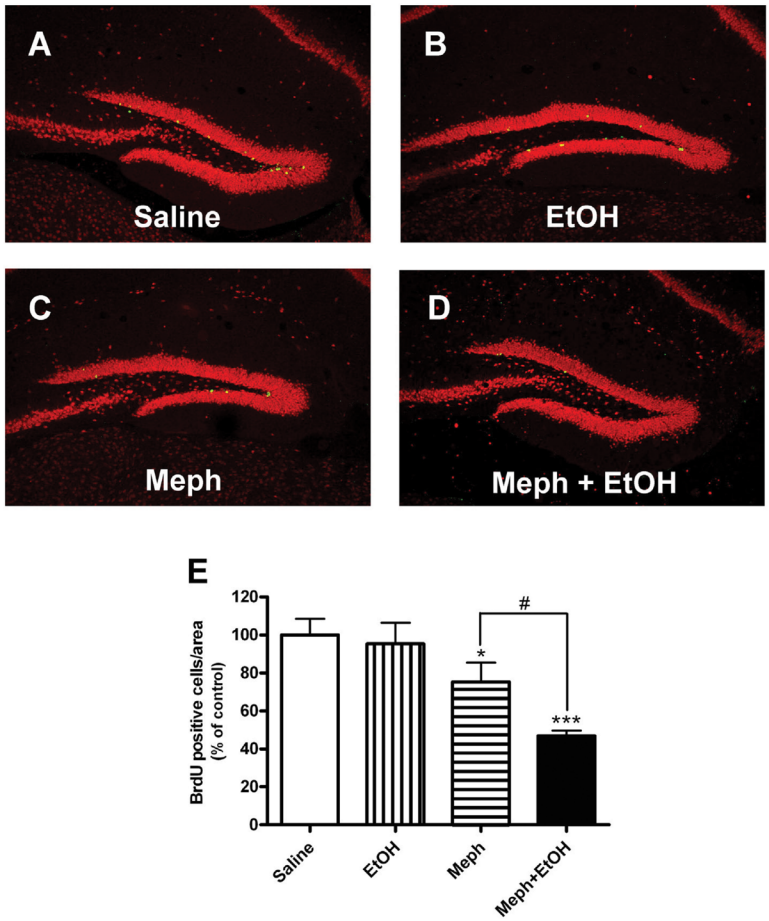

Fig. 5. Neurogenesis assessment in the dentate gyrus of the hippocampus of mice, 28 days after treatment with either saline, ethanol, mephedrone or their combination. Adolescent mice were treated as described in Materials and Methods, and received two injections of BrdU, 2 and 12 h after the last dose of treatment, respectively. Brains were fixed, sliced and immunostained for BrdU (proliferating cells) and NeuN (neuronal marker). Panels A-D show representative micrographs of the overlayed fluorescence for the two labels, where red corresponds to NeuN and green corresponds to BrdU. Due to the thickness and size of labeling, colocalization was individually assessed using higher magnification. Panel D shows overall quantification and means of BrdU-positive neurons and data are means ± SEM coming from 6 animals per group. *P < 0.05, ***P < 0.001vs. saline; #P < 0.05 between the indicated groups.

that ethanol (the psychoactive ingredient of alcoholic drinks) enhances the subjective effects of other drugs of abuse such as MDMA, and studies have shown that it increases its rewarding and psychostimulant effects (Jones et al., 2010). Similarly, our group reported a significant increase in mephedrone-induced conditioned place preference and psychostimulant properties (Ciudad-Roberts et al., 2015). Due to evidence pointing towards an increase in mephedrone's behavioral effects, it became essential to explore the potential enhancement of its neurotoxic effects.

In a previous work, three different treatment schedules were tested for neurotoxicity markers, showing a dose and time-dependent selective neurotoxicity of mephedrone in mice (Martinez-Clemente et al., 2014). Out of the three, the present schedule was selected for this study (four administrations of 25 mg/kg in one day, every 2 h, at a room temperature of 26 °C), as it showed to be the most equivalent to a typical recreational use; this same rationale was applied for the selection of the treatment schedule for ethanol (see methods for details).

Monoamine transporters such as DAT and SERT are primary targets of psychostimulants. Persistent decreases in transporter levels are generally related to neurotoxic effects of psychostimulants (Battaglia et al., 1987; Escubedo et al., 2005). Accordingly, as an initial approach, both SERT and DAT were measured in several areas of the brain 7 days after drug exposure. In the striatum, neither of both markers was modified by mephedrone nor ethanol, whereas SERT and DAT decreased in the hippocampus and frontal cortex, respectively. These effects were further potentiated by the concomitant administration of ethanol. It must be noted that DAT and SERT were not modified in the hippocampus and the cortex, respectively, pointing to a neurotransmitter/region-specific effect. This is in agreement with our previous reports (Martinez-Clemente et al., 2014). In fact, serotonergic and dopaminergic toxicities by other amphetamine derivatives (i.e. MDMA; Green et al., 2003; Yamamoto and Bankson, 2005) have also been reported to be region-specific and the extension or affectation of a given brain area also depend on the dosing schedule. In the case of mephedrone, affectation of serotonergic terminals in the frontal cortex only appeared in a two-day treatment (3 doses of 25 mg/kg per day), while dopaminergic impairment in the striatum was detected when increasing the dose of mephedrone (four doses of 50 mg/kg in one day) (Martinez-Clemente et al., 2014).

Given the marked decrease in transporter levels, we sought to measure, in the affected areas, the DA and 5-HT synthesis-limiting enzymes TH and TPH-2, specific neuronal markers. There was a good correlation between changes in enzymes and their respective neurotransmitter transporters: most importantly, ethanol was capable of potentiating, again, the decreases in enzyme levels. This points towards the possibility that changes in DAT and SERT are, in fact, due to a deleterious effect, rather than simply a homeostatic compensatory mechanism. This is supported by the fact that decreases persisted 7 days after treatment termination,

In an attempt to explain the effects on these DA- and 5-HT-related parameters, a series of oxidative stress markers were assessed, as this phenomenon is known to be responsible for the deleterious effects of multiple drugs of abuse (Yamamoto and Bankson, 2005). Oxidative stress can damage phospholipids, which are essential components of the cellular membrane, as well as other cellular structures, such as the nucleus and mitochondria, thus compromising cells viability. The complete degradation (i.e., peroxidation) of lipids is a hallmark of oxidative damage. Specifically, the polyunsaturated fatty acids present in the membranes' phospholipids are particularly sensitive to attack by hydroxyl radicals and other oxidants (Wu and Cederbaum, 2003).

In one of our previous studies, conducted in rats, we showed that mephedrone increases lipid peroxidation (Lopez-Arnau et al., 2015). Furthermore, ethanol has been described to increase ROS production through several mechanisms, such as the decrease in functional glutathione (GSH), the induction of the enzyme CYP E1 or the formation of ethanol-breakdown products (Montoliu et al., 1995; Lieber, 1997; Wu and Cederbaum, 2003).

In the present study, ethanol was capable of significantly increasing mephedrone-induced lipid peroxidation (by around 2-fold) in the frontal cortex and hippocampus, while the increase in TBARs that is induced when administered alone did not reach statistical significance. This is accompanied by the fact that this group did not suffer visible changes in oxidative stress-related enzymes (Gpx, SOD and CAT). Moreover, enzyme production was not further increased in the mephedrone + ethanol group. We hypothesize that all the above points to a potentiation in oxidative stress-related damage, where the effects of the drug combination exceed the antioxidant response leading to increased effect of generated ROS.

In this sense, GSH is believed to be the most important antioxidant present in cells (Wu and Cederbaum, 2003). When conjugated to GSH, H_2O_2 is converted into innocuous H_2O + glutathione disulfide through the enzymatic reaction mediated by Gpx. Ethanol has been shown to induce, in a dose-dependent manner, a depletion of GSH levels (Montoliu et al., 1995). It is feasible that, despite the increased amount of Gpx, ethanol causes a decrease in available GSH such that the generated H_2O_2 by the effect of mephedrone cannot be metabolized at a sufficient rate.

Due to the fact that neurotoxicity markers were consistently modified in the hippocampus and frontal cortex of mephedrone and mephedrone + ethanol exposed mice we sought to determine whether the assessed treatment was capable of causing significant differences in behavioral markers for memory and learning, as the hippocampus is the brain area most related with these functions (Squire, 1992). Interestingly, during the learning phase of the MWM protocol, animals treated with the drug combination presented significantly worse performance than control animals on the last three training days, being the only group that did not show a significant reduction in latency to platform on day 6 with respect to day 1. The learning curve of the mephedrone group is between those of the vehicle and the combination groups, indicating a lesser effect on learning. This is in agreement with the poor performance of the mephedrone + ethanol group on the probe test day, with respect to the group treated with mephedrone alone and points to a higher deleterious effect of the combination treatment on learning and memory.

Following the MWM test, neurogenesis was measured 28 days after treatment. There was a good correlation between the total amount of new cells and overall MWM performance, as only the groups treated with mephedrone showed a significantly lower cell count to that of saline; furthermore, there was a significant difference between them, the combination group exhibiting the lowest amount of new cells.

5-HT input to the hippocampus positively regulates adult neurogenesis (Brezun and Daszuta, 1999). In this sense, 5-HT reuptake inhibitors increase hippocampal neurogenesis (Malberg and Duman, 2003). Furthermore, repeated exposure to high doses of MDMA causes the opposite effect (Catlow et al., 2010). Similarly to what occurs with mephedrone in the present study, MDMA is known to produce a depletion of serotonergic markers in the hippocampus 7 days after repeated treatment (O'Shea et al., 1998); this 5-HT depletion can, in turn, cause decreased cell survival in the dentate gyrus (Brezun and Daszuta, 2000).

As far as ethanol alone is concerned, in the present study it caused a significant decrease in TH in the frontal cortex; a similar effect had been previously described by Landau et al. (2007), who administered an ethanol treatment consisting of 6 doses at 1 g/kg, causing a dramatic drop in TH, DA and its main metabolites HVA and DOPAC. The fact that no significant decrease was detected in DAT backs the possibility that the decrease in TH was in fact due to homeostatic regulations.

Although we detected changes in SERT, and TPH-2 after treating animals with ethanol alone, the behavioral consequences of its administration seem to only be apparent when given concomitantly with mephedrone. This could be due to the fact that these changes do not reflect a deleterious permanent injury when ethanol is administered alone. This is backed by the observation that ethanol alone did not cause significant changes in oxidative stress markers, which we hypothesize to be responsible for the nerve terminal damage and subsequent 5-HT and DA depletion in the mephedrone-treated groups. Finally, this hypothesis is further supported by the fact that, as mentioned above, serotonergic depletion in the hippocampus causes decreased neurogenesis, a phenomenon that has not been detected in the present study for the ethanol group, pointing to the possibility that serotonergic transmission was unaffected in these animals. Nonetheless, although it is beyond the scope of the present work, further studies are warranted to better explain the decreases in 5-HT and DA markers caused by this ethanol regimen.

To sum up, the co-administration of ethanol in adolescent mice potentiates the neurotoxic properties of a mephedrone treatment. We postulate that this phenomenon takes place through an increase in oxidative stress, which, in the hippocampus, is reflected by learning and memory deficits, as well as decreased neurogenesis.

All this suggests an increased risk if translated to humans. This is the first neurotoxicity study performed on polyabuse with cathinones, which are becoming increasingly popular among adolescents. Given that cathinones are mostly used in combination with alcoholic drinks, and that this new family of psychostimulants is generally regarded as "safe", this study is of crucial importance. Thus, an experimental-based warning concerning the risks regarding the combined consumption of these drugs should be conveyed to the population at large. Nonetheless, although adolescent brains are exceptionally vulnerable, from present data we cannot discern whether or not these effects are specific to this age window. A replica of this study using adult mice would be necessary to determine whether adults could be susceptible to changes of the same nature and degree.

Conflict of interest

None.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

We are grateful to Drs. Ester Piqué and Elisabet Teixidó, from the Laboratory of Toxicology of the Faculty of Pharmacy, for the advice in the determination of blood ethanol through gas chromatography. This work was supported by grants from the Spanish drug initiative "Plan Nacional sobre Drogas" (2012I102), "Generalitat de Catalunya" (2009SGR977) and from "Plan Nacional de Investigación Científica" (SAF2013-46135-P). Our group holds the Quality Mention from the "Generalitat de Catalunya" (2014SGR1081). None of the funding sources had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. AC is a recipient of a fellowship from the "Plan Nacional sobre Drogas".

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2015.12.019.

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7.2. Annex II

The combination of MDPV and ethanol results in decreased cathinone and increased alcohol levels

Study of such pharmacological interaction

López-Arnau, R., Buenrostro-Jáuregui, M., Muñoz-Villegas, P., Rodríguez-Morató, J., Ciudad-Roberts, A., **Duart, L.**, Camarasa, J., De la Torre, R., Pubill, D., Escubedo, E.

Progress in Neuro-Psychopharmacology & Biological Psychiatry 2017; 76; 19-28

Progress in Neuro-Psychopharmacology & Biological Psychiatry 76 (2017) 19–28

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

The combination of MDPV and ethanol results in decreased cathinone and increased alcohol levels. Study of such pharmacological interaction

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ARTICLE INFO

Article history:
Received 21 October 2016
Received in revised form 14 February 2017
Accepted 16 February 2017
Available online 17 February 2017

Keywords:
3,4-Methylenedioxypyrovalerone
Ethanol
Psychostimulant
Conditioning
Interaction

ABSTRACT

Methylenedioxypyrovalerone (MDPV) is a new psychostimulant cathinone acting as a selective dopamine transporter blocker. Due to the concomitant consumption of ethanol (EtOH) and new psychoactive substances, it is of interest to explore a possible pharmacological interaction between MDPV and EtOH. In locomotor activity assays, EtOH (1 g/kg i.p.) elicited a reduction in the stimulant effect induced by low doses of MDPV (0.1–0.3 mg/kg, s.c.) in rats, jointly with a decrease in blood and brain MDPV concentrations. Experiments in rat liver microsomes showed different effects depending on the [MDPV]/[EtOH] relationship, evidencing, at certain concentrations, the enhancing effect of EtOH on MDPV metabolism. These suggest that EtOH interacts with MDPV at microsomal level, increasing its metabolic rate. The interaction between both substances was also supported by results in plasma EtOH concentration, which were significantly increased by MDPV, in such a manner that EtOH elimination rate was significantly reduced. The possible toxicological impact of this phenomenon deserves further investigation. In contrast, the rewarding properties of MDPV were unaltered by EtOH. Microdialysis experiments verified that, in the NAcc, both substances could also act synergistically, in such a manner that extracellular dopamine concentrations are maintained. Finally, if the psychostimulant effect induced by MDPV decreased with EtOH, it could favor the boosting and re-dosing in search of the desired effects. However, as the rewarding effect of each dose of the substance would not decrease, the addictive liability could increase considerably. Moreover, we must warn about the increase in EtOH concentrations when consumed concomitantly with MDPV.

1. Introduction

The popularity of cathinones as recreational drugs has been increasing since they first appeared in the illicit drug market. 3,4-Methylenedioxypyrovalerone (MDPV) is a synthetic cathinone which shares pharmacodynamics and structural similarities with cocaine and MDMA (3,4-methylenedioxymethamphetamine). Some studies have demonstrated that MDPV is even more potent than cocaine in blocking the dopamine transporter (DAT), as well as in producing locomotor activation (Baumann et al., 2013; Cameron et al., 2013). Moreover, MDPV shows rewarding and reinforcing properties similar to those of cocaine (Baumann et al., 2013; King et al., 2015).

Recreational polydrug use is quite common (Pedersen and Skrondal, 1999). New psychoactive substances are also commonly combined with many other drugs, especially ethanol (EtOH) (Elliott and Evans, 2014). Many studies in rodents indicate that EtOH can alter the pharmacological profile of cocaine, and vice versa (Busse et al., 2005, 2004; Masur et al., 1989; Sobel and Riley, 1997). A dose-dependent attenuation of cocaine-induced hyperlocomotion when EtOH is administered prior to cocaine has been described (Dewey et al., 1997). In rats, EtOH-MDMA co-administration potentiates MDMA-induced hyperlocomotion and rewarding effect (Ben Hamida et al., 2007; Jones et al., 2010). In fact, in animal models, EtOH increases the concentrations of MDMA, and its main metabolite, in blood and brain depending on the administration regimen (Ben Hamida et al., 2007; Cassel et al., 2007). Thus, when investigating possible drug-drug interactions such as MDPV-EtOH, both pharmacokinetics and pharmacodynamics issues must be considered. Just a few studies have been published on MDPV metabolism in rats and humans (Anizan et al., 2014b; Meyer et al., 2010; Negreira et al.,

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http://dx.doi.org/10.1016/j.pnpbp.2017.02.011
0278-5846/

2015). In previous findings from our lab, we also have demonstrated in rats a correlation between MDPV brain concentrations and enhancement of the locomotor activity (Novellas et al., 2015).

The effects of EtOH on the brain are numerous and extremely complex. EtOH potentiates GABAergic transmission, but additional mechanisms elicited by EtOH can modulate this effect, triggering different consequences in the striatum (STR) and Nucleus accumbens (NAcc). Its metabolism involves alcohol-dehydrogenase (ADH) and microsomal oxidases (for review see (Cederbaum, 2012)).

To date, there is no report describing the pharmacokinetics and/or pharmacodynamics of MDPV in the presence of EtOH. Therefore, the aim of this study was to assess whether EtOH can modify the psychostimulant and/or conditioning effects of MDPV when administered concomitantly, as well as MDPV concentrations in blood and rat brain. Moreover, it is known that dopamine (DA) and the NAcc play a key function in the neural circuitry underlying psychostimulant action and acquisition of reward. Therefore, we investigated the effects of MDPV alone and combined with EtOH on the concentration of extracellular DA and its main metabolites in NAcc. The surprising results we obtained warranted a study aimed at determining the effects of MDPV on EtOH pharmacokinetics and pharmacodynamics.

2. Experimental procedures

2.1. Subjects and drugs

Male Sprague-Dawley rats (Charles River, Spain), weighing 250–300 g were used. All animal care and experimental protocols in this study complied with the guidelines of the European Community Council Directive (2010/63/EU) and ARRIVE, and were approved by the Animal Ethics Committee of the University of Barcelona.

MDPV and methylone were synthesized in racemic form as HCl salt in our laboratory as described previously (López-Arnau et al., 2012; Novellas et al., 2015). DA·HCl, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), NADPH, as well as chemicals for mobile phase and perfusion medium preparations were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat liver microsomes prepared from a pool of male Sprague-Dawley rats were purchased from Life Technologies Europe. Absolute EtOH was purchased from Scharlau (Barcelona, Spain) and diluted in saline at a concentration that never exceeded 20% (v/v) to avoid tissue irritation. Animals were randomly assigned to the following treatment groups: saline + saline, saline + EtOH, MDPV + saline or MDPV + EtOH, onwards saline, EtOH, MDPV or MDPV + EtOH groups.

2.2. Locomotor activity

After being assigned to the different treatment groups (N = 3–5/group) the animals received two habituation sessions (48 and 24 h before the experiment) in the activity box (LE8811, Panlab, Barcelona, Spain). On the testing day, the animals were injected with saline, MDPV (0.1, 0.3, 1 or 3 mg/kg, s.c.), EtOH (1 g/kg, i.p.) or their combination. Since EtOH, at certain doses, can impair or enhance locomotion, it was administered at doses reported to not affect basal activity (Cassel et al., 2004; Hodge et al., 2004). After drug administration, the animals were immediately placed in the activity box and horizontal locomotor activity (HLA) was monitored over a 20-min block during 60, 120 or 360 min. Occlusions of the photo beams (breaks) were recorded (SedaCom32, Panlab, Barcelona). Results are expressed as the area under the curve (AUC), which was measured as the total changes from baseline at each recording interval. This interval was taken from time 0 until hyperlocomotion vanished, that is, until activity was not significantly different from that of the saline group. Accordingly, the effect of 0.1 mg/kg MDPV ended after 60 min, that of 0.3 and 1 mg/kg after 120 min and that of 3 mg/kg after 360 min. The ANOVAs were thus performed between the values of the four groups at the same time chosen for each MDPV dose.

2.3. Conditioned place preference (CPP) test

The apparatus was composed of three distinct compartments (two compartments communicated by a central corridor) separated by manually operated doors. CPP was performed in three phases: preconditioning, conditioning and post-conditioning test. During the preconditioning phase (days 0 and 1), rats were placed in the middle of the corridor and had free access and roam among the three compartments of the apparatus for 20 min. The mean time spent in each compartment was recorded (Smart 3.0, Panlab, Barcelona, Spain).

During the conditioning phase (days 2, 4, 6 and 8), rats (N = 6–10/group) were treated with saline, MDPV (0.3 or 3.0 mg/kg s.c.), EtOH (1 g/kg i.p.) or both, and immediately confined into one of the two conditioning compartments for 30 min. We intended to use a dose of EtOH that did not produce CPP on its own (Tzschentke, 2007). On days 3, 5, 7 and 9 animals received saline and were confined to the opposite compartment. The animals were exposed to only one pairing per day and treatments were counterbalanced.

The post-conditioning test (day 10) was conducted identically to the pre-conditioning phase. A preference score was expressed in seconds and calculated for each animal as the difference between the time spent in the drug-paired compartment in the test minus the time spent in the same compartment in the pre-conditioning phase.

2.4. Surgery and microdialysis experiments

The microdialysis experiments were carried out on awake rats (N = 3–5/group) according to the protocol described by Kehr et al. (2011), with some modifications. Rats were anesthetized with ketamine (90 mg/kg i.p.) plus xylazine (10 mg/kg, i.p.) and placed in a stereotaxic frame. After exposure of the skull, a hole for a guide cannula and three holes for the anchor screws were drilled. Then, an intracerebral guide cannula (AT6.14.iC, Agntho's, Lidingö, Sweden) was surgically implanted and aimed at the NAcc, according to the coordinates: 1.6 mm lateral, 2.2 mm anterior to bregma and 6.0 mm ventral to the dura surface (Paxinos and Watson, 2007), and fixed to the skull using dental cement. Rats were allowed at least one week for recovery from surgery. On the evening before an experiment, a microdialysis probe (AT.6.14.2, Agntho's, Lidingö, Sweden; 2 mm membrane length with 15,000 Da cut-off) was inserted into the guide cannula and perfused overnight with artificial cerebrospinal fluid (aCSF) solution (148 mM NaCl, 2.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 1.2 mM Na_2HPO_4 and 0.3 mM NaH_2PO_4) at a flow rate of 0.6 μL/min. On the next day, the flow was changed to 1 μL/min and after a stabilization period of 2 h, the microdialysis samples were collected at 20 min intervals in plastic vials filled with 10 μL of an antioxidant mixture (0.1 M acetic acid; 0.27 mM Na_2EDTA; 0.5 mM ascorbic acid) (Thorré et al., 1997). The first three samples were used for estimation of basal levels of DA, DOPAC and HVA. Thereafter, saline, MDPV (0.3 or 3.0 mg/kg s.c.), EtOH (1 g/kg i.p.), or both were injected to separate groups of rats and the fractions were collected for 120 or 240 min and stored at − 80 °C before analysis. At the end of the experiments, the animals were perfused with paraformaldehyde and the brains were removed and examined for correct placement of the probe using Cresyl violet staining. Only the data from those rats with correct probe placements were included in the study.

2.5. Liquid chromatography/mass spectrometry determination of DA and metabolites in dialysate samples

An Agilent 1290 Liquid Chromatography (LC) system equipped with an autosampler and coupled to AB Sciex QTRAP 6500 mass spectrometer (MS) was used to quantify the corresponding monoamine and

metabolites. Chromatographic separation was achieved on a Discovery HS F5 (150 mm × 4 mm, 3 μm, Sigma-Aldrich, St. Louis, MO, USA) pentafluorophenyl column thermostated at 37 °C. The mobile phase was water (A) and methanol (B) with 0.1% of HCOOH in both solvents. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows, (0, 0), (0.5, 0), (5.90, 30), (6, 100), (9, 100), (9.10, 0), (10.0, 0) at a constant flow rate (500 μL/min). The flow was directed to waste for the first 2 min to prevent the inorganic ions of aCSF solution to enter the mass spectrometer. The microdialysate samples were refrigerated at 4 °C and 20 μL were injected, without sample pretreatment, into the LC-MS/MS system. Mass spectrometric quantification in positive ion mode was carried out using the following transitions: DA (m/z 154 → 137 and 154 → 91) and DOPAC (m/z 123 → 77). A negative ion mode was used in the analysis of HVA (m/z 181 → 122).

Six standards (from 0.1 nM to 10 nM for DA or from 10 nM to 1 μM for metabolites) were prepared daily in a solution composed by aCSF/antioxidant mixture (2:1) to obtain the calibration curve. The method showed linearity within the concentration range studied and the detection limit (signal-to-noise ratio = 3) for DA was 0.05 nM and for DOPAC and HVA was 1 nM. The accuracy of the assay was 85–115% and the intra- and inter-assay coefficients of variation were <15%. Analyst v1.4.2 software was used to calculate the areas of chromatographic peaks.

2.6. MDPV concentrations in blood and the STR

Before blood sampling, rats (N = 6–8/group) were implanted with an intravenous (i.v.) jugular catheter under isoflurane anesthesia (Caine and Koob, 1993). Blood samples (150–200 μL) were collected from awake rats through the catheter at 5, 10, 20, 30, 40, 60 and 120 min after MDPV injection (0.3 mg/kg, s.c.) alone or in combination with EtOH (1 g/kg, i.p.) and transferred to 300 μL tubes with EDTA. According to the method previously described (Novellas et al., 2015), 90 μL of plasma was mixed with 10 μL of an internal standard (IS) solution (methylone, 200 ng/mL). The mixture was extracted by adding 250 μL of methanol. After centrifugation (10,000 ×g, 5 min), 250 μL of supernatant were acidified with HCOOH (50%) (Sørensen, 2011). The mixture was ultrafiltered and 100 μL of the filtrate were transferred to an autosampler vial to quantify MDPV concentrations by LC-MS/MS (Novellas et al., 2015).

A PE Sciex API3000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was used to quantify the MDPV in brain and blood samples. Chromatographic separation was achieved on a Luna C18 (100 × 2.0 mm, 2.5 μm) column. The mobile phase was water (A) and methanol (B) with 0.1% of HCOOH 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 (27.5, 5), at a constant flow rate (150 μL/min). 5 μL of biological samples were injected into the LC-MS/MS system.

As there is a direct correlation between brain concentrations and hyperlocomotion elicited by MDPV (Novellas et al., 2015), we quantified MDPV in rat STR after its administration alone (0.3 and 3 mg/kg s.c.) or with EtOH (1 g/kg i.p.). To minimize the number of animals used in this study and according to the data of locomotor activity, the MDPV concentrations in STR were measured only at 20 min after drug administration. Moreover, a blood sample was also collected and MDPV plasma concentrations were quantified as above in order to calculate the brain/blood ratio at this time.

This experiment was carried out as described by Novellas et al., 2015, with minor modification. Briefly, rats STR (N = 5/group) were homogenized and centrifuged (1000 ×g, 20 min). The sample plus IS (methylone) was applied to a C8 Sep-Pak® SPE cartridges (Waters Corp., Milford, MA, USA). MDPV was eluted with methanol and transferred in an auto sampler vial to quantify MDPV concentrations by LC-MS/MS as above.

2.7. Blood EtOH concentration (BEC)

Blood samples (150–200 μL) from animals (N = 4/group) were collected through vein catheters at 5, 10, 20, 30, 40, 60 and 120 min after EtOH injection (1 g/kg i.p.) alone or in combination with MDPV (0.3 mg/kg, s.c.). 80 μL of plasma were transferred to sample microvials, combined with 20 μL of IS solution (1-propanol, 1 g/L) and placed in a water bath at 50 °C for 5 min. Then, 1 mL of the headspace gas was injected with a Hamilton syringe into the gas chromatograph (Agilent 7890A GC-System) equipped with a flame ionization detector (FID). EtOH and IS were separated on a capillary column (Supelcowax-10®, 30 m × 250 μm × 0.25 μm) using helium as a carrier gas (2.0 mL/min, split ratio 20:1). The column oven temperature was isothermal at 55 °C and the injector and the FID system were selected at 150 and 200 °C, respectively. BECs were quantified from linear standard curves in EtOH-free plasma (0.1–2 g/L EtOH) using the peak area ratios of EtOH to the IS using Agilent Chemstation software.

2.8. MDPV metabolism in rat liver microsomes

The evaluation of MDPV metabolism was carried out at 37 °C in a shaking bath. Preliminary experiments were performed to determine whether MDPV metabolism was linear with respect to time and to evaluate the percentage of MDPV metabolized. The final incubation mixtures (250 μL) contained MDPV (1 or 10 μM), EtOH (0, 0.1, 1.0, 10 or 100 mM), rat liver microsomes (0.5 mg protein/mL), and NADPH (1 mM) in 100 mM sodium phosphate buffer (pH 7.4). After a 5-min pre-incubation with NADPH, the reactions were initiated by the addition of rat liver microsomes and stopped 3 min later by the addition of 625 μL ice-cold methanol. A negative control in the absence of NADPH was employed to verify the NAPDH-dependent reaction. All the experiments were performed in triplicate. To each reaction mixture, 20 μL IS solution (methylone, 10 μg/mL) was added. The resulting mixture was centrifuged (10,000 × g, 5 min). The supernatant was filtered (cellulose acetate membrane pore size 0.22 μm), acidified with 100 μL HCOOH (50%) and centrifuged (12,000 × g, 10 min). The supernatants were then transferred to HPLC vials, and MDPV concentrations were analyzed by LC-MS/MS as previously described.

2.9. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences between groups were compared using a one- or two-way (repeated measures) analysis of variance (ANOVA) or Student's t-test for independent samples where appropriate. Significant differences ($p < 0.05$) were analyzed using the Tukey's post hoc test for multiple comparison measures (InVivoStat software package). In all two-way ANOVA analysis, the variable time and the interaction treatment × time were significant. To ease reading, the statistics of these variables were not showed.

3. Results

3.1. Effects on locomotor activity

HLA was monitored for 60, 120 or 360 min depending on the MDPV dose (see Table 1). The AUC analysis of variance demonstrated an overall significant effect of treatment variable ($AUC_{0-60\ min}$ $F_{3,12} = 9.73$, $p < 0.001$; $AUC_{0-120\ min}$ $F_{5,16} = 30.11$, $p < 0.001$; $AUC_{0-360\ min}$ $F_{3,12} = 8.33$, $p < 0.01$). The post hoc test revealed that MDPV increased the locomotor activity at all doses tested compared with the saline group. Moreover, at low doses of MDPV (0.1 and 0.3 mg/kg) co-administration of EtOH induced a significant decrease ($p < 0.01$) in HLA compared with MDPV alone (See Table 1). Nevertheless, at higher doses of MDPV (1 and 3 mg/kg) co-administration of EtOH did not produce any change.

Table 1
Effect of MDPV alone or in combination with EtOH on HLA in rats. Results are expressed as mean ± SEM and represent the measurement of the area under the curve (AUC).

Drug	AUC		
	0–60 min	0–120 min	0–360 min
Saline	10,565 ± 1809	14,359 ± 3233	15,485 ± 3164
EtOH 1 g/kg	13,433 ± 2228	13,827 ± 2300	15,085 ± 2239
MDPV 0.1 mg/kg	30,950 ± 5401**	–	–
MDPV 0.1 mg/kg + EtOH	9050 ± 2278##	–	–
MDPV 0.3 mg/kg	–	88,016 ± 10,189**	–
MDPV 0.3 mg/kg + EtOH	–	30,506 ± 6160#	–
MDPV 1 mg/kg	–	134,596 ± 17,712***	–
MDPV 1 mg/kg + EtOH	–	140,915 ± 13,917***	–
MDPV 3 mg/kg	–	–	202,924 ± 38,741*
MDPV 3 mg/kg + EtOH	–	–	254,328 ± 68,569**

* $p < 0.05$ vs. saline.
** $p < 0.01$ vs. saline.
*** $p < 0.001$ vs. saline.
# $p < 0.05$ vs. the corresponding MDPV group.
$p < 0.01$ vs. the corresponding MDPV group.

Fig. 1 shows the time course of the effects on locomotor activity elicited by EtOH and MDPV (0.3 mg/kg Fig. 1A, 3 mg/kg Fig. 1B). At the dose of 0.3 mg/kg, the post hoc test confirmed statistical significance between 0.3 mg/kg MDPV and 0.3 mg/kg MDPV + EtOH group, suggesting a blockade of the psychostimulant effect elicited by MDPV. However, no effects of EtOH on the hyperlocomotion induced by 3 mg/kg MDPV were found at any time point.

3.2. Effect of EtOH on the place conditioning induced by MDPV

On the test day (day 10, post-conditioning), results revealed a significant effect of treatment ($F_{5,36} = 6.94$, $p < 0.001$). MDPV treated animals acquired similar place preference after being conditioned with both doses. The concomitant administration of EtOH did not modify the rewarding effect induced by MDPV. Moreover, as we expected, EtOH did not exert any effect on preference score by itself (Fig. 2).

3.3. Effects of MDPV alone or with EtOH on DA, DOPAC and HVA concentrations in the NAcc

Fig. 3 shows the time course of the extracellular concentrations of DA in the NAcc of animals after an acute administration of MDPV 0.3 mg/kg (panel A) or 3 mg/kg (panel B), MDPV + EtOH, EtOH or saline. Two-way ANOVA revealed a significant effect of the treatment variable (panel A: $F_{3,11} = 8.210$, $p < 0.01$; panel B: $F_{3,10} = 14.58$, $p < 0.001$). However, the post-hoc analysis did not show any difference in DA concentrations between MDPV and MDPV + EtOH at any time point or MDPV dose tested. Administration of MDPV (0.3 and 3 mg/kg) caused a rapid increase in extracellular concentrations of DA, reaching changes in peak concentrations of 382.4 ± 74.1% (Fig. 3A, $p < 0.001$) and 1477.1 ± 524.9% (Fig. 3B, $p < 0.001$) compared to saline, at 20 and 40 min, respectively. Moreover, we observed a slight increase, although no significant, of about 50% in the extracellular concentrations of DA after EtOH (1 g/kg) administration.

The overall effects of saline, EtOH, MDPV and MDPV + EtOH on the DA concentrations, expressed as relative AUC values, are depicted in Figs. 4A and 5A. MDPV (0.3 and 3 mg/kg) caused an increase in total DA of 150.0 ± 35.1% ($p < 0.01$) and 891.1 ± 218.7% ($p < 0.01$), respectively, that was unchanged when EtOH was administered concomitantly.

The same samples were also analyzed for the quantification of DOPAC and HVA concentrations. Following MDPV administration, the DOPAC and HVA concentrations were significantly reduced (21.7 ± 0.2, and 17.4 ± 3.5%, respectively, Fig. 4B), and this decline was dampened by EtOH co-administration. In a similar way, when the high dose of MDPV was used, a significant reduction in the DOPAC concentrations of 23.1 ± 6.2% was found (Fig. 5B) without being significantly modifed

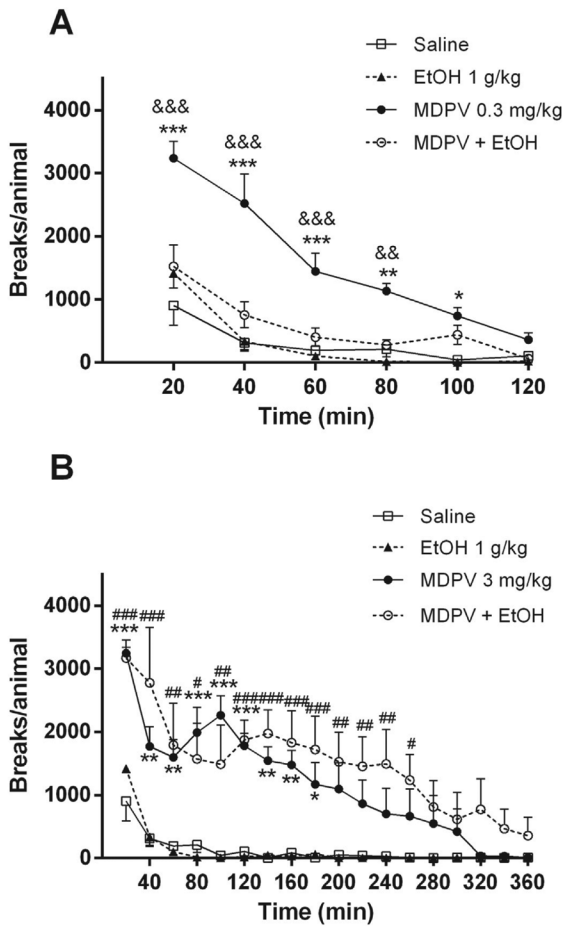


Fig. 1. Time course of locomotor activity induced by saline, EtOH, MDPV or MDPV plus EtOH administration. In panel A (MDPV 0.3 mg/kg), two-way ANOVA revealed the effect of the treatment variable ($F_{3,11} = 24.49$, $p < 0.001$). In panel B (MDPV 3 mg/kg), two-way ANOVA also denoted the effect of treatment ($F_{3,11} = 10.12$, $p < 0.01$). Data are expressed as the mean ± SEM. */#$p < 0.05$, **/##$p < 0.01$ or ***/###$p < 0.001$ vs. saline group at the corresponding time point; &&$p < 0.01$, &&&$p < 0.001$ vs. MDPV + EtOH group at the corresponding time point.

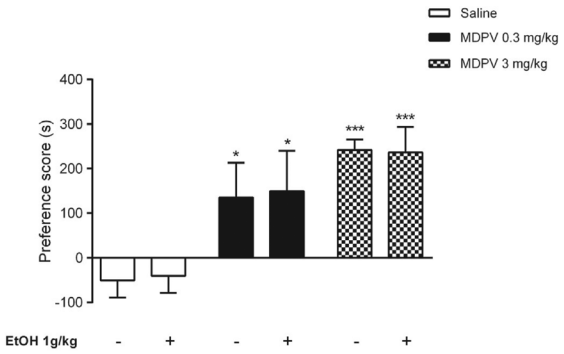


Fig. 2. Effect of EtOH on MDPV-induced conditioned place preference. Results are expressed as the mean ± SEM. $^{*}p < 0.05$ or $^{***}p < 0.001$ vs. saline group.

by EtOH. In addition, HVA concentrations remained unchanged (Fig. 5C).

3.4. Effect of EtOH on MDPV concentrations in blood and rat STR

We measured the plasma concentrations of MDPV over time after s.c. administration of 0.3 mg/kg alone or in combination with EtOH i.p. There was a significant reduction of MDPV concentrations during the first 20 min when combined with EtOH. As shown in Fig. 6, the statistical test revealed a significant effect of the treatment variable ($F_{1,14} = 5.969$, $p < 0.05$). Moreover, the $AUC_{5 \rightarrow 120}$ analysis also showed a significant decrease in the total amount of MDPV (Fig. 6 inset).

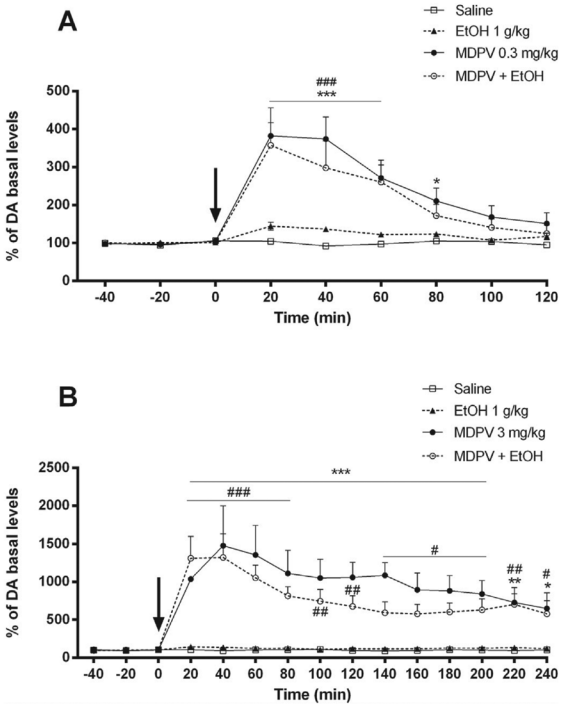


Fig. 3. Effect of saline, EtOH, MDPV alone (0.3 mg/kg, panel A, or 3 mg/kg, panel B) or in combination with EtOH on extracellular levels of DA in the NAcc of awake rats. The arrow indicates the time of drug or saline administration. Data are mean ± SEM and expressed as a percentage of preinjection baseline values (% basal). $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ MDPV vs. saline group. $\#p < 0.05$, $\#\#p < 0.01$ or $\#\#\#p < 0.001$ MDPV + EtOH vs. saline group.

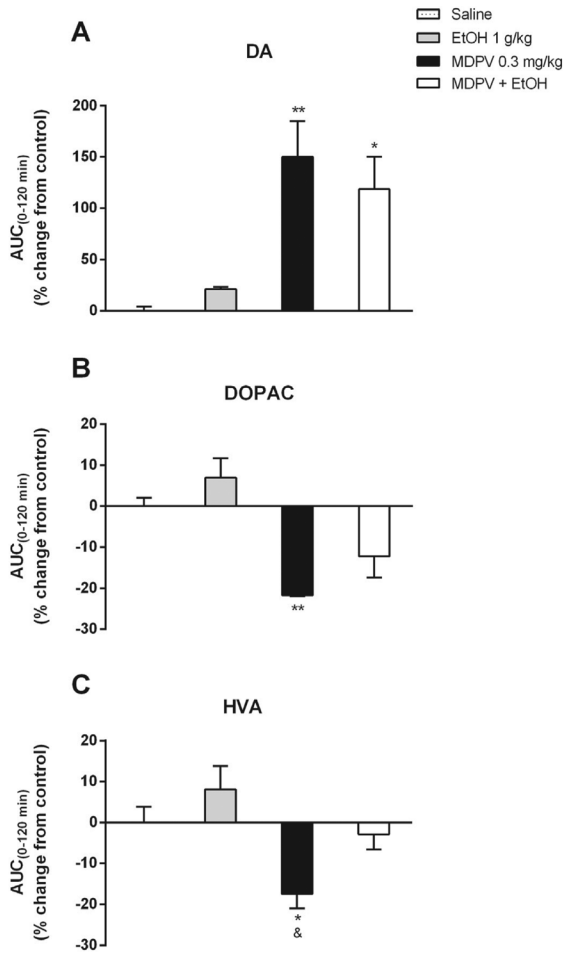


Fig. 4. Overall effects of saline, EtOH, MDPV alone (0.3 mg/kg) and plus EtOH on DA (panel A), DOPAC (panel B) and HVA (panel C) levels in the NAcc of awake rats. The columns represent the $AUC_{0-120\ min}$ values calculated as the differences in relative changes (%) in these compounds between the drug- and saline-treated groups. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. saline group; $\&p < 0.05$ vs. MDPV + EtOH group.

20 min after the MDPV (0.3 mg/kg) injection, the results in STR corroborated the decrease in MDPV plasma concentrations described above (MDPV 0.3 mg/kg = 133.6 ± 8.5 ng MDPV/g tissue; MDPV 0.3 mg/kg + EtOH 1 g/kg = 77.4 ± 10.4 ng MDPV/g tissue ($t = 4.189$, $p < 0.05$)). The assessed brain/blood ratio at 20 min was 2.2 ± 0.5 after MDPV administration and also 2.2 ± 0.5 after MDPV + EtOH.

Because the combination of EtOH with MDPV 3 mg/kg did not modify MDPV hyperlocomotion, we tested the blood and brain concentrations of this combination only at the time point of 20 min (maximal psychostimulant effect and maximal striatal concentrations; Novellas et al., 2015). Concerning plasma concentrations, no significant changes were observed between both groups (MDPV 3 mg/kg = 446.0 ± 39.5 ng/mL; MDPV 3 mg/kg + EtOH 1 g/kg = 385.8 ± 25.4 ng/mL; n.s.). As expected, the combination with EtOH also resulted in no different striatal concentrations (MDPV: 1266.5 ± 40.1 ng MDPV/g tissue vs MDPV + EtOH: 1179.8 ± 68.8 ng MDPV/g tissue; n.s.).

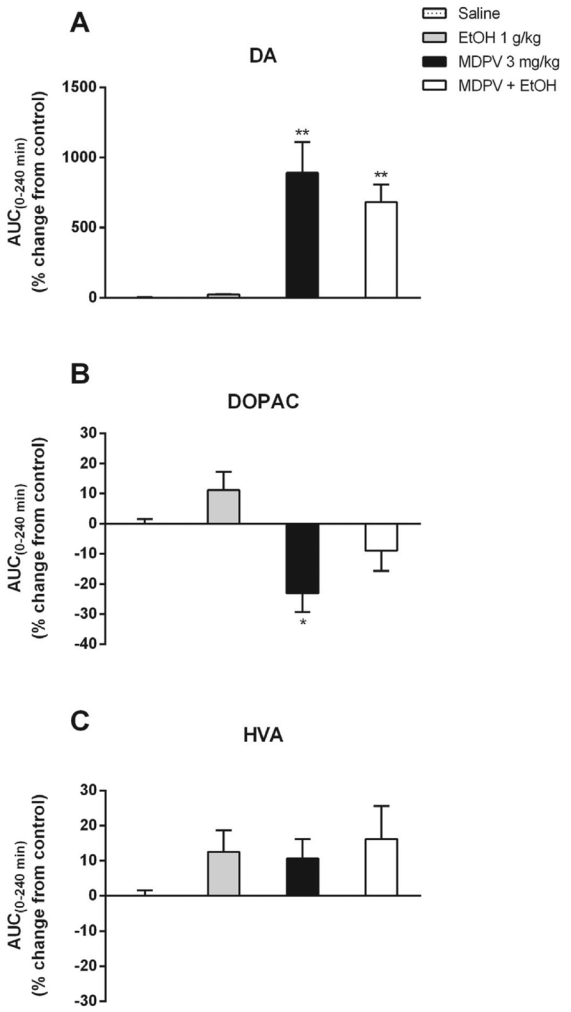


Fig. 5. Overall effects of saline, EtOH, MDPV alone (3 mg/kg) and plus EtOH on DA (panel A), DOPAC (panel B) and HVA (panel C) levels in the NAcc of awake rats. The columns represent the $AUC_{0-120\ min}$ values calculated as in Fig. 4. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. saline group.

3.5. MDPV metabolism in rat liver microsomes in the presence of EtOH

The metabolic rate of MDPV was determined using rat liver microsomes. The amount of MDPV decreased linearly in a time-dependent manner and at a very high velocity (63% of substrate metabolized at 15 min). MDPV metabolism was NADPH- and microsome-dependent. In order to avoid substrate depletion, the experiments were performed with an incubation time of 3 min (25% of MDPV metabolized). The amount of MDPV metabolized at 3 min of incubation is expressed as 100% of metabolic rate. The effects of increasing amounts of EtOH on MDPV metabolism were then assessed (Fig. 7).

When MDPV was assayed at high concentration (10 μM) (Fig. 7A), EtOH inhibited the MDPV metabolism in a concentration-dependent manner ($F_{4,10} = 40.56$; $p < 0.001$). Nevertheless, when using a lower MDPV concentration (1 μM), we demonstrated a biphasic effect of EtOH on microsomal activity ($F_{4,10} = 29.96$; $p < 0.001$) (Fig. 7B). EtOH 1 mM increased MDPV microsomal metabolism ($p < 0.01$), but at high concentrations (100 mM) an inhibition of the microsomal activity was found. Consequently, the enhanced MDPV metabolism induced by EtOH was evidenced only when combining MDPV and EtOH at concentrations of 1 μM and 1 mM, respectively. In these conditions, the metabolic rate of MDPV was increased from 100% to 145%.

3.6. Effect of MDPV on BECs

We measured the BECs over time after i.p. administration of EtOH alone or in combination with MDPV (0.3 mg/kg, s.c.) (Fig. 8). After comparing both BEC curves versus time, a significant effect of treatment ($F_{1,6} = 13.97$; $p < 0.001$) was observed. BECs were significantly higher during 60 min ($p < 0.01$) post-administration in rats given 0.3 mg/kg MDPV compared to those given 1 g/kg EtOH alone. Furthermore, the AUC analysis also supported these differences (Fig. 8 inset; $t = 3.833$, $p < 0.01$). Additionally, both BECs time course were adequately fitted to a mono-exponential decay model, allowing us to calculate the corresponding half-life values. MDPV increased the EtOH half-life from 23.0 ± 2.4 min to 64.7 ± 9.4 min ($t = 4.315$; $p < 0.01$).

4. Discussion

In the present study we have described a pharmacokinetic interaction between MDPV and EtOH, substances often consumed concurrently (Elliott and Evans, 2014), that could have a significant toxicological impact. The initial approach of this work was to assess the effect of EtOH on MDPV-induced changes in behavioral parameters for psychostimulant and conditioning properties. The chosen EtOH dose (1 g/kg, i.p.) yielded a BEC in the range of that observed after moderate alcohol drinking (Eckardt et al., 1998). Our initial finding was of great interest, as EtOH significantly reduced locomotor activity counts (70% and 65%) induced by low subcutaneous doses of MDPV (0.1 and 0.3 mg/kg, respectively). Being obtained the previous results on locomotor activity, our study was mainly focused on two different s.c. doses of MDPV, 0.3 and 3 mg/kg, which are equivalent to a 3 and 30 mg dose for a 60 kg person (Reagan-Shaw et al., 2008), respectively. Tentative estimations pointed towards threshold levels around 1–5 mg to "strong" effects between 10 and 25 mg (www.erowid.org). Interestingly, our subsequent experiments using the dose of 3 mg/kg MDPV showed that the locomotor effects of this higher dose were not significantly affected by EtOH.

Next, we assayed the effects of EtOH on the rewarding effects (CPP) induced by two different doses of MDPV (0.3 and 3 mg/kg). Both MDPV doses produced CPP, with a higher significance degree for the dose of 3 mg/kg, but not significantly different with respect to the dose of 0.3 mg/kg which showed an important SEM. This probably denotes an effect bordering on significance. By contrast, the result of the dose of 3 mg/kg is more robust and supports a high rewarding effect of this drug at this dose. In fact, DA release in the NAcc, which is directly related with rewarding effects, was about three-fold higher after the dose of 3 mg/kg. This points to a dose-response relationship in the rewarding effect regardless the lack of significance between doses on CPP. Accordingly, other authors (i.e. King et al., 2015) reported that MDPV not always shows a clear dose-response effect in the CPP paradigm.

Conversely to the effect of EtOH on MDPV-induced hyperlocomotion, the rewarding properties of MDPV were unaffected by EtOH. If the psychostimulant effect of the cathinone decreased with alcohol, it could favor the boosting and re-dosing in search of the lost effects. These are typical behaviors followed by consumers of such substances (Ross et al., 2012). However, as the reinforcing effect of each dose of the substance would not decrease, the addictive liability could increase.

In an attempt to shed a light on the potential underlying mechanisms for this phenomenon, MDPV concentrations were determined both in plasma and STR after a single administration of MDPV alone or in combination with EtOH. It is known that MDPV crosses the blood brain barrier (BBB) through passive diffusion and active transport.

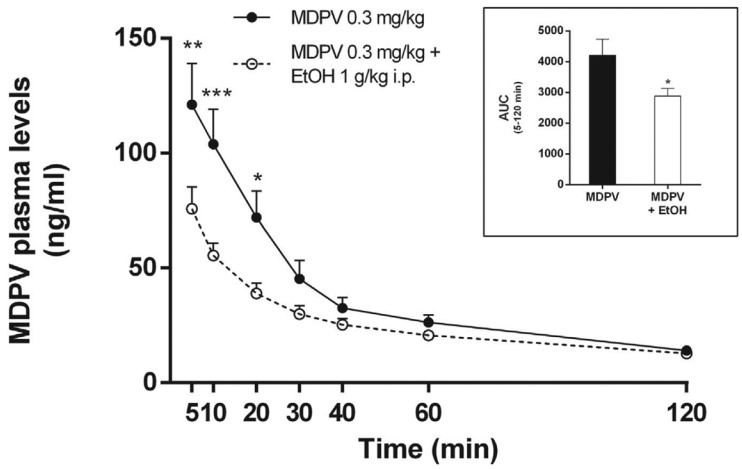


Fig. 6. Time-course of MDPV plasma levels after s.c. administration of MDPV alone (0.3 mg/kg) (filled circles) or in combination with EtOH (open circles). Data are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. MDPV + EtOH group at the corresponding time point. Inset: Overall effect of EtOH on AUC values of MDPV plasma levels. $^{*}p < 0.05$ vs. MDPV group.

This last feature is a differential trait of this compound with respect to other synthetic cathinones (Simmler et al., 2013). Thus, we initially hypothesized that EtOH could be disrupting the active transport of MDPV through the BBB. In agreement with the registered psychostimulant effect, the results revealed a significant reduction (of around 50%) in MDPV concentrations in both blood and brain when the cathinone, only at the lowest dose tested (0.3 mg/kg, s.c.), was combined with EtOH. Consequently, no blood/brain ratio disruption was observed.

With these results, taking into account the reported extensive metabolism of MDPV (Anizan et al., 2014a,b; Meyer et al., 2010; Negreira et al., 2015; Uralets et al., 2014), the high liposolubility of this drug and the frequent interactions of EtOH with other drugs' metabolism, the most likely explanation for this effect could be a metabolic interaction. Consequently, we carried out the in vitro studies with rat liver microsomes to test the possibility of an interaction under some specific concentrations of MDPV and EtOH. Interestingly we found two opposite effects, depending on [MDPV]/[EtOH] relationship. When MDPV was assayed at a high concentration (10 μM), EtOH inhibited the MDPV metabolism in a dose-dependent manner. When using a lower MDPV concentration (1 μM), we demonstrated a biphasic effect of EtOH. The enhancing effect of EtOH on MDPV metabolism was mainly evidenced when combining MDPV 1 μM and EtOH 1 mM.

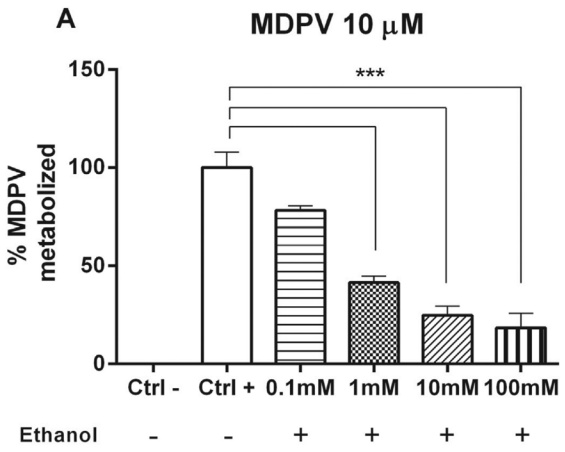
Therefore, the stimulation of MDPV metabolism induced by EtOH is a reasonable explanation for the decrease in MDPV concentrations observed in vivo after concomitant administration of EtOH and low doses of MDPV. In fact, a similar interaction on microsomal metabolism had already been described for other substances. Linnoila et al. (1990) studied plasma concentrations of adinazolam and EtOH when administered jointly. They observed higher BEC when administering adinazolam after EtOH, while concentrations of the adinazolam main metabolite were higher. Moreover, Hellum and Nilsen (2007) studied the effect of EtOH on the CYP2D6 mediated metabolism of dextromethorphan. They clearly demonstrated a biphasic effect of EtOH on CYP2D6. EtOH, at concentrations from 0.5% to 1.1%, increased CYP2D6 activity as compared to control. For EtOH concentrations exceeding 1.5%, a linear inhibition of the CYP2D6 activity was found.

The main enzymes responsible for the transformation from MDPV into its metabolites, in rats, are CYP2D1 (rat orthologue of human CYP2D6), CYP2C19 and CYP1A2 (Meyer et al., 2010; Negreira et al., 2015). CYP2C19 could be inhibited by low concentrations of EtOH (Busby et al., 1999). Therefore, CYP2D1 seems to be the main candidate to show a stimulant effect of EtOH over MDPV metabolism. However, this aspect deserves further investigation and requires extending the study also to the human liver microsomes.

Given the effect of EtOH on locomotor activity, as well as on MDPV concentrations, it proved interesting to look for an explanation to the fact that MDPV conditioning properties were not affected by the combination. For this purpose, microdialysis experiments were performed and changes in DA, DOPAC and HVA in NAcc were assessed. We found that MDPV produces a rapid dose-dependent elevation of extracellular DA, as expected (Schindler et al., 2015). As previously described for a DAT blocker (Kalivas and Duffy, 1990; Müller et al., 2004; Shimada et al., 1996), in parallel with an increase in DA concentration, a slight decrease in DA metabolites was found. This occurs because DA metabolism by MAO takes place inside the nerve terminal. The resulting DOPAC is actively transported to the synaptic cleft (Miyamoto et al., 1991). As MDPV inhibits DAT, DA metabolism is reduced. Similar effects were observed at 3 mg/kg, except for HVA, which can be explained by the high extracellular DA concentrations reached (1500%, Fig. 3B) allowing metabolism by COMT, which is localized in the plasma membrane (Trendelenburg, 1990).

Similarly, EtOH-treated animals showed a slight increase, although no significant, in DA concentrations. It is known that EtOH modulates the function of $GABA_A$ receptors, reducing DA release. In the VTA, however, EtOH decreases rather than increases GABAergic neurotransmission (Stobbs et al., 2004; Xiao et al., 2007), leading to increased mesoaccumbal DA release (Martí-Prats et al., 2015; Melendez et al., 2003; Yoshimoto et al., 1992). Thus, while in the NAcc this substance increases the release of DA, in the dorsal STR it does just the opposite (Budygin et al., 2001). This is the reason why the effect of EtOH on motor activity is more likely to vary depending on the different experimental conditions, whereas its reinforcing and rewarding effect are clear and reproducible (Acevedo et al., 2013; Cunningham et al., 2002; Durcan and Lister, 1988; Frye and Breese, 1981; Milton et al., 1995; Morales et al., 2012; Robledo et al., 1991; Wróbel, 2011).

Interestingly, the increase in DA concentrations induced by MDPV was not significantly modified by EtOH. In fact, EtOH may increase DA release. Although this increase was not statistically significant when administered alone, significant levels could be reached when combined with the DA uptake inhibition induced by MDPV. This should result in increased extracellular DA levels and potentiation of the effects of MDPV. However, the pharmacokinetic interaction with 1 g/kg EtOH observed at 0.3 mg/kg MDPV produces decreased blood and brain MDPV levels, so the effect of MDPV should be lower than expected when



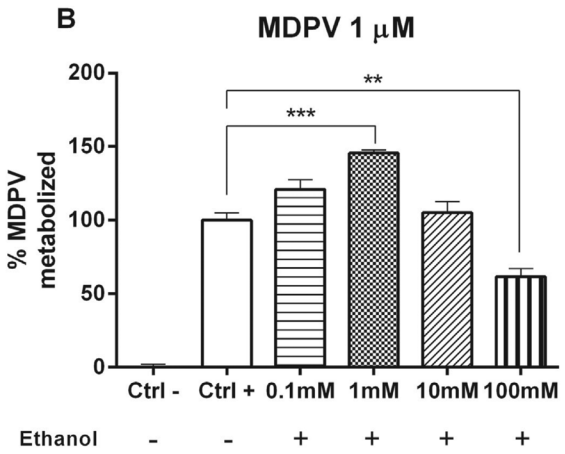


Fig. 7. In vitro metabolism of MDPV by rat liver microsomes. MDPV 10 μM (panel A) or 1 μM (panel B) was incubated with rat liver microsomes in the presence of different EtOH concentrations. A negative control (Ctrl −) in the absence of NADPH was included. The amount of MDPV metabolized at 3 min of incubation is expressed as 100% of metabolic rate (positive control, Ctrl +) and the rest of results are normalized by this value. Data are the mean ± SEM of the percentage of MDPV metabolic rate. $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. Ctrl +.

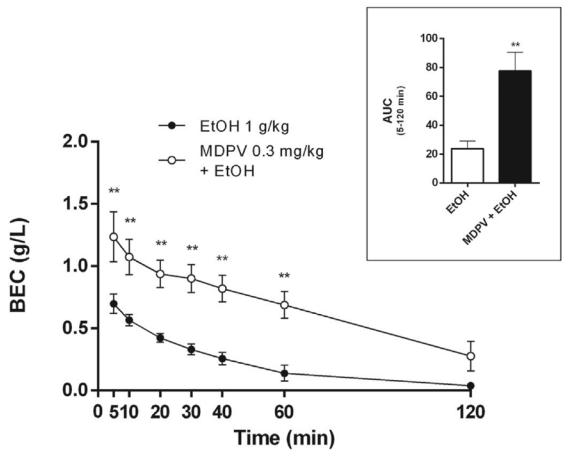


Fig. 8. Time course of BEC's after the administration of EtOH alone (filled circles) or in combination with MDPV (open circles). Data are expressed as the mean ± SEM. $^{**}p < 0.01$ vs. EtOH group at the corresponding time point. Inset: Overall effect of MDPV on AUC values of BEC's.

combined with EtOH, making a potentiation more unlikely. In our experiments, such decreased effect of MDPV appears to be compensated by the DA release induced by EtOH which may act synergistically with the uptake inhibition. At 3 mg/kg, the increase in DA concentration induced by MDPV is so high that probably could mask the enhancing effect of EtOH, which cannot be appreciated.

At that moment, we also wondered if, in addition to MDPV concentrations, those of EtOH could be modified when combining both drugs. Interestingly, BEC's were very significantly increased by MDPV, in such a manner that its elimination rate was extremely reduced (half-life increased from 23.01 ± 2.35 min to 64.69 ± 9.36, $p < 0.01$). For this reason, it is really feasible that the decrease in DA (as a consequence of lower amounts of brain MDPV) was compensated by the increase in DA elicited by higher EtOH concentrations, yielding similar conditioning effects. Accordingly, although synaptic DA concentrations were similar, the amount of DA taken up into the terminal (and thus metabolized into DOPAC and HVA) was higher, as expected for a milder blockade of DAT, due to lower concentrations of MDPV.

The effects on locomotor activity must be discussed apart, as it results from increased DA not only in NAcc but also in other brain areas where EtOH inhibits DA release, so the psychostimulant effect of MDPV can be modified in a different way than the mesoaccumbal reinforcing effect.

Due to the possible toxicological impact, it would be interesting to study the exact mechanism responsible of increased BEC by the presence of MDPV. On one hand, Meyer et al. (2012) suggest that ADH has a large impact on the β-keto group reduction of cathinones (Meyer et al., 2012). On the other hand, studies carried out in urine of MDPV users demonstrate the absence of the metabolic product of this β-keto reduction, likely due to both pyrrolidine- ring and methylenedioxy-group of MDPV (Uralets et al., 2014). Therefore, MDPV could not act as a substrate of ADH but might inhibit ADH activity, impairing ethanol metabolism. However, this hypothesis requires a thorough study of the effect of MDPV on ADH.

Finally, we would like to stress that under certain conditions the combination of MDPV plus EtOH reduces the psychostimulant effect of the cathinone, whereas its reinforcing effect is maintained. This would lead to repeat or increase drug intake in search of the desired stimulation, while the accompanying extra reinforcement could induce higher abuse liability. Due to the possible toxicological impact, further research focused on finding out the exact mechanism by which MDPV increases EtOH half-life and whether this phenomenon takes also place in humans is warranted. Moreover, from the present results is mandatory to warn about the risk of EtOH intoxication when this is consumed concomitantly with MDPV.

5. Conclusions

In summary, EtOH, at low-moderate doses, seems to trigger a strong decrease in overall concentrations of MDPV, when low doses of cathinone are administered, which dampens the MDPV-induced hyperlocomotion. This phenomenon could be explained by a pharmacokinetic interaction in the metabolic process. Therefore, MDPV and EtOH can interact at microsomal level, increasing the metabolic rate of MDPV. The interaction between both substances was also supported by results on BEC, which were significantly increased by MDPV, in such a manner that EtOH elimination rate was significantly lowered. In the NAcc, both substances could also act synergistically, in such a manner that although

brain MPDV concentrations are lower, the synaptic DA concentration is maintained high enough by EtOH to elicit a similar reinforcing effect.

Disclosure statement

All authors disclose any actual or potential conflict of interest including financial, personal or other relationships with other people or organizations that could inappropriately influence the present work.

Contributors

RL and MB performed microdialysis and blood kinetics experiments. PM was involved in conditioned place preference experiments. AC and LD accomplished brain kinetics. JR and RT performed microsome experiments. JC and DP performed locomotor activity experiments and analyzed the data of behavioral experiments. DP wrote the first draft of the manuscript. EE designed the study, undertook statistical and nonlinear regression analyses and wrote the final version of the manuscript. All authors contributed to and approved the final manuscript.

Role of funding source

Funding for this study was provided by Ministerio de Economia y Competitividad (grants number SAF2013-46135-P and SAF2016-75347-R) and CIBER de Fisiopatología de la Obesidad y Nutrición (CB06/03/0028) (CIBEROBN, Instituto de Salud Carlos III, Madrid, Spain). MB-J and PM-V are post-doctoral fellows (CONACyT post-doctoral grants 263473 and 265633). The funding sources had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Acknowledgement

R. López-Arnau position was funded by an institutional program of the Universitat de Barcelona in collaboration with Obra Social de la Fundació Bancària La Caixa.

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7.3. Annex III

Exposure of adolescent mice to 3,4-methylenedioxypyrovalerone increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood

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British Journal of Pharmacology 2017; 174:1161-1173

British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 1161–1173 1161

RESEARCH PAPER

Exposure of adolescent mice to 3,4-methylenedioxypyrovalerone increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood

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Received 18 December 2016; **Revised** 21 February 2017; **Accepted** 24 February 2017

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BACKGROUND AND PURPOSE

3,4-Methylenedioxypyrovalerone (MDPV) is a synthetic cathinone with powerful psychostimulant effects. It selectively inhibits the dopamine transporter (DAT) and is 10–50-fold more potent as a DAT blocker than cocaine, suggesting a high abuse liability. The main objective of the present study was to assess the consequences of an early (adolescence) MDPV exposure on the psychostimulant, rewarding and reinforcing effects induced by cocaine in adult mice.

EXPERIMENTAL APPROACH

Twenty-one days after MDPV pretreatment (1.5 mg·kg^{-1}, s.c., twice daily for 7 days), adult mice were tested with cocaine, using locomotor activity, conditioned place preference and self-administration (SA) paradigms. In parallel, dopamine D_2 receptor density and the expression of c-Fos and ΔFosB in the striatum were determined.

KEY RESULTS

MDPV treatment enhanced the psychostimulant and conditioning effects of cocaine. Acquisition of cocaine SA was unchanged in mice pretreated with MDPV, whereas the breaking point achieved under a progressive ratio programme and reinstatement after extinction were higher in this group of mice. MDPV decreased D_2 receptor density but increased ΔFosB expression three-fold. As expected, acute cocaine increased c-Fos expression, but MDPV pretreatment negatively influenced its expression. ΔFosB accumulation declined during MDPV withdrawal, although it remained elevated in adult mice when tested for cocaine effects.

CONCLUSION AND IMPLICATIONS

MDPV exposure during adolescence induced long-lasting adaptive changes related to enhanced responsiveness to cocaine in the adult mice that seems to lead to a higher vulnerability to cocaine abuse. This particular behaviour correlated with increased expression of ΔFosB.

Abbreviations

CPP, conditioned place preference; DAT, dopamine transporter; HLA, horizontal locomotor activity; MDPV, 3,4-methylenedioxypyrovalerone; NPS, new psychoactive substances; SA, self-administration

DOI:10.1111/bph.13771

Tables of Links

TARGETS
GPCRs
Dopamine D_2 receptor

LIGANDS
Cocaine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

In recent years, the illicit drug-market has changed remarkably and several new psychoactive substances (NPS), such as the synthetic cathinones, have been identified. The popularity of synthetic cathinones has increased due to their ease of access, price and initial legal status (Bijlsma *et al.*, 2015; Katselou *et al.*, 2015). 3,4-Methylenedioxypyrovalerone (MDPV) is considered as one of the most abused synthetic cathinone and the main ingredient of 'bath salts' (Zuba and Byrska, 2013; Johnson and Johnson, 2014) and able of triggering powerful psychostimulant effects (Baumann *et al.*, 2013).

Cocaine is also a powerful psychostimulant, and its repeated use could lead to a substance use disorder and is often associated with other severe psychiatric and medical complications (Pozzi *et al.*, 2008; Walsh *et al.*, 2009). Despite the invasion of the illegal market by the NPS, the illicit use of cocaine is still a persistent health problem worldwide (UNODC, n.d.). Similarly, MDPV shows cocaine-like properties and selectively inhibits dopamine (DAT) and noradrenaline transporters, being 10- to 50-fold more potent than cocaine, as a DAT blocker (Simmler *et al.*, 2013; Baumann *et al.*, 2013). Furthermore, it shows rewarding and reinforcing effects (King *et al.*, 2014), pointing to a similar abuse liability to that of cocaine.

Although MDPV use could be considered as a transient trend in drug abuse, the long-lasting consequences of its repeated consumption are still unknown. Considering this, it is relevant to determine whether the use of MDPV will lead to an increased sensitivity and subsequent vulnerability to cocaine abuse. Adolescents and young adults use MDPV as a cheaper and easily obtained alternative to classical psychostimulants. Conversely, cocaine is a more widely and currently used psychostimulant and is generally consumed in adulthood. Consequently, the main objective of the present study was to assess the consequences of early and repeated MDPV exposure on the responses of adult mice to the psychostimulant cocaine.

A repeated (7 days) moderate dose (1.5 $mg{\cdot}kg^{-1}$, twice, daily) of MDPV eliciting hyperlocomotion was chosen for this study. After this MDPV schedule, adult mice were tested to cocaine responses. Hence, hyperlocomotion to an acute dose of cocaine was assayed as an indicative of its psychostimulant effect. In a second experiment, we investigated whether MDPV schedule could enhance the rewarding effects of cocaine, using the conditioned place preference (CPP). Next, we evaluated the reinforcing cocaine effects on the self-administration (SA) paradigm. Earlier studies have shown that dopamine D_2 receptors played a role in the development and expression of behavioural sensitization (Thompson *et al.*, 2010). Moreover, the expression of c-Fos and deltaFosB (ΔFosB) in some brain areas is induced by acute or chronic exposure to virtually all drugs of abuse and regulates their psychomotor and rewarding effects. Therefore, we have assessed the D_2 receptor density and the expression of c-Fos and ΔFosB in dorsal and ventral striatum.

Hence, we have performed behavioural procedures (hyperlocomotion, CPP and SA) and biochemical analyses that allowed us to characterize the liability for cocaine abuse shown by adult mice who were pretreated, as adolescents, with MDPV.

Methods

Animals

All animal care and experimental protocols were approved by the Animal Ethics Committee of the University of Barcelona and PRBB, respectively, under the supervision of the Autonomic Government of Catalonia, following the guidelines of the European Community Council (2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Animals were housed four per cage (polycarbonate with wood-derived bedding) at 22 ± 1°C under a 12 h light/dark cycle with free access to food and drinking water.

Our model was based on the following considerations. Adolescence is a period of particular vulnerability to drug addiction (Cass *et al.*, 2013), being a period of life in which different psychiatric disorders emerge (Paus *et al.*, 2008). For this reason, we used for our study adolescent (PND 41–44) male Swiss CD-1 mice (Charles River, Spain), which are equivalent to the beginning of peri-adolescence (Spear and Brake, 1983), and assayed cocaine effects after 21 days of withdrawal, when animals had reached adulthood. The CD-1 mouse strain was selected for its optimal sensitivity to the reinforcing and psychostimulating effects of cocaine (McKerchar *et al.*, 2005).

Drug administration protocols and experimental design

In administration regime A, MDPV (1.5 mg·kg^{-1}) or saline (5 mg·kg^{-1}) was injected s.c. to mice twice daily (4 h apart) for three consecutive days and, thereafter, they were exposed to the cocaine-sensitization protocol (Figure 1A). In administration regime B, animals were also treated with MDPV (1.5 mg·kg^{-1}) or saline (5 mL·kg^{-1}) twice daily for seven consecutive days and were then housed in their home cages until reaching adulthood (PND 69–72), when they were tested for cocaine-induced horizontal locomotor activity (HLA), CPP and SA experiments as described below (Figure 1B). This dose is equivalent to a dose in humans of about 6 mg twice a day (Reagan-Shaw *et al*., 2008), in which threshold dosages are around 1–5 mg and strong effects are shown with 10–25 mg (2014). Re-dosing is a typical pattern of consumption followed by consumers of such substances to avoid an unpleasant comedown (Ross *et al*., 2012).

Sensitization to the locomotor responses induced by a repeated cocaine administration

Locomotor activity was evaluated by placing the mice individually in the actimeter boxes (24 × 24 × 24 cm) (LE881 IR, Panlab, Barcelona, Spain) provided with 14 axes (Y and X) in a low-luminosity room. Animals were treated with saline (n = 10) or MDPV (n = 11), according to the administration regime A. The sensitization procedure consisted of three phases over 14 days: habituation, treatment and challenge. In the habituation phase (Days 6–7), mice were placed on the actimeter boxes for 30 min immediately after an i.p. saline injection. Treatment phase consisted of five sessions (Days 8–12). In each session, mice received daily an i.p. injection of cocaine (7.5 mg·kg^{-1}) immediately before being placed in the apparatus for 15 min. Finally, following a 7 day drug-free period after the last cocaine injection, mice were tested (Day 19 – challenge phase) with a cocaine injection (7.5 mg·kg^{-1}, i.p.) in the same actimeter boxes, and the locomotor activity was registered for 15 min.

Cocaine-induced HLA

The HLA response induced by a single cocaine injection was video-monitored (Smart 3.0, Panlab, s.l.u., Barcelona, Spain) for 15 min in a black Plexiglass open field arena (25 × 25 × 40 cm) under low-light conditions. Two days before testing, the animals were handled for 10 min and placed in the black arena for habituation. Two groups of animals (n = 12 per group) were pretreated in their home cage according to administration regime B, and after 21 days of withdrawal, when they reached adulthood (PND 69–72), both groups were challenged with saline (5 mL·kg^{-1}, i.p.) (Day 27) and cocaine (7.5 mg·kg^{-1} i.p.) (Day 28), and locomotor activity was registered.

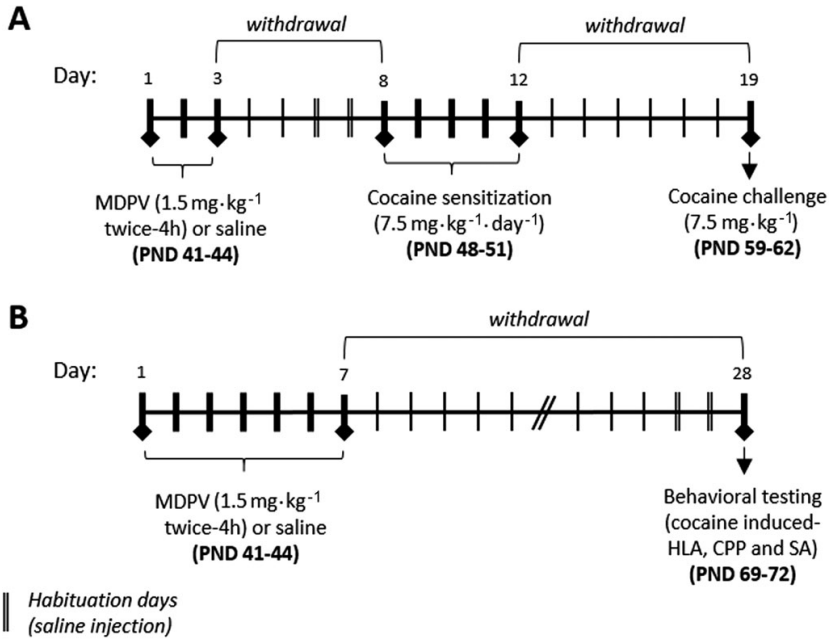

Figure 1

Drug exposure protocol and experimental design. (A) In administration regime A, animals were treated with MDPV or saline twice daily for 3 days and, 5 days after, they were exposed to a cocaine sensitization protocol. (B) In administration regime B, animals were treated with MDPV or saline twice daily for 7 days and, 21 days later, cocaine-induced HLA, CPP and SA experiments were performed.

Cocaine-induced CPP

The cocaine potential to induce approaching behaviours toward drug-related stimuli was determined using an unbiased place conditioning paradigm, as described by Soria *et al*. (2006). The apparatus consisted of two main conditioning compartments (30 × 29 × 35 cm) connected by a smaller, central compartment (Cibertec S.A., Madrid, Spain). The conditioning compartments were disposed with differences in visual and tactile cues. All the compartments were equipped with infrared emitter/detector pairs along the length of the box.

Saline- and MDPV-pretreated animals according to administration regime B (n = 16 per group) were subjected to the CPP procedure after a 21 day long drug-free period. During the preconditioning phase (day 28), initial unconditioned preference for the stimulus alternatives was determined. In this test, mice were placed in the central compartment and had free access to both compartments of the apparatus for 18 min. During the conditioning phase (days 29–34), mice received an i.p. injection of cocaine 10 mg·kg^{-1} immediately before being placed into one of the two conditioning compartments for 20 min on days 29, 31 and 33. On the alternate days (30, 32 and 35), mice were placed in the other compartment for 20 min after being given a saline injection. Treatments were counterbalanced as much as possible between compartments. Control animals received saline every day. The preference test was conducted exactly as the preconditioning phase. A CPP score was calculated for each subject as the difference between times spent in the drug-paired and the saline-paired compartments during the pre-conditioning and the preference tests.

Cocaine operant SA

Acquisition of cocaine SA. The SA experiments were carried out in eight mouse operant chambers (Model ENV-307A-CT, Med Associates, Inc. Cibertec S.A., Madrid, Spain), as previously described by Soria *et al*. (2006). Saline- and MDPV-pretreated mice according to administration regime B (n = 16 per group) were trained for 2 h·day^{-1} to nosepokes in order to receive 1 mg·kg^{-1} cocaine infusions on 10 consecutive days under a fixed ratio 1 (FR1).

Surgical implantation of the catheter into the jugular vein was performed following anaesthesia with a mixture of ketamine (100 mg·mL^{-1}) and xylazine (20 mg·kg^{-1}). The anaesthetic solution was injected in a volume of 0.15 mL·10 g^{-1}, i.p. (Soria *et al*., 2006; Tourino *et al*., 2012). Mice were housed individually and allowed to recover for at least 3 days. During recovery, mice were treated daily with an analgesic (meloxicam 0.5 mg·kg^{-1}, injected in a volume of 0.1 mL·10 g^{-1}, i.p.) and an antibiotic solution (enrofloxacin 7.5 mg·kg^{-1}, injected in a volume of 0.03 mL·10 g^{-1}, i.p.). The home cages were placed upon thermal blankets to avoid post-anaesthesia hypothermia.

SA procedures started 21 days after the last day of administration regime B (Day 28). Active and inactive nosepokes were assigned randomly. Cocaine was delivered in a 20 μL injection for 2 s via a syringe mounted on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA) connected to single-channel liquid swivel (375/25, Instech Lab, Plymouth Meeting, PA, USA) and the mouse's intravenous catheter. All FR1 sessions started with a cocaine priming infusion. When mice responded on the active hole, the stimulus lights (one located inside the nosepoke and the other above it) lit up for 4 s and a cocaine infusion was delivered automatically. Each infusion was followed by a 30 s time-out period in which a nosepoke on the active hole had no consequences. Mice were considered to have acquired stable SA behaviour when the following criteria were met in two consecutive FR1 sessions: (a) 80% stability in reinforcements (the number of reinforces in each day deviated by <20% from the mean number of reinforces in two consecutive days); (b) ≥ 65% of responses were received at the active hole; and (c) a minimum of five responses in the active hole. After 10 days of training (Day 38), mice that achieved the acquisition criteria (n = 9 per group) were moved to a progressive ratio (PR) session. In the PR session (2 h), the response requirement to earn an injection escalated throughout the following series: 1–2–3–5–12–18–27–40–60–90–135–200–300–450–675–1000.

Extinction and reinstatement. All the animals that reached the acquisition criteria were subjected to an extinction phase. The extinction procedure was adapted from Soria *et al*. (2008). Nosepokes in the active hole produced neither cocaine infusion nor stimulus light presentation. Extinction sessions (2 h) were conducted once a day, 5 days·week^{-1} until reaching the extinction criteria. These criteria were achieved when mice made a mean number of responses in two consecutive extinction sessions of less than 40% of the responses performed during the last day of the cocaine-training phase. Twenty-four hours after achieving the extinction criteria, mice underwent a cocaine-primed reinstatement session, as previously described (Soria *et al*., 2008). In order to recover the extinguished cocaine-seeking behaviour, saline- and MDPV-pretreated mice (n = 9 per group) were confined to the operant chambers for 2 h immediately after receiving an i.p. injection of cocaine 10 mg·kg^{-1}. Nosepokes had no consequences in any of the holes.

Tissue sample preparations

Mice pretreated according to the administration regime B were killed by cervical dislocation 24 h after the treatment (Day 8) or after saline/cocaine challenge (Day 28) for the analysis of ΔFosB expression and D_2 receptor density or 2 h after saline/cocaine challenge for the determination of c-Fos expression. The inclusion of a saline challenge group allowed us to study also the long-term effects of MDPV treatment. Ventral (including NAcc), dorsal or the whole striatum, when appropriate, were quickly dissected out and stored at −80°C until use.

Tissue samples for Western blot analysis were processed as described (Pubill *et al*., 2013), with minor modifications. Briefly, for nuclear c-Fos Western blot analysis, dorsal striatum tissue samples were homogenized at 4°C in 400 μL of buffer (5 mM Tris–HCl, 320 mM sucrose) with the protease inhibitor cocktail. The homogenates were centrifuged at 1000 × g for 15 min at 4°C, and the pellets were resuspended in buffer (Tris–HCl 50 mM) with the protease inhibitor cocktail.

For ΔFosB Western blot analysis, ventral striatum tissue samples were thawed and homogenized at 4°C in 20 volumes of lysis buffer (20 mM Tris–HCl, pH = 8, 1% NP40, 137 mM NaCl, 10% glycerol, 2 mM EDTA) with the protease inhibitor cocktail. The homogenates were shaken and rolled for 120 min at 4°C and centrifuged at 15 000 × *g* for 30 min at 4°C. Aliquots of resulting supernatants (total lysate) were stored at −80°C until use.

For [^{3}H]raclopride binding assays, crude membrane preparation from the whole striatum was prepared as described by Martínez-Clemente *et al*., (2012). Briefly, tissue samples were thawed and homogenized at 4°C in 20 volumes of buffer (5 mM Tris–HCl, 320 mM sucrose) with the protease inhibitor cocktail. The homogenates were centrifuged at 15000 × *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, and the final pellets were resuspended in the appropriate buffer and stored at −80°C until use. Protein content was determined using the Bio-Rad Protein Reagent (BioRad, Inc., Madrid, Spain).

Western blotting and immunodetection

ΔFosB and c-Fos Western blot analyses were performed as described by Buenrostro-Jáuregui *et al*. (2016) with minor modifications. Briefly, for each sample, 10 or 20 μg of protein was mixed with loading 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 PVDF sheets (Immobilion-P, Millipore). PVDF membranes were blocked for 1 h at room temperature with 5% defatted milk in Tris-buffer plus 0.05% Tween-20 and incubated overnight at 4°C with mouse primary antibody anti-FosB (1:250) or rabbit primary antibody anti-c-Fos (1:200). After washing, membranes were incubated for 1 h at room temperature with a peroxidase-conjugated (1:2500) antimouse or antirabbit (1:2000) IgG antibody. Immunoreactive protein was visualized using a chemoluminescence-based detection kit (Immobilion Western, Millipore) and a BioRad ChemiDoc XRS gel documentation system (BioRad, Inc., Madrid, Spain). Scanned blots were analysed using BioRad Image Software, and dot densities were expressed as a proportion of those taken from control. As a control for load, β-tubulin (1:2500) or GAPDH (1:5000) antibody was used.

D_2 receptor density

The density of D_2 receptors in striatal membranes was measured by [^{3}H]raclopride binding assays as described (Martínez-Clemente *et al*., 2014). Assays 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. The incubation was ended by rapid filtration under vacuum through Whatman GF/B glass fibre filters. The radioactivity in the filters was measured by liquid scintillation spectrometry.

Data acquisition and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al*., 2015). Data were expressed as mean ± SEM. Data from biochemical analyses were normalized with 100% defined as the mean of the technical replicates in the control group, and the SEM was normalized appropriately. Animals were randomly assigned to an experimental group. During the behavioural manipulations, researchers were not aware of the pretreatment that each animal had received.

Differences between groups were compared using two-way ANOVA or Student's *t*-test for independent samples where appropriate. The α error probability was set at 0.05. Significant differences ($P < 0.05$) were analysed using the Tukey's *post hoc* test for multiple comparison measures (InVivoStat software package) only if F achieved the necessary level of statistical significance ($P < 0.05$) and no significant variance inhomogeneity was observed. The exact group size for the individual experiments is shown in the corresponding figure legends. To analyse the acquisition of cocaine SA during the 10 day training, extinction of the operant behaviour and reinstatement, a three-way ANOVA was calculated, with nosepoke (active or inactive), treatment with MDPV or saline, and day (or session) as factors of variation. Subsequent Tukey's *post hoc* tests were calculated when required. Breaking point achieved at the end of the PR sessions was analysed using the non-parametric Mann–Whitney *U*-test.

Materials

Pure racemic MDPV·HCl was synthesized and characterized in our laboratory as described (Novellas *et al*., 2015). Cocaine was provided by the Spanish National Institute of Toxicology. MDPV and cocaine solutions for injection were prepared in 0.9% NaCl (saline, pH = 7.4) immediately before administration. Mouse monoclonal ΔFosB antibody and the protease inhibitor cocktail were purchased from Abcam (Cambridge, UK) and rabbit polyclonal c-Fos antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [^{3}H] raclopride was obtained from Perkin Elmer Life Sci. (Boston, MA, USA). Ketamine was from Rhône Merieux (Lyon, France). Xylazine, sulpiride and all buffer reagents (analytical grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Results

Sensitization to the hyperlocomotor responses induced by repeated cocaine administration

To evaluate the behavioural sensitization to cocaine 5 days after MDPV pretreatment (regime A), we assessed the hyperlocomotion induced by repeated cocaine administration (Figure 2). During the treatment phase (Figure 2 inset), cocaine induced an acute hyperlocomotion that increased with repeated daily exposure. Two-way ANOVA revealed effect of the *day* ($F_{4,76} = 8.791$) and the *pretreatment* factor ($F_{1,19} = 6.025$,) without *interaction* between factors.

When analysing the differences between the first day of the treatment (Day 8) and the cocaine challenge (Day 19) (Figure 2), two-way ANOVA also demonstrated a significant effect of the *day* ($F_{1,19} = 9.909$) and the *pretreatment* factor ($F_{1,19} = 9.504$) without the *interaction between pretreatment and day.*

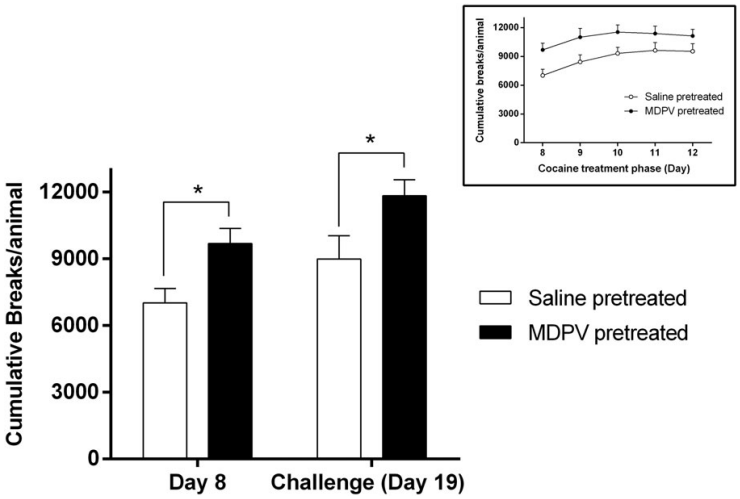

Figure 2

Effect of MDPV treatment (regime A) on cocaine-induced sensitization. Bars represent mean (±SEM) of cumulative breaks per animal after cocaine (7.5 mg·kg^{-1}, i.p.) injection on the first day of the sensitization protocol (Day 8) and the challenge day (Day 19) (saline-pretreated group, $n = 10$ and MDPV-pretreated group, $n = 11$). *$P < 0.05$, compared with the saline-pretreated group. Inset: Cumulative breaks per animal during the treatment phase of the cocaine-induced sensitization protocol in saline- and MDPV-pretreated mice.

Interestingly, mice pretreated with MDPV showed a significant increase in the hyperlocomotor activity induced by cocaine on both, the first day of cocaine administration (Day 8) and the challenge day (Day 19) compared with saline-pretreated mice. Overall, these results indicate that pre-exposure to MDPV is able to enhance the response to cocaine, without affecting the acquisition of sensitization.

Cocaine-induced HLA

The HLA was monitored for 15 min after a single saline (5 mg·kg^{-1}) or cocaine (7.5 mg·kg^{-1}) i.p. injection to mice previously pretreated with saline or MDPV according to administration regime B (Figure 3). This dose of cocaine elicited a significant psychostimulant effect on saline- and MDPV-pretreated animals, although a higher hyperlocomotor response was observed in the group pretreated with the psychostimulant. Two-way ANOVA revealed significant effect of the cocaine *challenge* ($F_{1,44} = 53.60$) and *interaction between challenge and pretreatment* ($F_{1,44} = 4.58$), but no effect of the *pretreatment* factor was found. This means that MDPV pretreatment, by itself, is not capable of increasing locomotor activity when mice are challenged with saline, but only after challenge with cocaine.

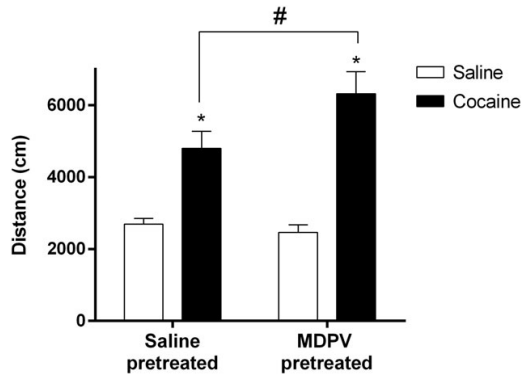

Figure 3

Effect of MDPV treatment (regime B) on cocaine-induced HLA. Bars represent mean (±SEM) of the distance travelled after a single saline (5 mL·kg^{-1}, i.p.) or cocaine (7.5 mg·kg^{-1}, i.p) injection 21 days after treatment ($n = 12$ per group). *$P < 0.05$, compared with the corresponding saline injection. #$P < 0.05$, compared with the saline-pretreated group.

Cocaine-induced CPP

As shown in Figure 4A, saline- and MDPV-pretreated mice (regime B) presented no preference for any of the compartments (Figure 4A). Only one animal from the saline-pretreated group was withdrawn from the study due to an initial preference for one of the compartments (>65% of the total session time spent in one compartment). The repeated administration of cocaine (10 mg·kg^{-1}, i.p.) produced a preference for the cocaine-paired compartment (Figure 4B). Two-way ANOVA demonstrated a significant effect of the *pretreatment* factor ($F_{1,58} = 15.0$), *treatment* effect ($F_{1,58} = 114.4$) and *interaction between treatment and pretreatment* ($F_{1,58} = 7.075$). Accordingly, MDPV-pretreated mice showed an increased expression of the cocaine-induced CPP compared with the saline-pretreated group (Figure 4B).

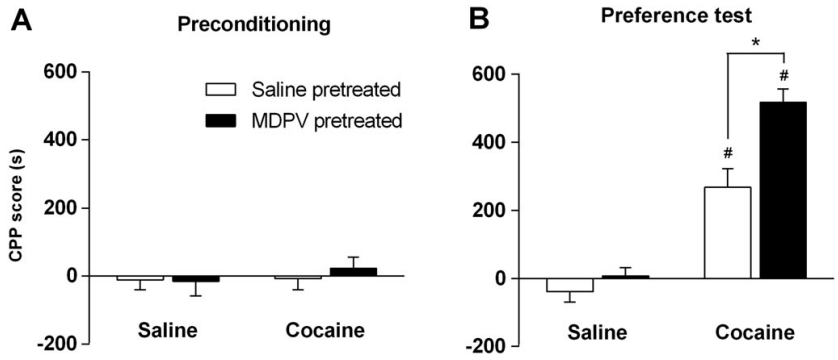

Figure 4

Effect of MDPV treatment (regime B) on cocaine-induced CPP. Bars represent mean (±SEM) of CPP score (see Methods for details) during preconditioning ($n = 16$ per group) (Panel A) and preference test ($n = 16$ per group) (Panel B). *$P < 0.05$, compared with saline-pretreated group; #$P < 0.05$, compared with its respective control group (conditioned with saline).

Self-administration reinforced with cocaine

Acquisition of cocaine SA. The effect of the MDPV pretreatment (regime B) on the reinforcing properties of cocaine was evaluated in the SA procedure. First, both saline- and MDPV-pretreated mice were trained to self-administer cocaine (1 mg·kg^{-1} per infusion) during 10 days under a FR1 schedule of reinforcement. The criteria acquisition rates were equal for both groups: 56%. All the statistical analyses and data representations were performed only with mice that acquired the learning criteria. Three-way ANOVA (*pretreatment* × *day of training* × *nosepoke*) of infusions on both nosepokes given along the FR1 sessions yielded no significant effects of the *pretreatment* factor ($F_{1,9} = 0.56$) (Figure 5A), thus indicating a lack of effect of MDPV pretreatment on the acquisition of cocaine SA behaviour. The operant procedure produced the acquisition of cocaine SA as revealed by the *day of training* factor ($F_{9,9} = 2.138$). Animals were able to discriminate between active and inactive nosepokes as indicated by the *nosepoke* factor ($F_{1,9} = 108.8$). An interaction between *day of training* and *nosepoke* factors was also found ($F_{9,9} = 4.45$). No other interactions were found. A significant effect of MDPV pretreatment was revealed in the breaking point achieved on the PR session (Mann–Whitney U = 15.5) (Figure 5B), indicating that MDPV pretreatment increased the value of cocaine as reinforcer.

Extinction and cocaine-primed reinstatement. Three-way ANOVA (*pretreatment* × *extinction day* × *nosepokes*) showed that groups extinguished cocaine SA behaviour among the extinction sessions, as the factor *extinction day* had a significant effect ($F_{11,11} = 14.78$) (Figure 6A). However, the factor *pretreatment* had no significant influence over nosepokes along the extinction phase ($F_{1,11} = 0.057$). Animals were able to discriminate between *nosepokes* ($F_{1,11} = 284.6$), but as the interaction between this factor and *pretreatment* revealed ($F_{1,11} = 16$), both groups stopped discriminating between nosepokes in different days. Subsequent *post hoc* analyses indicated that MDPV-pretreated animals discriminated between nosepokes until day 7 (Tukey), whereas SAL-pretreated animals discriminated until day 6 (Tukey). A significant interaction

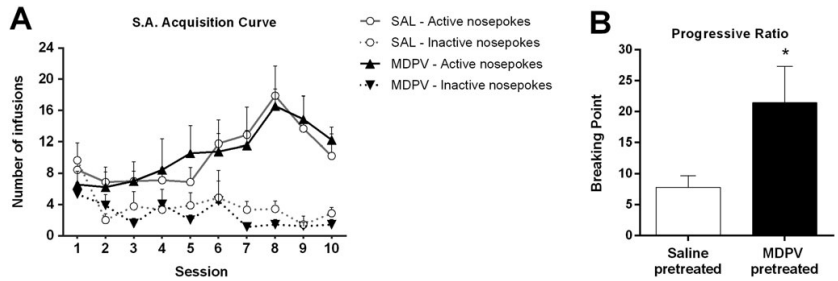

Figure 5

(Panel A) Effect of MDPV treatment (regime B) on the acquisition of cocaine SA behaviour for 2 h daily FR1 sessions during 10 days of training ($n = 9$ per group). (Panel B) Effect of MDPV treatment (regime B) on SA behaviour breaking point in a PR schedule of reinforcement ($n = 9$ per group). Data represent the mean (±SEM) of the last ratio achieved in the PR session during 2 h. *$P < 0.05$, compared with the saline-pretreated group.

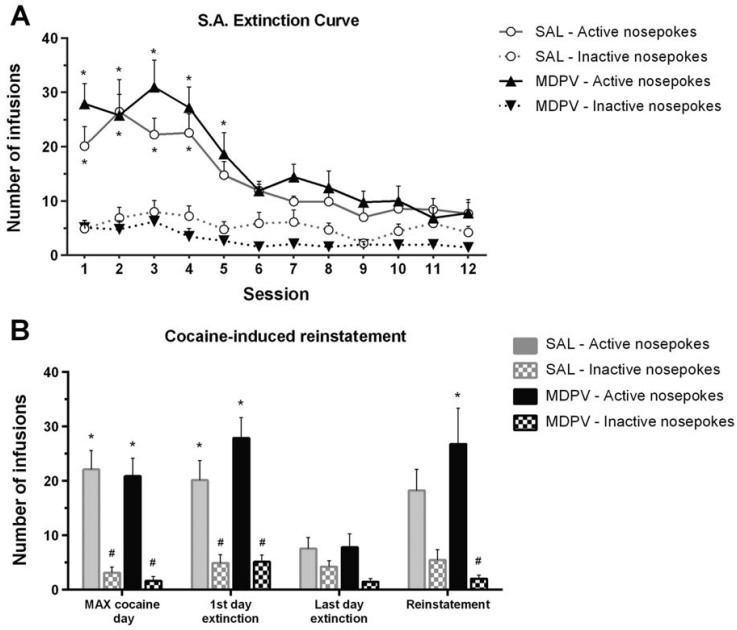

Figure 6

(Panel A) Extinction of cocaine SA behaviour for 2 h daily sessions during 12 days (n = 9 per group). *P < 0.05, compared with the inactive nosepokes performed by the same group in the same day of extinction phase. (Panel B) Cocaine-primed, drug-induced reinstatement of cocaine SA behaviour (n = 9 per group). The different phases of the experiment are showed in the X axis. *P < 0.05, compared with the active nosepokes performed by the same group in the last day of extinction. #P < 0.05, compared with the active nosepokes of the same group in the same experimental phase. Data represent the mean of nosepokes ± SEM in the active and inactive holes.

between *extinction day* and *nosepokes* ($F_{11,11}$ = 7645) was found. No other interactions were found.

After extinction of cocaine SA, mice that acquired the extinction criteria were submitted to a cocaine-primed (10 mg·kg^{-1}), drug-induced reinstatement session (Figure 6B). Three-way ANOVA (*pretreatment* × *session* × *nosepokes*) showed a significant effect of *day* ($F_{3,3}$ = 8.263) and *nosepokes* ($F_{1,3}$ = 116.9), without *pretreatment effect* ($F_{1,3}$ = 0.477). Significant interactions were found between *days* and *nosepokes* ($F_{3,3}$ = 6.128), and *pretreatment* and *nosepokes* ($F_{1,3}$ = 3.979). *Post hoc* analyses indicated that both groups of pretreatments did not differ in the number of active nosepokes in the reinstatement session. However, MDPV-pretreated mice, unlike SAL-pretreated, reinstated their previously extinguished cocaine-SA behaviour after being drug-primed (cocaine 10 mg·kg^{-1}). Therefore, SAL-pretreated mice did not reinstate previous extinguished cocaine-SA behaviour (Tukey). Interestingly, MDPV-pretreated mice achieved a significant difference between active nosepokes in reinstatement session versus the last day of extinction (Tukey) (Figure 6B).

c-Fos and ΔFosB expression

Because we had seen that the pretreatment with MDPV modified the response to cocaine, we wanted to determine how the MDPV pretreatment influenced the expression of certain factors that are known to be associated with the effects of cocaine, such as c-Fos and ΔFosB. Expression of the transcription factor ΔFosB in the brain also controls the responsiveness of an animal to the rewarding and locomotor-activating effects of cocaine. Following regime B, we investigated the expression of ΔFosB 24 h after saline or MDPV pretreatment (Day 8) (Figure 7A) and after saline or cocaine challenge (Day 29) (Figure 7B) and also c-Fos 2 h after saline or cocaine challenge (Day 28) (Figure 7C).

As shown in Figure 7A, mice pretreated with MDPV (regime B) showed a significant increase of ΔFosB expression, by 300% compared with saline-treated mice, when measured 24 h after finishing the treatment. At day 29, 24 h after receiving the saline/cocaine challenge, the statistical analysis showed a significant effect of the *pretreatment factor* ($F_{1,18}$ = 5.976; Figure 7B). Therefore and although the high ΔFosB expression declined during withdrawal, this factor still remained apparently elevated (132% animals pretreated with MDPV and challenged with saline). In addition, cocaine challenge also produced an increase in ΔFosB expression compared with saline injection ($F_{1,18}$ = 4.699).

The c-Fos expression has been used as a marker for neuronal activity. In this context, two-way ANOVA revealed effect of the *challenge factor* ($F_{1,18}$ = 54.21; Figure 7C). Cocaine induced a significant increase in c-Fos expression in both groups, saline- and MDPV-pretreated mice (320% and 220% respectively). Accordingly, statistical analysis also disclosed the effect of the *pretreatment factor* ($F_{1,18}$ = 8.497). Thus, MDPV

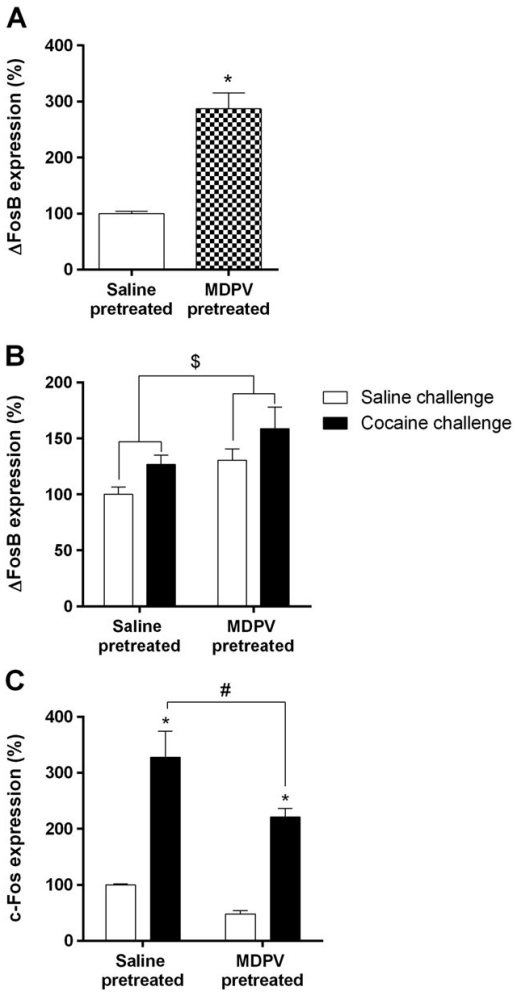

Figure 7

Effect of MDPV treatment (regime B) on ΔFosB expression 24 h after treatment ($n = 6$ per group) (Panel A) or saline and cocaine challenge (saline-pretreated group, $n = 5$ per group and MDPV-pretreated group, $n = 6$ per group) (Panel B). *$P < 0.05$, compared with the saline-pretreated group. $$P < 0.05$, two-way ANOVA *pretreatment factor* ($F_{1,18} = 5.976$). (Panel C) Effect of MDPV treatment (regime B) on c-Fos expression 2 h after saline and cocaine challenge (saline-pretreated group, $n = 5$ per group and MDPV-pretreated group, $n = 6$ per group). *$P < 0.05$, compared with its corresponding saline-challenge group. #$P < 0.05$, compared with the saline-pretreated group. Results are expressed as mean ± SEM.

pretreatment reduces the cocaine-induced expression of this marker. Moreover, after saline challenge, MDPV-pretreated mice showed a decrease (52%) in c-Fos expression compared with SAL-pretreated mice, although such differences did not reach statistical significance. Therefore, it seemed that, at the same time when ΔFosB was increasing, c-Fos expression was reduced.

D_2 receptor density

To determine the involvement of striatal dopamine D_2 receptors, we measured [^{3}H]raclopride binding in this brain area 24 h after treatment (Figure 8A) or saline and cocaine challenge (Figure 8B). Twenty-four hours after MDPV pretreatment, the D_2 receptor density was significantly decreased ($t_{18} = 2.613$). However, 24 h after saline or cocaine challenge, two-way ANOVA only revealed differences of the *challenge factor* ($F_{1,30} = 4.179$). However, *post hoc* analysis did not demonstrate statistical significance.

Discussion

Cocaine abuse represents a heavy burden of disease in many countries and has become a global problem. Any factor that increases the vulnerability to cocaine abuse must be carefully evaluated. In the present study, we demonstrate that MDPV enhances the responsiveness to cocaine in all tested aspects.

In a first study (regime A), we investigated the influence of a short-term MDPV exposure on the behavioural sensitization induced by cocaine. MDPV produced a gradual increase of cocaine hyperlocomotor effects and a high

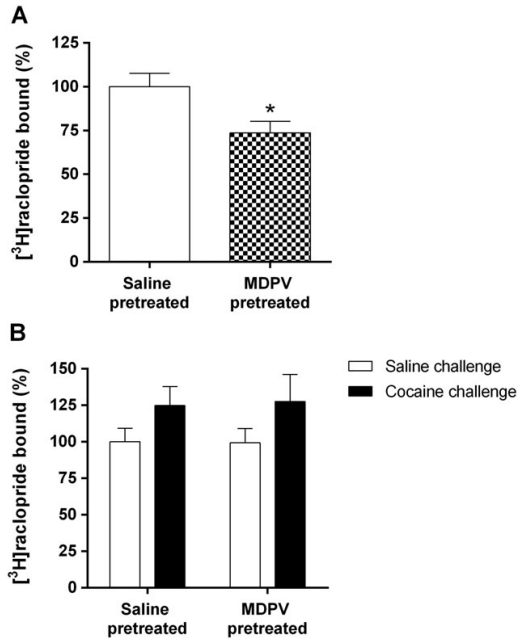

Figure 8

Effect of MDPV treatment (regime B) on D_2 receptor density 24 h after treatment (saline-pretreated group, $n = 9$ and MDPV-pretreated group, $n = 11$) (Panel A), and after saline or cocaine challenge (saline-pretreated group, $n = 8$ per group and MDPV-pretreated group, $n = 9$ per group) (Panel B). D_2 receptor density was measured as [^{3}H]raclopride bound in the mouse striatum. Results are expressed as mean ± SEM. *$P < 0.05$, compared with the saline-pretreated group.

reactivity to a cocaine challenge after 7 days of cocaine withdrawal. However, mice exposed to MDPV showed a higher response to cocaine when they received this psychostimulant for the first time, and this increased hyperlocomotor effect was maintained throughout all the sensitization procedure.

In a second set of experiments, we carried out a repeated treatment (regime B) with a moderate dose of MDPV, in adolescent mice, and we investigated the influence on the acute effects of cocaine on adulthood, including motor responses, rewarding effects on the CPP, and the cocaine-induced reinforcing effects on the SA paradigm.

We found that mice exposed to MDPV were more reactive to cocaine but not to saline injection. So this observed response was independent of the environmental context because MDPV treatment was administered in the home cages. Accordingly, an MDPV treatment with low doses (0.3 or 0.5 mg·kg^{-1}) during 5–7 days increased responsiveness to acute cocaine (5–10 mg·kg^{-1}) after a 10–11 days drug-free period (Berquist *et al.*, 2016; Buenrostro-Jáuregui *et al.*, 2016).

We also sought to find out if MDPV exposure resulted in increased responsiveness to cocaine in reward and reinforcing effects on mice. Our results revealed that repeated administration of MDPV in adolescent mice led to a long-term increase of cocaine-induced behavioural adaptations, related to its abuse potential, when tested into adulthood in the CPP. This paradigm is used to reflect the degree by which drugs of abuse establish approaching behaviours toward drug-related stimuli (Bardo and Bevins, 2000). The observed increase in the robustness of the conditioned responses in the CPP expression seems to reflect an increase in the abuse liability of cocaine after exposure to MDPV. Thus, our findings demonstrate that MDPV exposure during adolescence can effectively potentiate cocaine abuse liability in adulthood by sensitizing the neural circuitry underlying associations between cocaine and its related stimuli. To our knowledge, this is the first study that evaluates the long-term effects of MDPV treatment on cocaine-induced CPP. However, similar effects of MDPV pretreatment on the development of psychostimulants-induced reinforcing effects have been recently reported. For instance, MDPV (1.8 mg·kg^{-1} during 5 days) attenuated the taste avoidance induced by cocaine (18 mg·kg^{-1}), but not those induced by LiCl, in rats (Woloshchuk *et al.*, 2016). As mentioned above, MDPV also sensitized to cocaine locomotor response after a drug washout period (Berquist *et al.*, 2016; Buenrostro-Jáuregui *et al.*, 2016). Moreover, an intermittent repeated exposure to MDPV (1 mg·kg^{-1} during 5 days) produced sensitization to methamphetamine challenge (0.5 mg·kg^{-1}) in rats (Watterson *et al.*, 2016). Nevertheless, no study has yet evaluated global MDPV effects on cocaine-induced abuse liability and, in particular, evaluated its effects on cocaine-induced reinforcing effects on the SA paradigm, as we present in the present study. In fact, mice repeatedly exposed to MDPV during adolescence made greater efforts to obtain a cocaine infusion in a PR schedule of reinforcement when tested in adulthood. The PR schedule of reinforcement requires behaviours specially linked to motivational functions, such as instrumental learning, execution of efforts and sustained engagement (Randall *et al.*, 2012). The lack of difference between mice exposed to MDPV or to saline during the acquisition phase of SA could be attributed to the fact that this phase was performed under an FR1 schedule of reinforcement that seems not to be appropriate to determine the reinforcement efficiency (Richardson and Roberts, 1996). In this sense, it is a common feature of rodent cocaine SA studies to find a certain degree of inconsistency between results on FR1, PR schedules and reinstatement sessions depending on the experimental conditions (Homberg *et al.*, 2002; Morgan *et al.*, 2005; Zhang *et al.*, 2005; España *et al.*, 2011).

In addition, MDPV-pretreated mice delayed extinction after acquisition of the operant behaviour to cocaine and reinstated after a cocaine priming injection. Reinstatement of SA behaviour reflects the reinforcing properties of drugs and its pharmacological manipulations (Shaham *et al.*, 2003; Soria *et al.*, 2008; Bossert *et al.*, 2013). We have also found a drug-seeking reinstatement behaviour in mice previously exposed to MDPV, suggesting a higher level of craving than in control mice. Thus, we suggest that MDPV exposure during adolescence will strengthen the efforts to obtain cocaine in adulthood, and this effect leads to an enhanced vulnerability to reinstate cocaine SA behaviour once extinguished.

These results lead us to hypothesize that MDPV administration induces long-lasting adaptive changes, leading to a greater response to cocaine. ΔFosB, c-Fos and D_2 receptors are factors involved in the acute and long-lasting effects of cocaine (Larson *et al.*, 2010; Lee *et al.*, 2013). For instance, studies performed using positron emission topography have consistently shown that drug abuse is accompanied by a decrease in striatal D_2 receptor availability. Furthermore, studies in drug-naïve, non-human primates suggest that D_2 receptor availability is predictive of drug-seeking behaviour (Nader *et al.*, 2006). In the present study, we have found that repeated MDPV exposure was associated with a decreased striatal density of D_2 receptors, probably reflecting a neuroadaptive effect in response to MDPV dopaminergic stimulation, but this effect is only transient as it did not remain after 21 days of withdrawal, when D_2 receptor population was observed to return to initial values. Additionally, we determined D_2 receptor levels after the challenge of cocaine. As expected, an acute dose of this drug modulated D_2 receptors, although without influence of MDPV pre-exposure.

There is growing evidence for an important role of ΔFosB in animal models of drug addiction (Nestler, 2008). The Fos family of proteins is rapidly and transiently induced in the striatum after acute administration of several drugs of abuse (Graybiel *et al.*, 1990; Hope *et al.*, 1992; Young *et al.*, 1991). Although most of them are highly unstable, ΔFosB is progressively accumulated after repeated drug exposure. This accumulation has been linked to cocaine-induced reward, locomotor sensitization and SA behaviour (Colby *et al.*, 2003; Kelz *et al.*, 1999; McClung *et al.*, 2004), which together suggest a role in the neural mechanisms involved in transitioning between recreational use and abuse phenomenon. In the present study, an increased expression of ΔFosB was found 24 h after the end of the MDPV exposure and levels of this factor remained raised throughout the period of abstinence. Therefore, it is reasonable to propose that the increased level of this transcription factor responded

to MDPV treatment, leading to an increased responsiveness to cocaine effects. Changes in ΔFosB seem to extend the regulation of drug sensitivity toward more complex behaviours (Colby *et al*., 2003). Thus, according to our findings, mice overexpressing ΔFosB work harder to self-administer cocaine in PR schedule of reinforcement in SA assays, suggesting that ΔFosB may sensitize animals to the incentive motivational properties of cocaine and thereby leading to a propensity for relapse after drug withdrawal.

Numerous putative targets for ΔFosB have been identified in brain, and some of these target genes have been related to the cellular and behavioural effects of this transcription factor (McClung *et al*., 2004). One of these target genes is *c-Fos*. Induction of c-Fos protein is considered an early marker of neural activation, and it is also important for behavioural responses to cocaine (Zhang *et al*., 2006). This factor is markedly activated by acute administration of psychostimulants, but only weakly after repeated exposure (Hope *et al*., 1992; Persico *et al*., 1993), when levels of ΔFosB are high. In this sense, Renthal *et al*. (2008) demonstrated that ΔFosB mediates epigenetic desensitization of the *c-Fos* gene after chronic exposure to a psychostimulant. In our study, a significant increase of c-Fos expression appeared a short time after cocaine challenge that undoubtedly reflects neuronal activation by the drug. However, this protein expression was related to MDPV pretreatment. Thus, levels of c-Fos seemed to be inversely associated with MDPV pretreatment, when ΔFosB is significantly expressed. Results demonstrate a negative association between both factors, in accordance with the findings of Renthal *et al*., (2008).

In summary, MDPV increased most of the behavioural responses related to cocaine effects, including locomotor sensitization, reward and the strength of cocaine as reinforcer in a SA procedure. It is noteworthy that MDPV increased capability to reinstate cocaine SA behaviour once extinguished, presumably indicating increased craving, after MDPV exposure during adolescence. These behavioural alterations were associated with an accumulation of ΔFosB, a sustained molecular switch for cocaine addiction, providing a possible mechanism by which molecular changes induced by MDPV can persist for weeks after withdrawal and supporting the deleterious effects of MDPV on cocaine abuse liability. Therefore, these results suggest that consumption of MDPV during adolescence induces long-lasting adaptive changes leading to a higher response to cocaine in the adulthood, predisposing to a higher vulnerability to abuse of this drug. From a clinical point of view, this feature represents a basic step to provide new knowledge about factors involved in the vulnerability to cocaine addiction.

Acknowledgements

This study was supported by Ministerio de Economia y Competitividad (grant numbers SAF2016-46135-R, SAF2016-75966-R-FEDER and SAF2013-41761-R-FEDER), by the European Union's Horizon 2020 research and innovation programme 2014–2020 under grant agreement no 634143, Spanish Ministry of Health (RD/12/0028/0024-FEDER and Plan Nacional sobre Drogas #2014/020). L.D.C. and M.A.L. received FPU grants from the Ministerio de Economía y Competitividad (15/02492). J.C., D.P. and E.E. belong to 2014SGR1081, and O.V. to 2014SGR34. R.L.-A. position was funded by an institutional program of the Universitat de Barcelona in collaboration with Obra Social de la Fundació Bancària La Caixa.

Author contributions

O.V. and E.E. were responsible for the study concept and design. R.L.A., M.A.L. and L.D.C. carried out the experimental studies. J.C. participated in the data analysis and D.P. in immunoassays methodology. R.L.A. and M.A.L. drafted the manuscript. J.C. and D.P. participated in the interpretation of findings. O.V. and E.E. provided a critical revision of the manuscript for intellectual content. All authors critically reviewed the content and approved the final version for publication.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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7.4. Annex IV

Effects of MDPV on dopamine transporter regulation in male rats

Comparison with cocaine

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Psychopharmacology (BerI) 2019; 236:925-938

Psychopharmacology
https://doi.org/10.1007/s00213-018-5052-z

ORIGINAL INVESTIGATION

Effects of MDPV on dopamine transporter regulation in male rats. Comparison with cocaine

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Received: 30 May 2018 / Accepted: 24 September 2018

Abstract

Rationale MDPV (3,4-methylenedioxypyrovalerone) is a synthetic cathinone present in *bath salts*. It is a powerful psychostimulant and blocker of the dopamine transporter (DAT), like cocaine. It is known that acute exposure to psychostimulants induces rapid changes in DAT function.

Objectives To investigate the effects of MDPV on DAT function comparing with cocaine.

Methods Binding of [^{3}H]WIN 35428 was performed on PC 12 cells treated with MDPV and washed. Rat striatal synaptosomes were incubated with MDPV or cocaine (1 μM) for 1 h and [^{3}H]dopamine (DA) uptake was performed. Also, different treatments with MDPV or cocaine were performed in Sprague-Dawley rats to assess locomotor activity and ex vivo [^{3}H]DA uptake.

Results MDPV increased surface [^{3}H]WIN 35428 binding on PC 12 cells. In vitro incubation of synaptosomes with MDPV produced significant increases in V_{max} and K_M for [^{3}H]DA uptake. In synaptosomes from MDPV- (1.5 mg/kg, s.c.) and cocaine- (30 mg/kg, i.p.) treated rats, there was a significantly higher and more persistent increase in [^{3}H]DA uptake in the case of MDPV than cocaine. Repeated doses of MDPV developed tolerance to this DAT upregulation and 24 h after the 5-day treatment with MDPV, [^{3}H]DA uptake was reduced. However, a challenge with the same drugs after withdrawal recovered the DAT upregulation by both drugs and showed an increased response to MDPV vs the first dose. At the same time, animals were sensitized to the stereotypies induced by both psychostimulants.

Conclusions MDPV induces a rapid and reversible functional upregulation of DAT more powerfully and lasting than cocaine.

Keywords Bath salts · Cathinones · Cocaine · Dopamine transporter · Dopamine uptake · MDPV · Upregulation

Introduction

Membrane neurotransmitter transporters play a key role in the regulation of neural transmission as they are responsible for removal of neurotransmitters released into the synaptic cleft upon neuronal stimulation. Thus, the dopamine transporter (DAT) is crucial for dopaminergic transmission in the nigrostriatal and mesocorticolimbic dopaminergic pathways (see Lohr et al. 2017 or Torres et al. 2003 as reviews) and modifications in its density or transport rate can reduce or enhance the effects of a given amount of dopamine (DA).

DAT, as other monoamine transporters, exhibits a great ability to modify its density in the plasma membrane through a very fast trafficking through internalization and recycling in a protein kinase C (PKC)-regulated manner (Loder and Melikian 2003; Ramamoorthy et al. 2011; Schmitt and Reith 2010).

Psychostimulants increase synaptic monoamine levels by acting on their transporters, either by directly binding and inhibiting their function (e.g., cocaine, Ritz et al. 1987) or through a combined mechanism consisting in entering the cell as a transporter substrate and reversing the transport of cytosolic monoamines (e.g., amphetamine) which are previously displaced from synaptic vesicles by the drug (Sulzer and Galli 2003).

Apart from these acute effects on monoamine uptake, rapid and reversible compensatory changes in transporters and their

Elena Escubedo and David Pubill contributed equally to this work.

This article belongs to a Special Issue on Bath Salts.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00213-018-5052-z) contains supplementary material, which is available to authorized users.

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Published online: 04 October 2018

function have been reported as well. Generally speaking, using samples from which residual drug has been washed away, psychostimulants that act as substrates of the transporters (e.g., amphetamine and analogues) induce an acute decrease in uptake, whereas blockers (e.g., cocaine or methylphenidate) tend to increase it. For example, an acute injection of methamphetamine (METH) to rats reversibly decreases plasmalemmal DA uptake in striatal synaptosomes (Fleckenstein et al. 1997). Similarly, uptake of serotonin is affected by METH treatment (Haughey et al. 2000). Moreover, in vitro incubation of striatal synaptosomes with METH followed by drug washout, rapidly decreased DAT activity, but not total WIN 35428 binding sites (Escubedo et al. 2005; Pubill et al. 2005; Sandoval et al. 2001). Similar effects were reported for MDMA (3,4-methylenedioxymethamphetamine) (Chipana et al. 2006; Hansen et al. 2002). It has been reported that PKC-mediated DAT phosphorylation contributes to this in vitro amphetamine-induced deficit (Cervinski et al. 2005; Giambalvo 2003; Sandoval et al. 2001) which is accompanied by transporter internalization (Saunders et al. 2000; Johnson et al. 2005).

Regarding cocaine, published results from animal in vivo and human postmortem studies are variable depending on the subjects and experimental design. Most reports demonstrate that chronic exposure to cocaine increases DAT binding sites and function (Little et al. 1999; Mash et al. 2002; Staley et al. 1994), but others show a decrease or no change in the striatum following cocaine exposure (i.e., Farfel et al. 1992; Izenwasser and Cox 1990; Letchworth et al. 1997; Peraile et al. 2010; Samuvel et al. 2008; Wilson and Kish 1996). However, in vitro studies in hDAT-transfected cells generally report that acute cocaine treatment produces increases in surface [^{3}H]WIN 35428 binding and [^{3}H]DA uptake, with no change in DAT total protein or mRNA, but through induction of the trafficking from endosomes to the plasma membrane by a mechanism that remains to be elucidated (Little et al. 2002; Zahniser and Sorkin 2009).

Synthetic cathinones have become a very popular group of illicit psychostimulants as substitutes of other amphetamine derivatives such as methamphetamine or MDMA (NIDA 2018: https://www.drugabuse.gov/publications/drugfacts/synthetic-cathinones-bath-salts). One such synthetic cathinone, MDPV (3,4-methylenedioxypyrovalerone), shares pharmacodynamics with cocaine. It is a common ingredient of the so-called *bath salts* (Zuba and Byrska 2013; Johnson and Johnson 2014), which are commercialized by certain Internet sites, although it is currently banned in many countries because its consumption has led to several life-threatening medical consequences (Kesha et al. 2013; Wright et al. 2013). It is a highly selective and potent reuptake inhibitor at monoamine transporters, with greater specificity for the DAT and norepinephrine transporters, compared to that for serotonin transporters. In vitro experiments have shown that MDPV is a more potent reuptake inhibitor than cocaine at DAT (Baumann et al. 2013; Cameron et al. 2013; Simmler et al. 2013), while in vivo studies have found that MDPV has is at least 10-fold more potent at increasing extracellular DA than cocaine (Baumann et al. 2013). Moreover, MDPV robustly increases locomotion, traveled distance and stereotypic movements in rats and mice and produces more intense locomotor and rewarding effects compared to cocaine (Aarde et al. 2013; Gatch et al. 2013; López-Arnau et al. 2017).

Considering the persistence of synthetic cathinone abuse, especially MDPV (Alvarez et al. 2017; Bade et al. 2017), it is necessary to increase the knowledge of its neuropharmacological profile to better understand the effects and risks of this drug. To our knowledge, there are no previous reports of the effects of MDPV or any other synthetic cathinone on DAT regulation. Therefore, the aim of this work is to investigate the effects of MDPV on DAT density and function, using in vitro (PC12 cells and striatal synaptosomes) and ex vivo models (striatal synaptosomes from treated rats) after an acute exposure to the drug and a sensitization regime. Parallel experiments with cocaine were also run in order to compare their effects.

Materials and methods

Drugs and reagents

MDPV was synthesized in racemic form as hydrochloride in our laboratory as described previously (Novellas et al. 2015) and with permission from the University of Barcelona. Cocaine-HCl was provided by the Spanish National Institute of Toxicology. MDPV and cocaine solutions for injection were freshly prepared in saline (0.9% NaCl, pH = 7.4) every day before administration.

[^{3}H]DA and [^{3}H]WIN 35428 were from PerkinElmer (Boston, MA, USA). Dulbecco's modified Eagle's medium (DMEM), culture serums, and antibiotics were purchased from GIBCO (Invitrogen Corp., Paisley, UK). Pargyline, bupropion, HEPES sodium, and ascorbic acid were purchased from Sigma-Aldrich Company Ltd. All other reagents were of analytical grade and obtained from several commercial sources.

PC12 cell culture and treatment

The rat pheochromocytoma PC 12 cell line has been reported as a model to study DAT trafficking (Loder and Melikian 2003) and we used it to investigate the effects of amphetamine derivatives on DA transport (Chipana et al. 2008). For these reasons, we considered these cells a suitable model for this study. PC 12 cells were routinely cultured in 92-mm dishes (Nunc) coated with collagen and maintained in DMEM supplemented with heat-inactivated 5% fetal bovine serum, 10%

horse serum, 10 mM HEPES, 2 mM glutamine, 25 UI/ml penicillin, and 25 μg/ml streptomycin. Cells were cultured to semi-confluency in a humidified 5% CO_2 atmosphere at 37 °C, and medium was changed every 2–3 days. For splitting, cells were dislodged from the dish using a pipette with medium, with a portion of these replated onto new culture dishes. Cells were used between passages 15 and 25.

To ensure their proper differentiation, cells were mechanically dislodged and seeded (200×10^3 cells per well) onto collagen-coated 24-well plates (Nunc) in medium containing 50 ng/ml nerve growth factor (NGF, Upstate Biotechnology, Lake Placid, NY), 1% horse serum, 10 mM HEPES, and 2% glutamine in DMEM. Under these conditions, the cells developed a neuronal phenotype with neurite outgrowth that was already apparent 24 h after seeding (Garcia-Rates et al. 2007).

Treatments were performed 48 h after seeding in differentiation medium. MDPV was dissolved in DMEM and added to the corresponding wells in a volume of 10 μl to reach the desired final concentration. The concentration of MDPV used (0.1 μM) was chosen from preliminary experiments showing that higher concentrations required many washes to be removed, thus compromising the attachment of the cells. Previous reports stated that MDPV can reach peak concentrations of around 1 and 4 μM in plasma and striatum, respectively, after subcutaneous administration (Novellas et al. 2015). Control wells received 10 μl of DMEM. The plates were returned to the incubator for the desired time, until the binding experiment was performed.

Binding of [^{3}H]WIN 35428 to PC12 cells

[^{3}H]WIN 35428 binding was used to label membrane DAT in differentiated PC 12 cells. After incubation with MDPV, the medium was carefully removed and the cells were washed twice with 1 ml of warm Dulbecco's phosphate-buffered saline (PBS, Biological Industries Inc.). Then, 500 μl of [^{3}H]WIN35428 (final radioligand concentration 5 nM) diluted in 0.32 M sucrose-supplemented PBS was added to each well. A parallel set of wells also contained 30 μM bupropion to assess non-specific binding. The plates were placed on ice, and incubation was performed for 2 h at 4 °C. Binding was terminated by detaching the cells by pipetting and filtering through Whatman GF/B glass-fiber filters pre-soaked in 0.5% polyethyleneimine, followed by three 1 ml washes of wells and filters with ice-cold buffer. The radioactivity retained in the filters was measured through liquid scintillation spectrometry. Each experiment was run in triplicate wells.

Animals and treatment

The experimental protocols concerning the use of animals in this work were approved by the Animal Ethics Committee of the University of Barcelona under supervision of the Autonomous Government of Catalonia, following the guidelines of the European Communities Council (86/609/EEC). Efforts were made to minimize suffering and reduce the number of animals used.

Male adult Sprague-Dawley (SD) rats (aged 10–12 weeks and weighing 250–350 g; Harlan Ibérica, Barcelona, Spain) were used for synaptosome assays. They were housed at 22 °C under a 12-h light/dark cycle with free access to food and water. For in vitro incubations with the drugs, animals (2 rats per experiment) were anesthetized and sacrificed and synaptosomes were obtained. For ex vivo experiments with synaptosomes originating from treated animals, MDPV (1.5 mg/kg) and cocaine (30 mg/kg) were dissolved in saline and administered subcutaneously (s.c.) or intraperitoneally (i.p.), respectively, in a volume of 1 ml/kg. Then, the animals were anesthetized and sacrificed and synaptosomes were obtained after 1, 3, or 16 h. The dose of MDPV used in this study (1.5 mg/kg) is equivalent to a dose of approximately 15 mg in humans (Reagan-Shaw et al. 2008; Novellas et al. 2015) which is in the middle range of the doses most commonly used by consumers. The dose of cocaine of 30 mg/kg has been chosen on the basis that MDPV is more potent (10–30-fold) than cocaine (Baumann et al. 2013; Kolanos et al. 2013; Simmler et al. 2013), and the psychomotor stimulation induced by 30 mg/kg of cocaine was not statistically different from that induced by 1.5 mg/kg of MDPV (see Figs. 6 and 7).

The repeated administration procedure consisted in a daily administration of saline, MDPV, or cocaine for 5 consecutive days, followed by 10 days of withdrawal and, 1 day after, a challenge of saline (1 m/kg), MDPV (1.5 mg/kg, s.c.) or cocaine (30 mg/kg, i.p.) was injected. A similar procedure was reported by Gregg et al. (2013) and our group, producing a robust psychomotor sensitization to MDPV and cocaine in mice (Buenrostro-Jáuregui et al. 2016). One set of animals received only the first dose to obtain the uptake values 1 h after the acute dose (day 1, $n = 4$ for each treatment). Two other sets of rats were sacrificed 1 and 24 h after the fifth dose to obtain the results of day 5 and day 6 ($n = 4$ and 6 for each treatment group, respectively). Finally, another set of six animals per group was sacrificed 1 h after the challenge.

Obtention of striatal synaptosomes

Striatal synaptosomes (P2 fraction) were obtained as described by Sandoval et al. (2001), with minor modifications. Rats were decapitated under isoflurane anesthesia, their brains rapidly removed and the striatum was dissected out on ice, weighed, and placed in 20 volumes of cold homogenization buffer (5 mM Tris-HCl and 320 mM sucrose). Tissues were homogenized on ice using a borosilicate glass tube fitted with a motor-driven Teflon pestle and centrifuged twice to obtain the P2 fraction (synaptosomes). In the case of synaptosomes originating from treated rats, the pellet was resuspended in

5 ml of Tris-sucrose buffer and re-centrifuged three times, in order to wash out the residual drugs that could interfere with the experiments.

Finally, the synaptosome fraction was resuspended in Hank's Balanced Salt Solution (HBSS, Biological Industries, Inc.) supplemented with 5.5 mM glucose and 20 mM HEPES-sodium (HBSS/G/H, pH 7.4) for [^{3}H]DA uptake experiments or in 0.1/0.32 M sodium phosphate/sucrose buffer (pH 7.9) for [^{3}H]WIN 35428 binding. The resuspension was done to reach a final protein concentration of approximately 0.1 mg/ml. Protein concentration was determined using the Bio-Rad Protein Reagent (Bio-Rad Labs., Inc., Hercules, CA, USA) according to the manufacturer's instructions and using bovine serum albumin as a standard.

[^{3}H]DA uptake

For in vitro treatment experiments, synaptosomes were obtained as described above and distributed in 1 ml aliquots in centrifuge tubes to perform the incubation with MDPV or cocaine in a shaking water bath at 37 °C for 1 h. Ten microliters of MDPV or cocaine solution or buffer was added to each tube to reach the desired final concentration (1 μM). After incubation, synaptosomes were centrifuged at 13,000×*g* for 20 min and washed three more times with 5 ml of Tris-sucrose buffer followed by centrifugation. The final pellets were resuspended in uptake buffer (HBSS/G/H buffer plus 10 μM pargyline and 1 mM ascorbic acid). For experiments with tissue from treated rats, striatal synaptosomes were obtained as described above and, after the three washes, directly resuspended in uptake buffer.

Reaction tubes consisted in 0.125 ml of uptake buffer, 0.1 ml of synaptosome suspension and 0.025 ml of [^{3}H]DA added at the start of incubation. The remaining synaptosomes from each pellet were kept for further protein concentration assessment. Tubes were warmed 5 min at 37 °C before the addition of [^{3}H]DA after which incubation was carried out for a further 5 min. Uptake reaction was stopped by rapid filtration as described for binding experiments. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Non-specific uptake was determined at 4 °C in parallel samples containing 100 μM cocaine. Specific DA uptake was calculated subtracting non-specific uptake values from those of total uptake (37 °C). Each experiment was run in duplicate tubes.

Specific DA uptake for each condition was normalized by dividing by the protein concentration and expressed as percentage of uptake with respect to control tubes.

We could not perform reliable [^{3}H]DA uptake in differentiated PC 12 cells with our means due to methodological issues (i.e., the need to perform repeated washes to remove free [^{3}H]DA, leading to cell loss by detachment or, alternatively, to mechanically detach the cells and filtering which provokes indeterminate neurite and cell breaking with substrate leakage).

Locomotor activity recording

The locomotor responses induced by MDPV (1.5 mg/kg, s.c.) or cocaine (30 mg/kg, i.p.) were assessed in black Plexiglas open-field arenas ($l \times w \times h$: 45 × 45 × 40 cm) under low-light conditions. Two days before testing, the animals were handled for 10 min, administered saline (1 ml/kg), and placed in the arena for habituation for 30 min each day. On the test day, the rats were administered the assigned treatment and placed in the arenas and their horizontal traveling were video-monitored by a zenithal video-camera coupled to a computer running a tracking software (Smart 3.0, Panlab, S.L.U., Barcelona, Spain) for 60 min. Total traveled distances were obtained and analyzed.

Stereotypy scoring

Stereotyped behaviors were scored by two observers blind to treatment condition using the method of Creese and Iversen (1974). Briefly, video recordings (60 min) were divided into 10-min segments. Behavior during the first minute of each segment was rated on a 0–6 scale: 0 = asleep or lying down, 1 = predominantly slow locomotor activity with non-stereotyped rearing and sniffing, 2 = predominantly rapid locomotor activity with bursts of stereotyped rearing and sniffing, 3 = predominantly stereotyped rearing and sniffing with some locomotor activity, 4 = stereotyped rearing and sniffing maintained in a small area of the enclosure, 5 = stereotyped behavior maintained in a small area with oral stereotypies (licking or gnawing), 6 = assumption of awkward or bizarre posture. A score between 0 and 2 is, generally, considered a normal behavior. Both raters were trained on the same set of sample videos and achieved an inter-rater reliability of at least 0.90 before scoring experimental recordings. The score for each rat and experimental day is the average sum of the scores obtained in each recording block by both observers.

Data analysis

One-way ANOVA followed by Tukey's post hoc test or Student's *t* test was used to analyze data from the binding experiments with cells and synaptosomes. Paired data were applied when comparing K_M and V_{max} of control and MDPV from different experiments, as each one was performed with the same synaptosomal preparation. A two-way ANOVA was used to compare the effects of MDPV, cocaine, and saline on DA uptake (treatment × time factors), as well as to compare the effects of the first dose with those of the challenge (treatment × dose factors) in the sensitization schedule. Finally, a two-way (day and treatment) ANOVA with repeated

measures was used to analyze the temporal evolution of locomotion and stereotypies during the sensitization procedure. When the overall ANOVA yielded significant effects, Tukey's post hoc tests were applied for comparisons between groups.

Results

MDPV treatment increases [^{3}H]WIN 35428 binding on the surface of differentiated PC12 cells

After preliminary trials, we assessed the effect of the exposure to 0.1 μM MDPV on [^{3}H]WIN 35428 binding to intact NGF-differentiated PC12 cells, as a measure of DAT present in the cell membrane. Incubation with the cathinone was performed for 30 min, 1, 3, and 24 h. As can be seen in Fig. 1, a short (30 min–1 h) exposure to MDPV was able to increase superficial DAT expression by 120% compared to control (DMEM alone). This elevation was also evident at 3 h, but was no longer different from control at 24 h (one-way ANOVA: $F_{6,14} = 8297$, $p < 0.001$).

In vitro effects of MDPV and cocaine on [^{3}H]DA uptake in striatal synaptosomes

The effects observed on [^{3}H]WIN 35428 binding in differentiated PC12 cells encouraged us to test if this increased binding was accompanied by increased function of DAT (increased DA uptake). Unfortunately, due to technical issues, we could not perform uptake assays with these cells (see "Materials and Methods"). For this reason, we chose an alternative experimental model and investigated whether in vitro incubation of striatal synaptosomes with MDPV was able to modify DA uptake, as occurs with other amphetamine derivatives, and how such modification was influenced by substrate concentration. Due to its similar mechanism of action, we also sought to compare these effects with those of cocaine. For this reason, we performed kinetic [^{3}H]DA uptake experiments with synaptosomes pre-incubated with either MDPV or cocaine (both at 1 μM) for 1 h and compared the values with those obtained from control (pre-incubated in the absence of drug) synaptosomes.

For MDPV, significant increases in both K_M (62%) and V_{max} (57%) were obtained, showing higher uptake values in the MDPV group as long the substrate concentration was raised (Fig. 2). Cocaine induced a more modest increase in V_{max} (30%), without significantly modifying the K_M value (Fig. 3).

We also performed experiments measuring [^{3}H]DA uptake after incubating striatal synaptosomes for 1 h with increasing concentrations of MDPV or cocaine. In this case, we chose a constant [^{3}H]DA concentration of 0.2 μM because differences between control and drug-treated kinetic curves were more apparent from and above this concentration. One-way ANOVA for each drug reported statistically significant effects

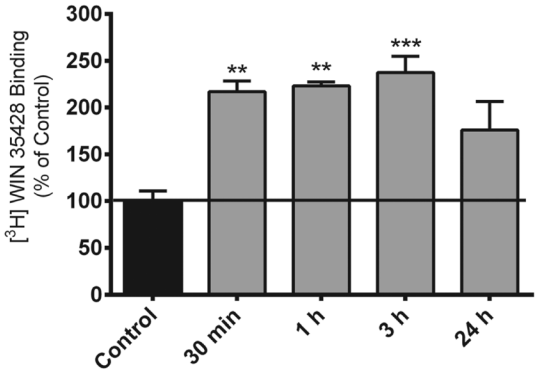

Fig. 1 Effect of MDPV (0.1 μM) during different incubation times on [^{3}H]WIN 35428 binding to intact differentiated PC12 cells. Results are the means of two independent experiments carried out in triplicates and are expressed as the mean ± SEM of the percentage from the binding obtained in medium-treated cells (control).**$p < 0.01$; ***$p < 0.001$ vs. control

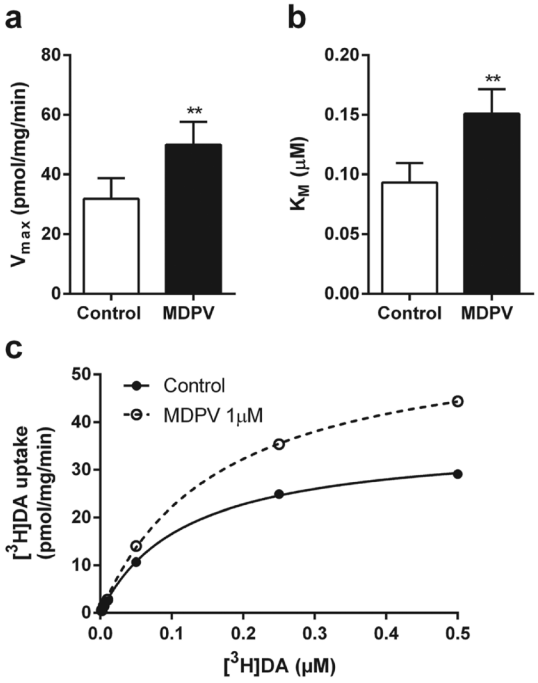

Fig. 2 Effect of pre-incubation with MDPV (1 μM) on [^{3}H]DA uptake kinetics in rat striatal synaptosomes. Panel **a** shows the values of V_{max}, while panel **b** shows those of K_M. Results are the means ± SEM from five experiments carried out on duplicates. Panel **c** depicts a representative experiment of such kinetics. **$p < 0.01$ vs. control

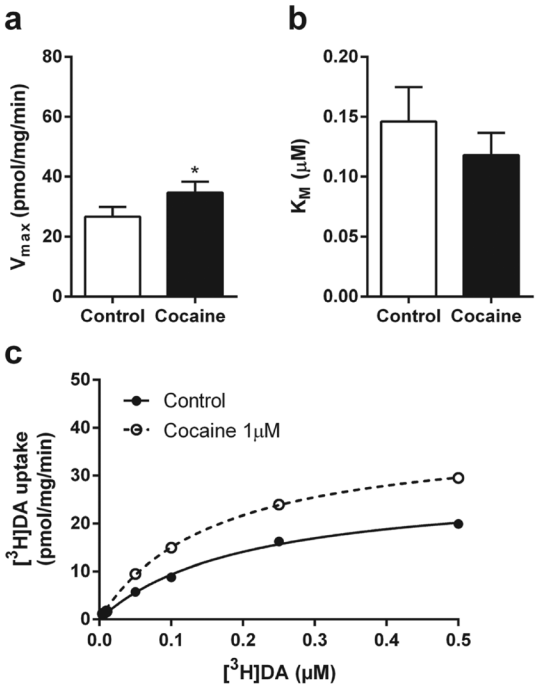

Fig. 3 Effect of pre-incubation with cocaine (1 μM) on [^{3}H]DA uptake kinetics in rat striatal synaptosomes. Panel **a** shows the values of V_{max}, while panel **b** shows those of K_M. Results are the means ± SEM from five experiments carried out on duplicates. Panel **c** depicts a representative experiment of such kinetics. *$p < 0.05$ vs. control

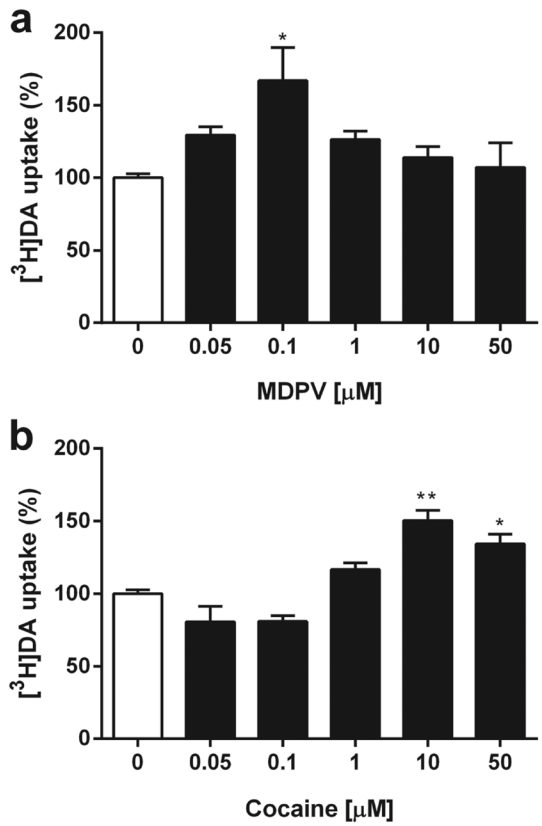

Fig. 4 Effect of increasing MDPV (**a**) and cocaine (**b**) concentrations on [^{3}H]DA uptake in rat striatal synaptosomes. Results are the means ± SEM from four experiments carried out on duplicates. *$p < 0.05$; **$p < 0.01$ vs. control (0 μM)

of drug treatment (MDPV: $F_{5,12} = 3.667$, $p < 0.05$; cocaine: $F_{5,12} = 19.20$, $p < 0.0001$). As depicted in Fig. 4a, MDPV produced a statistically significant increase in uptake at a concentration of 0.1 μM whereas the differences in uptake at the rest of concentrations did not reach statistical significance. On the other hand, cocaine started showing a tendency to upregulate uptake at a concentration of 1 μM, which became statistically significant at concentrations of 10 and 50 μM (Fig. 4b).

Administration of a single dose of MDPV upregulates DA uptake more potently and longer than cocaine

After the in vitro results, we investigated whether acute administration of MDPV to rats was able to similarly increase DA uptake. Accordingly, we treated rats with saline, MDPV (1.5 mg/kg, s.c.) or cocaine (30 mg/kg, i.p.) and sacrificed them after 1, 3, and 16 h (Fig. 5). Striatal synaptosomes were immediately obtained and washed and uptake of [^{3}H]DA (0.2 μM) was performed. This substrate concentration was chosen after the in vitro experiments showing that the differences between groups were more apparent at higher [^{3}H]DA concentrations.

The two-way ANOVA of the results showed the existence of significant differences for treatment ($F_{2,27} = 60.09$, $p < 0.0001$) and time ($F_{2,27} = 25.86$, $p < 0.0001$), as well as a significant interaction between them ($F_{4,27} = 13.30$, $p < 0.0001$). MDPV administration induced a significant increase in [^{3}H]DA uptake (around 120%, $p < 0.001$) measured 1 h after administration which persisted until 3 h post-treatment. Cocaine treatment also induced a significant, but smaller, increase than MDPV in [^{3}H]DA uptake (around 75%, $p < 0.001$) when assessed 1 h after administration, but it was no longer evident 3 h post-treatment.

Effects of a repeated administration of MDPV or cocaine on DA uptake

In view of the effects of an acute in vivo administration of MDPV (1.5 mg/kg), we investigated the consequences of its daily administration for 5 days, followed

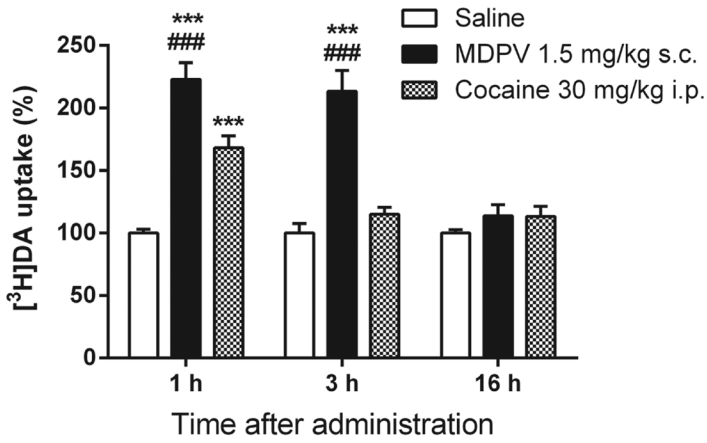

Fig. 5 [^{3}H]DA uptake in striatal synaptosomes from rats treated acutely with saline (1 ml/kg, s.c.), MDPV (1.5 mg/kg, s.c.), or cocaine (30 mg/kg, i.p.) and sacrificed after 1, 3, or 16 h. Uptake values are normalized as percentage of control (saline group, 100%) and are the means ± SEM of those from four animals per group and time. ANOVA post hoc comparisons: $^{***}p < 0.001$ vs. saline at the same time point; $^{###}p < 0.001$ vs. cocaine at the same time point

by 10 days of withdrawal, on the changes in DA uptake induced by a challenge with the drug (1.5 mg/kg, s.c.). One set of rats was sacrificed 1 h after the first administration (acute, day 1), and another set 1 h after the challenge dose (challenge day). The same schedule was concomitantly carried out with cocaine (30 mg/kg) for comparison. Moreover, we sacrificed one set of rats 1 (day 5) and 24 h (day 6) after receiving the fifth dose of the assigned treatment in order to study how the DA uptake at these time points was affected. The results are depicted in Fig. 6.

The two-way ANOVA showed significant effects of drug treatment ($F_{2,48} = 95.00$, $p < 0.0001$) and time ($F_{3,48} = 74.85$, $p < 0.0001$), as well as an interaction between these factors ($F_{6,48} = 24.84$, $p < 0.0001$). The results and post hoc tests showed that there was an increase in DA uptake after the challenge in the striatum of rats which had received the repeated administration of MDPV and that this increase was higher than that produced by a single administration ($p < 0.05$). As occurred with the acute dose, the effects of cocaine, although significant, were less than those of MDPV and, interestingly, the increase in DA uptake produced by the challenge with cocaine did not significantly differ from that of the acute dose (day 1).

One hour after the fifth dose (day 5), DA uptake was increased in the animals receiving drugs, especially those given MDPV, whose increase was significant with respect to saline. The increases, however, were not as high as those induced by the first doses (day 1). Moreover, 24 h after the fifth dose (day 6), the uptake levels were significantly reduced in the case of MDPV-treated rats, whereas those treated with cocaine did not show significant differences with respect to saline.

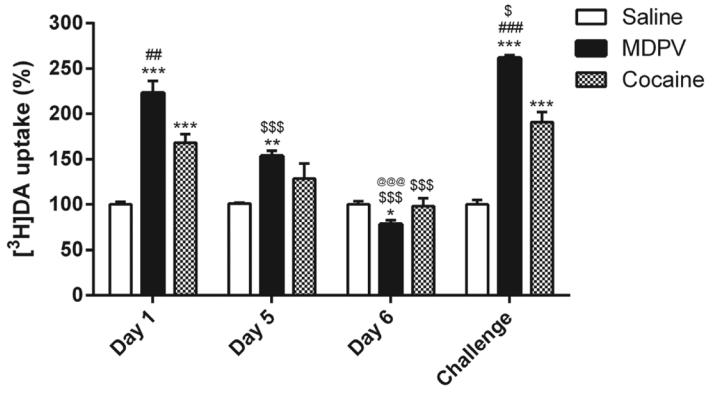

Fig. 6 Effects of repeated daily administration of MDPV or cocaine on striatal DA uptake at different time points. Results are expressed as percentage of control (saline group, 100%) and are the mean ± SEM ($n = 4$–6 per group). Tukey's post hoc test: $^{***}p < 0.001$, $^{**}p < 0.01$ vs. saline at the same time point; $^{###}p < 0.001$, $^{##}p < 0.01$ vs. cocaine at the same time point; $^{$$$}p < 0.001$, $^{$}p < 0.05$ with respect to the same treatment group at day 1; $^{@@@}p < 0.001$ with respect to the same treatment group at day 5

Comparative effects of MDPV and cocaine in hyperlocomotion and stereotyped behavior during the repeated administration procedure

Locomotor activity of rats was measured during the 5 days of administration and the challenge day in order to assess whether locomotor sensitization had also occurred. A two-way ANOVA with repeated measures revealed significant effects of treatment ($F_{2,15} = 38.21$, $p < 0.0001$) and day ($F_{4,60} = 4.17$, $p < 0.01$), but no interaction between these variables. MDPV (1.5 mg/kg) and cocaine (30 mg/kg, which is considered a high dose) induced similar increases in locomotor activity (Fig. 7a). Interestingly, over the daily administration period, locomotor activity decreased in the case of MDPV or did not significantly change in the case of cocaine. Moreover, on the challenge day, the two-way ANOVA revealed significant effects of treatment ($F_{1,10} = 17.79$, $p < 0.01$) and day ($F_{2,20} = 27.49$, $p < 0.0001$) as well as an interaction between the two variables ($F_{2,20} = 7.952$ $p < 0.01$). The locomotor activity induced by both drugs was significantly less than that induced on the first day (Fig. 7b).

However, we observed an increase in stereotypies over the administration days, including repetitive head weaving, sniffing, and rearing, which could account for the reduction in ambulation. To explore this hypothesis, we re-analyzed the videos and scored the stereotyped behavior. The results are displayed in Fig. 7c, d, and the two-way ANOVA with repeated measures revealed a significant effect of treatment ($F_{2,15} = 74.40$, $p < 0.0001$) and day ($F_{4,60} = 43,87$, $p < 0.0001$) and a significant interaction between these factors ($F_{8,60} = 10.33$, $p < 0.0001$). The stereotypy score increased along the 5 days of treatment (Fig. 7c) and there was a sensitization of the stereotyped behavior on the challenge day for both drugs ($p < 0.001$, Fig. 7d). Again, the stereotypy score on the challenge day for MDPV was higher than that of cocaine. The overall two-way repeated measures ANOVA comparing the stereotypies on day 1 and after the challenge showed significant effects of treatment ($F_{2,15} = 91.33$, $p < 0.0001$) and day

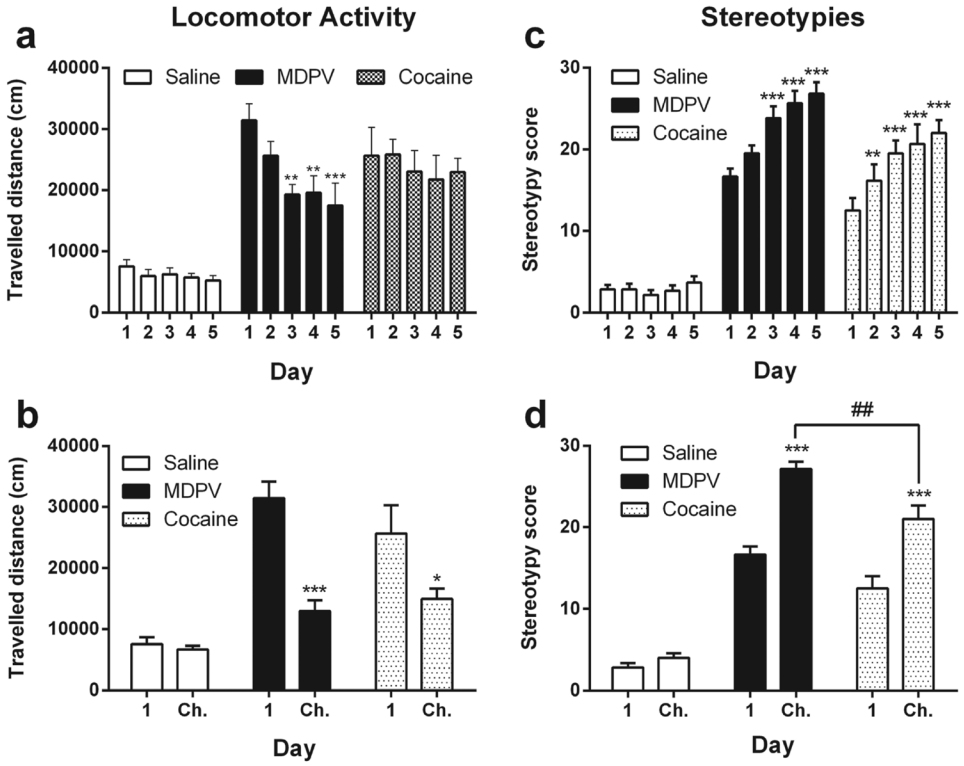

Fig. 7 **a** Locomotor activity during daily administration of saline (1 ml/kg), MDPV (1.5 mg/kg, s.c.), or cocaine (30 mg/kg, i.p.) to rats for 5 consecutive days. **b** Locomotor activity induced by a challenge with saline (1 ml/kg, s.c.), MDPV (1.5 mg/kg, s.c.), or cocaine (30 mg/kg, i.p.) after 10 days of withdrawal, compared with the distance recorded on day 1. Panels **c** and **d** show the stereotypy scores assigned from the corresponding recordings to the data depicted in A and B, respectively. Data represent the mean ± SEM of the values from six rats per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the corresponding value on day 1. $^{\#\#}p < 0.05$ between the indicated groups

($F_{1,15} = 149.1$, $p < 0.0001$), as well as an interaction between these factors ($F_{2,15} = 26.56$, $p < 0.0001$).

Finally, to assess whether the increase in stereotypies was correlated with the decreased locomotion, we carried out the correlation analysis between stereotypy score and distance (shown in Fig. 8). As can be observed, there was a very significant negative correlation between stereotypy score and distance in the case of MDPV, while the analysis corresponding to cocaine did not reach statistical significance.

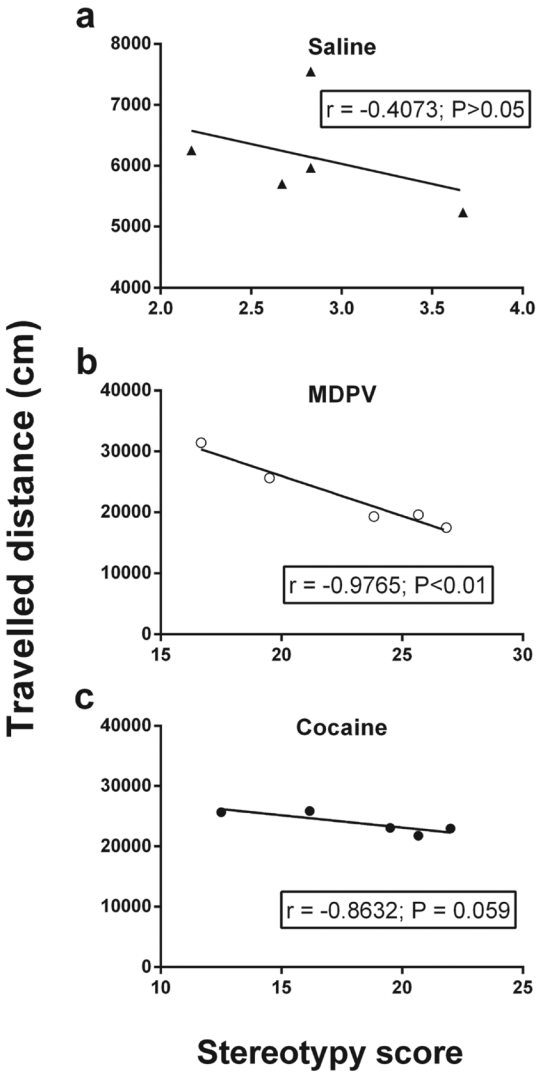

Fig. 8 Correlation analyses between the means of the stereotypy score and the corresponding traveled distance during the consecutive 5 days of treatment with saline (1 ml/kg), MDPV (1.5 mg/kg, s.c.), or cocaine (30 mg/kg, i.p.)

Discussion

In the present work, we studied the regulatory effects of MDPV on DAT density and function in vitro as well as DAT function after an acute administration and during a repeated schedule, followed by drug removal. As cited in the "Introduction," apart from the effects produced when they reach the synapses, psychostimulants can rapidly induce either up- or downregulation of DAT depending on their mechanism of action. MDPV (like cocaine) is a non-substrate DAT blocker, (Baumann et al. 2013; Simmler et al. 2013), and therefore an upregulation of this transporter would be expected. A small DA-releasing effect of MDPV at very low concentrations (1 nM) has also been reported (Shekar et al. 2017). However, the concentrations of MDPV reached in CNS after an average dosing are much higher (Novellas et al. 2015) and, therefore, a predominance of blockade is expected. Nevertheless, due to the higher affinity and potency on DAT when compared with cocaine, the study of such effects of MDPV are of great interest in order to increase the knowledge about this new drug and to predict possible side effects after its consumption.

To start with, we tested the effects of MDPV exposition on surface DAT density ([^{3}H]WIN 35428 binding) in NGF-differentiated PC 12 cells. This cell line develops a phenotype of dopaminergic neurons upon differentiation and, therefore, is a good model to study dopaminergic drugs and DAT (Greene and Tischler 1976; Kittner et al. 1987; Chipana et al. 2008). We must point out that binding was carried out on intact plate-attached cells, in order to avoid binding to intracellular forms of DAT which would have masked any change in the membrane population. Using this experimental model, we found that MDPV, at a concentration within those achieved in brain and plasma after recreational administration (Novellas et al. 2015), produces a rapid upregulation of [^{3}H]WIN 35428 binding sites which persists for at least 3 h. These results are in agreement with those reported by other groups using cell lines transfected with hDAT and exposed to cocaine (Daws et al. 2002; Little et al. 2002). The decline in DAT after 24-h incubation might have several explanations that require further investigation. One possibility could be the spontaneous degradation of MDPV or cell metabolism as PC 12 have been reported to express several xenobiotic metabolizing cytochrome P450 isoforms (Kashyap et al. 2011). The decrease in MDPV concentration may lead to a process of restoration of DAT levels.

Recently, Colon-Perez et al. (2018) reported a rapid internalization of fluorescence-labeled DAT after 1 h exposure to MDPV (0.3 μM) in transfected HEK cells, whereas we found an increase in surface [^{3}H]WIN 35428 binding. A possible explanation to this discrepancy is the fact that the model they used is not a neural derived cell line, does not constitutively express DAT, and does not contain dopamine or other

monoamines. By contrast, PC 12 cells and striatal synaptosomes express DAT and might be equipped with all the machinery that may be involved in DAT trafficking and regulation in dopaminergic neurons under physiological conditions (i.e., secretory vesicles, dopamine receptors, and dopamine itself) whereas the cell line used by Colon-Perez et al. might not. In fact, Little et al. (2002) used a neuronal cell line (N2A) for similar purposes and reported, as we do, an upregulation of DAT upon incubation with cocaine, which is in line with our results and others'.

The next step was to investigate whether this binding upregulation was accompanied by increased DA uptake. For this reason, we moved to the in vitro model of striatal synaptosomes. This model had been successfully used by our group and others for measuring the effects of an acute exposure to METH and MDMA (Chipana et al. 2006; Escubedo et al. 2005; Hansen et al. 2002; Pubill et al. 2005; Sandoval et al. 2001). In these experiments, we studied the kinetics of DA uptake in striatal synaptosomes treated with buffer, MDPV or cocaine at the same concentration for 1 h. MDPV significantly increased V_{max}, as cocaine, and increased $K_{M,}$ whereas cocaine did not significantly modify this parameter. The increase in V_{max} is compatible with the increased [^{3}H]WIN 35428 binding we found in PC12 cells treated with MDPV and that reported in hDAT-transfected cell lines treated with cocaine (Little et al. 2002; Zahniser and Sorkin 2009) and indicates that there exists a rapid functional upregulation of DAT upon acute exposition to MDPV and cocaine. The fact that K_M remained unchanged after treatment with cocaine is in agreement with the results from Little et al. (2002) using transfected cells. To our knowledge, this is the first time that such upregulation is reported for MDPV and for cocaine in rat striatal synaptosomes. Moreover, the fact that these effects are produced in vitro in synaptosomes suggests that this upregulation takes place rapidly, at the nerve terminals, without the need of increased gene translation or de novo protein synthesis. This is in line with the results reported using transfected cell lines, where no changes in DAT total protein or mRNA were found (Little et al. 2002; Zahniser and Sorkin 2009), suggesting a fast effect on DAT trafficking from endosomes to the plasma membrane by a mechanism that remains to be elucidated. The greater effects of MDPV compared with cocaine could be explained by its higher affinity for DAT (Baumann et al. 2013; Simmler et al. 2013).

The relevance and relative impact of both increased K_M and V_{max} for DA uptake in the case of MDPV deserves further investigation. An interesting point is that significant increases in these two parameters have been reported in the nucleus accumbens of post-mortem brains from schizophrenic patients (Haberland and Hetey 1987). In fact, the reported increase in K_M was about twofold, whereas what we found was about 50%. The changes in DAT function could be responsible, at least in part, of the psychiatric effects observed in some MDPV abusers (Schmoll et al. 2017).

In addition, we assessed the effect of an in vitro incubation (1 h) with increasing concentrations of MDPV and cocaine on DA uptake. At a substrate concentration of 0.2 μM, MDPV significantly increased uptake only at 0.1 μM while the concentrations of 0.05 and 1 μM only reached statistical significance when compared with control using a Student's t test (data not shown). Interestingly, the uptake values at concentrations of 1 μM and above were lower than at 0.1 μM, regardless increased V_{max} was found thanks to using higher substrate concentrations. An explanation to this point could be the high affinity of MDPV for DAT, which is probably accompanied by a very slow dissociation rate. This slow dissociation makes necessary to perform repetitive washes to remove all drug residuals and reveal the transporter upregulation. In fact, the MDPV concentrations of 10 and 50 μM initially produced uptake downregulation when performing three washes (not shown), whereas performing one additional wash resulted in an uptake value higher than 100%. This phenomenon could also explain the increase in K_M produced by MDPV, which suggests that, despite the upregulation of transporters, a higher amount of substrate would be necessary to reach half the V_{max} because the function of part of the DAT population might be impaired by residual MDPV or previous exposition to high concentrations of drug. Conversely, the highest concentrations of cocaine produced an increase in uptake, with a steady maximum effect, which was lower than that of MDPV and in line with an increased V_{max} without changes in K_M.

Given the in vitro effects of MDPV, we further investigated whether an in vivo acute dose of this drug induced such upregulation and compared its effects with those of cocaine. In this case, we used doses of both drugs that produced similar psychomotor effects and measured uptake at a single concentration of [^{3}H]DA, which according to the previous kinetic experiments showed the highest difference between groups. The upregulation of DA uptake also appeared in drug-treated animals, and the percentages of increase were higher than those found in vitro (V_{max}), indicating that the in vivo conditions facilitate the observed effects. Again, MDPV showed a higher potency than cocaine at upregulating DA uptake. Moreover, its effects lasted at least 3 h post-administration, whereas the effects of cocaine were not significantly elevated at this time point. In both cases, the effect was reversible and no differences were found 16 h later. The longer persistence of MDPV effects could be explained by its long half-live in the brain, as significant levels of this drug can be found in striatum 3 h after administration (Novellas et al. 2015). Moreover, MDPV seems to have a slower dissociation from DAT, which implies the need to perform several washes of the preparation to remove residual drug, which can still be detected by HPLC-

MS in synaptosomes even after tissue homogenization and centrifugation (data not shown). On the other hand, cocaine has been reported to disappear more rapidly from the brain, so that its concentration in rat brain is very low at 1 h after administration (Bowman et al. 1999). This could explain why its effects on DAT are less persistent after a single administration. An aspect to consider is how long DAT is upregulated once the drug concentration has declined in brain, because an increased number of DAT in the absence of blocker could produce a hypodopaminergic status that may lead to search for drug re-dosing.

The next experiments focused on DA uptake after a repeated administration of these psychostimulants. Mash et al. (2002) reported a parallel increase in B_{max} of [^{3}H]WIN 35428 binding and V_{max} for [^{3}H]DA uptake in postmortem synaptosomes from cocaine abusers, indicating functional upregulation of DAT following chronic cocaine use. Another group (Samuvel et al. 2008) described that rats which self-administered cocaine followed by a 3-week abstinence showed a higher level of surface DAT and DA uptake in the striatum.

No previous reports exist concerning the effects of MDPV on DAT density and function, so we investigated the function of DAT after a repeated administration regime with MDPV or cocaine, followed by a period of withdrawal and a challenge with the drug. Interestingly, we found a sensitization of the DA uptake upregulation induced by MDPV, but not by cocaine. This indicates that MDPV, at the dose used, affects the population of DAT or its trafficking mechanisms differently, leading to increased upregulation by a re-exposure to the drug after a period of withdrawal. Further investigations are warranted in order to determine what mechanisms are involved in such effect. In line with these findings, when we assessed DA uptake 24 h after the fifth daily dose, we observed that uptake levels of cocaine-treated rats did not differ from controls, whereas those of the MDPV-treated group were significantly lower. This finding evidences again the differences between MDPV and cocaine. A recent publication by Colon-Perez et al. (2018) also reports DAT downregulation 24 h after the administration of MDPV to rats. Moreover, we had previously reported a decrease in [^{3}H]WIN 35428 binding in the striatum of mice that had received a repeated administration of MDPV (1.5 mg/kg) for 7 days after 21 days of withdrawal (preliminary results presented as a communication at the Neuroscience 2017 congress by Duart-Castells et al. 2017). Although in the present work, we did not measure DAT function after withdrawal, just before the challenge, these previous results suggest that DAT would be still downregulated at this time point (10 days after). A reduced DAT population would also explain, at least in part, why the challenge with the same dose of MDPV increases the stereotypies, as the blockade of DA uptake would be higher, leading to a hyperdopaminergic status. Consequently, a higher uptake upregulation would be expected as an attempt to restore normal DA synaptic levels.

DAT downregulation may be a homeostatic response once MDPV disappeared from the brain and left an increased population of free transporters that would dramatically reduce DA in the synapses if it was not normalized. The fact that DA uptake levels have returned to control values 16 h after an acute administration backs this argument. Moreover, 1 h after the fifth dose, DA uptake was significantly increased but to a lesser extent with respect to the first dose in MDPV rats, and closer to controls in the cocaine group. This suggests the development of a tolerance to the upregulation of DAT that may reflect a reduction in rapidly available DAT after the repeated administration. This could also account in some extent, to the subsequent reduction in DAT and to the sensitization of the homeostatic mechanism to respond to further exposures to the drug.

Another interesting issue was the behavioral responses to this repeated administration regime. Behavioral sensitization to psychostimulants can be attributed not only to a direct pharmacological action of the drug but also to environmental conditions associated with the drug experience (Pierce and Kalivas 1997). It has been suggested that behavioral sensitization could be involved in the development and maintenance of drug addiction (Tzschentke and Schmidt 2000) through enhanced incentive salience (Robinson and Berridge 1993). When we measured locomotor activity every day after the drug administration, we observed a progressive decrease in the locomotor response to the drug in the case of MDPV and a non-significant change in the case of cocaine. This response was also reflected on the challenge day, when both drug-treated groups showed a reduced locomotor activity after the drug administration when compared to that induced by the first dose, and very significant in the case of MDPV. These behaviors, however, were correlated with an increase in stereotypies, especially in the case of MDPV. Stereotypies appear after administration of a number of psychotropic drugs and become more apparent at higher doses in the case of psychostimulants, to an extent that they can preclude hyperlocomotion and mask it when measured as an indicator of psychostimulation (Creese and Iversen 1974; Pritchard et al. 2012). It has been suggested that stereotyped behavior is correlated with an imbalance between the medial prefrontal and sensorimotor circuits of the basal ganglia resulting in a loss of control of motor behavior (Aliane et al. 2009). Given the higher potency of MDPV, relatively low doses (if compared with cocaine) are able to produce stereotypies in rats and sensitization to these effects (Gregg et al. 2016). Similar effects had already been described for cocaine (Aliane et al. 2009; Souza et al. 2014). Our results are in line with those reported by Watterson et al. (2016), who used a 5-day daily or every 48 h administration of MDPV (1 or 5 mg/kg) followed by 5 days of withdrawal to assess motor sensitization. With this schedule, they only found motor sensitization to MDPV in the case of administering the drug every 48 h and at the dose

of 1 mg/kg and attributed the lack of effect of 5 mg/kg to the strong stereotyped behavior that this dose induces, although they did not quantify stereotypies. In our case and theirs using the daily administration of 1 mg/kg, sensitization to stereotypies might have developed more easily, thus precluding the locomotor sensitization.

A reduced population of DAT at the time of challenge could account for an increased psychostimulant effect of MDPV leading to more intense stereotypies. Thus, the increased upregulation of DAT with respect to day 1 could obey an attempt to reduce the excess of DA in the synapses after the intense blockade by MDPV.

In summary, we demonstrate here that acute exposure to MDPV induces rapid and reversible upregulation of DAT function, probably as a response to the intense blockade exerted by the drug. This effect takes place at the nerve terminal, without the need of gene transcription or de novo protein synthesis, and is higher than that exerted by an equimolar (in vitro) or an equipotent (in vivo) dose of cocaine. Moreover, after repeated administration of MDPV, sensitization occurs to this effect. Further studies are needed to assess the impact of this regulation in the addictive properties of this drug, as well as to investigate the molecular mechanisms involved.

Acknowledgments We are grateful to Dr. Anthony L. Riley for helpful critical reading of the manuscript. We also acknowledge Nacho Fargas for eventual technical support.

Funding information This study was supported by grants from Ministerio de Economia y Competitividad (grant SAF2016-75347R) and Plan Nacional sobre Drogas #2014I020, #2016I004). LDC received FPU grants from the Ministerio de Economía y Competitividad (15/02492). JC, LDC, EE, RLA, and DP belong to the quality mentioned group 2017SGR979 by Generalitat de Catalunya. RLA position was funded by an institutional program of the Universitat de Barcelona in collaboration with Obra Social de la Fundació Bancària La Caixa.

Compliance with ethical standards

The experimental protocols concerning the use of animals in this work were approved by the Animal Ethics Committee of the University of Barcelona under supervision of the Autonomous Government of Catalonia, following the guidelines of the European Communities Council (86/609/EEC).

Conflict of interest The authors declare that there is no conflict of interest.

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7.5. Annex V

Behavioural and molecular effects of a high fat diet and maternal binge-like alcohol consumption on female mice offspring

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Psychoneuroendocrinology 2020 [submitted]

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ABSTRACT

Prenatal alcohol exposure is a leading cause of neurobehavioural and neurocognitive deficits collectively known as foetal alcohol spectrum disorders (FASD), including eating disorders and increased risk for substance abuse as very common issues. In this context, the present study aimed to assess the interaction between alcohol exposure during gestation and lactation periods and a high fat diet (HFD) during adolescence. Accordingly, pregnant C57Bl/6 mice underwent a procedure for alcohol binge drinking during gestation and lactation periods. Subsequently, prenatal and lactation alcohol-exposed (PLAE) female offspring were fed with a HFD for 8 weeks and thereafter, some nutrition-related parameters as well as their response to cocaine were assessed.

In our model, feeding young females with a HFD increased their triglyceride blood levels but did not induce an overweight compared to those fed with a standard diet. Moreover, PLAE affected how females responded to the fatty diet as they consumed less amount of food than water-exposed offspring, consistent with their lower gain weight. HFD reduced dopamine uptake and, only in non-alcohol-exposed animals, increased the psychostimulant effect of cocaine. Surprisingly, PLAE attenuated body weight gain and prevented the enhanced psychostimulant response to cocaine and the overexpression of cannabinoid 1 receptors induced by a HFD. PLAE also induced an alteration of biomarkers in the prefrontal cortex and this effect was mitigated by a HFD-based feeding. Therefore, in female offspring, several effects triggered by one of these factors, PLAE or a HFD, were blunted by the other, noting some interactions between them.

INTRODUCTION

Prenatal alcohol exposure is a leading cause of significant neurobehavioural and neurocognitive deficits. For instance, it induces serious neurophysiological and neuropsychological sequelae that contribute to increased risk for substance abuse problems among people with foetal alcohol spectrum disorders (FASD) (Grant et al., 2013). In this sense, Barbier et al. (2008) reported that alcohol-exposed male offspring are more sensitive to the anxiolytic effect of ethanol, a feature that could partially explain the altered pattern of consumption of ethanol observed in these animals. Furthermore, it has been described that maternal binge-like alcohol consumption during gestation and lactation alters sensitivity to the reinforcing effects of cocaine and thus, enhances vulnerability to cocaine addiction in adult mice (Cantacorps et al., 2019).

Additionally, prenatal alcohol exposure increases the hypothalamic-pituitary-adrenal (HPA) axis tone, resulting in HPA dysregulation throughout life. More concretely, foetal programming of the HPA axis by prenatal alcohol exposure alters neuroadaptive mechanisms that mediate the stress response, thus sensitizing the organism to stressors encountered later in life, and mediating, at least partly, the increased vulnerability to mental illness in individuals with FASD (Hellemans, et al., 2010). Nevertheless, the potential consequences of maternal alcohol exposure for eating behaviours, nutritional status, as well as on other nutritional issues of offspring during childhood are relatively under-researched. In this regard, it is known that alcohol may be associated with altered acquisition and distribution of body mass with increasing age (Spohr et al., 2012; Klug et al., 2002). Likewise, eating disorders seem to be common, thereby children with FASD show increased feeding behaviours with a surrounding lack of

satiety that suggest an altered food intake self-regulation. Indeed, rates of overweight and obesity are increased among children with FASD diagnosis, particularly in females (Werts et al., 2014). Altogether, evidence is suggestive of possible metabolic and/or endocrine disruptions in people with FASD. Furthermore, responses to drugs (therapeutic and recreational) are also profoundly impacted by eating conditions, including body weight and type of food, and such changes in drug sensitivity might be long-lasting (Baladi et al., 2010).

In this context, the present study aims to assess the interaction between alcohol exposure during gestation and lactation and a high fat diet (HFD) during the female offspring's adolescence and their consequences on the psychostimulant-induced effects of cocaine (both behavioural and molecular) and on nutrition-related parameters.

It is important to highlight that the experimental model used in the present study intends to mimic as much as possible real-life consumption patterns. Hence, C57Bl/6 female mice were exposed to alcohol following the drinking-in-the-dark (DID) paradigm during the period that mimics the entire gestational period. Subsequently, prenatal and lactation alcohol-exposed (PLAE) female offspring were fed *ad libitum* with a HFD for 8 weeks. Thereafter, they were assessed for the psychostimulant, sensitizing and rewarding effects of cocaine using the horizontal locomotor test and the conditioned place preference (CPP) paradigm. In parallel, cocaine-related factors regarding dopaminergic neurotransmission and neuronal damage were studied in specific brain areas: the ventral striatum (VS) and the prefrontal cortex (PFC), respectively. Additionally, nutrition-related parameters such as food intake, glucose and triglyceride blood levels were measured, and the expression levels of ghrelin-related genes were determined

in the hypothalamus. Importantly, we could observe that several effects triggered by PLAE were blunted by a HFD, and vice versa, evidencing an interaction between both factors.

MATERIALS AND METHODS

Animals

Twelve-week-old male and female C57BL/6 inbred mice were purchased from Charles River (Barcelona, Spain) and shipped to our animal facility (UBIOMEX, PRBB) to be used as breeders. Upon arrival, they were housed in standard cages at constant temperature (21 ± 1ºC) and humidity (55 ± 10%), under a reversed light-dark cycle (white lights on 20:00-08:00 h). After one week of acclimatization, breeding pairs were mated, and pregnant females were observed daily for parturition. For each litter, the date of birth was designated as PND 0. Pups remained with their mothers for 21 days and were then weaned (PND 21). Food and water were available *ad libitum* except during the DID procedure, as described below. Every effort was made to minimize the number of animals used and their suffering. All animal care and experimental procedures were approved by the local ethics committee (CEEA-PRBB) and conducted in accordance with the European Union Directive 2010/63/EU guidelines on the protection of animals used for scientific purposes. ARRIVE guidelines for reporting animal research have been followed. For all the experiments, animals were randomly assigned to an experimental group. During the behavioural manipulations, researchers were not aware of the treatment that each animal had received.

The C57Bl/6 mouse strain was selected as it drinks alcohol voluntarily and in large quantities (Yoneyama et al., 2008). Moreover, it is a particularly good model of diet-induced obesity and has become one of the most important tools for understanding the interplay of HFD and the development of obesity (Wang and Liao, 2013).

Materials

Ethyl alcohol was purchased from Merck (Darmstadt, Germany) and diluted in tap water in order to obtain a 20% (v/v) alcohol solution for the Drinking-in-the-dark test. Cocaine hydrochloride was purchased in Alcaliber S.A. (Madrid, Spain) with the authorization of the "Agencia Española del Medicamento y Productos Sanitarios" (Ministerio de Sanidad, Consumo y Bienestar Social) and prepared in 0.9% NaCl pH=7.4 (saline) immediately before administration.

The protease and phosphatase inhibitor cocktail was purchased from Abcam (Cambridge, UK). [^{3}H]DA was from Perkin Elmer (Boston, USA). Pargyline, HEPES sodium and ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents were of analytical grade and purchased from several commercial sources.

Experimental design

For a better understanding, the experimental design and the four experimental groups involved in the study are depicted in Figure 1 and explained in more detail below.

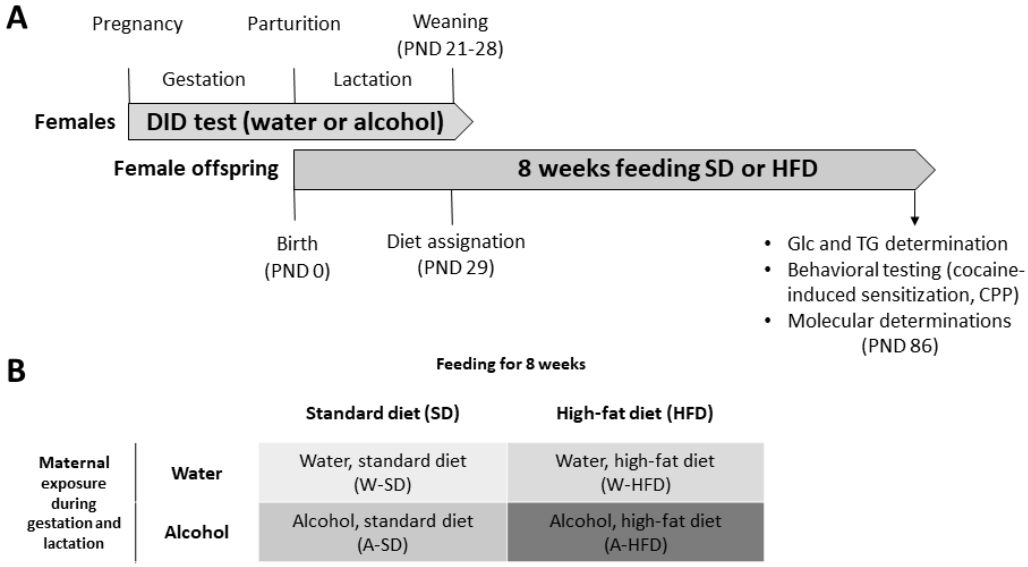

Figure 1. *(A) Experimental design. Pregnant C57BL/6 females were exposed to alcohol or water (control) during the gestation and lactation periods, following the drinking-in-the-dark (DID) procedure. After weaning (PND 21-28), female offspring were assigned a standard (SD) or a high-fat diet (HFD). After feeding for 8 weeks, their glucose (Glc) and triglyceride (TG) blood levels were determined and, thereafter, mice were tested either for cocaine-induced sensitization, cocaine-induced conditioned place preference (CPP) or for molecular determinations. (B) Experimental groups of the study: there are four experimental groups according to the exposure of their mothers to water (W) or alcohol (A) during the gestation and lactation periods, and the diet they were fed (SD or HFD). PND: postnatal day.*

Drinking-in-the-dark (DID) test

To reproduce an episodic pattern of excessive alcohol drinking (binge-like alcohol consumption), pregnant and nursing C57Bl/6 mice were exposed to alcohol following the DID paradigm (Rhodes et al., 2005), which has been proposed as a useful binge drinking model of FASD (Boehm et al., 2008). The procedure was conducted as previously reported (Cantacorps et al.,

2017; 2019), starting two days after mating. Pregnant females were randomly assigned to two groups: alcohol and water-exposed (control). Briefly, water bottles were replaced with 10-ml graduated cylinders fitted with sipper tubes containing either 20% (v/v) alcohol in tap water or only tap water 3 h after the lights were turned off. Following a 2 h-access period, individual intake was recorded, and the original water drinking bottles were returned to the home cage. During this period, female mice were individually housed, and each corresponding male breeding pair was removed from the home cage for the DID procedure. This procedure was repeated on days 2 and 3 and fresh fluids were provided each day (from Monday to Wednesday). On day 4 (Thursday), alcohol or water cylinders were left for 4 h and fluid intakes were recorded. Food was removed during the alcohol exposure periods and then, returned to the home cage. Two empty control cages (water and alcohol) were placed in the rack to measure general liquid loss (leakage/evaporation) and drip values were subtracted from the drinking values. Fluid intakes (g/kg body weight) were calculated based on average 2-day body weight values, as dams were weighed at 2-day intervals (Mondays and Wednesdays). The procedure was maintained throughout the 3-week gestation and the 3-week lactation periods as most mothers who drink during pregnancy continue doing so when breastfeeding and, therefore, they transfer alcohol to their offspring through their milk (Haastrup et al., 2014). Moreover, the maturational rate of mice does not linearly correlate with humans, it occurs 150 times faster during the first month of life and 45 times faster over the next five months (Flurkey et al., 2007). Considering this, the first 10 postnatal days in rodents are approximately equivalent to the third human gestational trimester (Alfonso-Loeches and Guerri, 2011; Patten et al., 2014). Accordingly, in our study, we completely covered the entire human-equivalent gestational period.

Feeding conditions

Two different types of diet were assigned to female offspring: a standard diet (SD, 831193 RM1; Special Diets Services, Essex, UK) and a high-fat diet (HFD, D05122301; Research Diets, Inc., USA). The SD has an energy density of 3.52 kcal/g (energy contribution from 75.1% carbohydrates, 17.49% protein and 7.42% fat). The HFD has an energy density of 4.73 kcal/g (35% carbohydrate energy, 20% protein energy and 45% fat energy). The fat source is composed of 91% hydrogenated coconut oil and 9% soybean oil.

On PND 29, female offspring were randomly divided in four experimental groups and assigned either a SD or a HFD (Figure 1B). Animals were fed with both diets from the weaning (3-4 weeks-old) up to sacrifice, being fed for at least 8 weeks before performing the behavioural tests and molecular determinations. Therefore, all experiments were carried out during the youth of the animals (Flurkey et al., 2007). The weight of the animals and the food intake were monitored weekly during the whole feeding period. The weight gain throughout the feeding period was calculated for each animal as the difference of weight between the last and the first week of diet exposure, expressed in grams.

Glucose and triglyceride determination

Fasting triglycerides and glucose levels were measured following 8 weeks of SD or HFD feeding, before the performance of the behavioural tests and molecular determinations. In brief, mice were fasted for 6 h and blood samples were extracted from the tail between 3 pm and 4 pm. The tests were performed in a quiet room using an appropriate measuring device (Accutrend Plus® system Cobas, Roche, Spain).

Behavioural Tests

Cocaine-induced locomotor sensitization

Female offspring mice were tested for the psychostimulant and sensitizing effects of cocaine as previously described by Duart-Castells et al., 2019b, with minor modifications (Figure 4A). Briefly, on day -2, all mice received a single dose of cocaine 8 mg/kg i.p. and were immediately placed into the open field arena where their horizontal locomotor activity (HLA) was recorded by a computerized monitoring software (Smart 3.0 Panlab, Barcelona, Spain) for 30 min. Three days later, animals were given cocaine (15 mg/kg i.p.), once daily for five consecutive days (days 1-5). After ten days of withdrawal (day 15), all mice were given a challenge of cocaine (8 mg/kg i.p.) and their HLA was registered again for 30 min, as performed on day -2. Even though HLA was only recorded on days -2 and 15, mice were placed in the open field arenas for 1 h after every injection during the treatment.

Cocaine-induced conditioned place preference (CPP)

Female offspring mice were tested for the rewarding properties of cocaine using an unbiased CPP paradigm, as previously described (Luján et al., 2019). The CPP procedure consisted on three different phases: preconditioning, conditioning and testing day. During preconditioning, mice could freely explore both compartments for 20 min. Mice showing strong unconditioned aversion (<35% of the session time) or preference (>65%) for either compartment were excluded from the study. The conditioning phase consisted of four pairings: mice received an i.p. injection of 15 mg/kg cocaine immediately prior to confinement to the drug-paired compartment for 30 min on days 2, 4, 6 and 8, while on alternate days (3, 5, 7 and 9)

mice received physiological saline before being confined to the vehicle-paired compartment for 30 min. Treatments were counterbalanced between compartments. Non-drug paired mice were administered saline prior to confinement to one of the two compartments every day. Finally, the testing session was conducted under the same conditions as in the preconditioning phase. The time spent in each compartment during the preconditioning and testing sessions, as well as the distance travelled, were recorded by computerized monitoring software (CIBERTEC APL software). A CPP score was calculated for each subject as the difference between the time spent in the drug-paired compartment during the testing and pre-conditioning sessions.

Molecular determinations

Tissue samples preparation

Mice were sacrificed by cervical dislocation to perform biomolecular determinations. The whole striatum, ventral striatum, prefrontal cortex or hypothalamus, when appropriate, were quickly dissected out and, except the whole striatum, they were stored at -80ºC until use.

For the [^{3}H]DA uptake experiments, mice synaptosome suspensions from fresh striatums were prepared as described by Pubill et al., 2005.

Total protein extracts were isolated from ventral striatum and prefrontal cortex and processed as described by Pubill et al., 2013, with minor modifications. Briefly, tissue samples were thawed and homogenized through sonication at 4ºC in 20 volumes of lysis buffer (20 mM Tris-HCl, 1% NP40, 137 mM NaCl and 2 mM EDTA, pH=8) containing a protease

and phosphatase inhibitor cocktail. Thereafter, the homogenates were shaken and rolled for 2 h at 4°C and subsequently centrifuged at 15,000 xg for 30 min at 4°C. Protein content of the supernatants was determined using the Bio-Rad Protein Reagent (Bio Rad, Inc. Spain).

[^{3}H]DA uptake in striatal synaptosomes

Reactions tubes were composed of 25 μl of the radioligand [^{3}H]DA (final concentration 5 nM), 100 μl of the synaptosome suspension and 125 μl of Hank's HEPES-buffered solution containing pargyline (20 mM) and ascorbic acid (1 mM). The incubation was performed for 5 min at 37 °C. Uptake reactions were terminated by rapid vacuum filtration through Whatmann GF/B glass fibre filters (Whatman Intl Ltd, Maidstone, UK) pre-soaked with 0.5% polyethyleneimine. Tubes and filters were washed three times with ice-cold 50 mM Tris-HCl. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Non-specific uptake value was determined in parallel samples containing cocaine (final concentration 300 μM) at 4 °C. The non-specific uptake value was subtracted from the data to yield specific uptake.

Western blot analysis

A general Western blotting protocol was used as described by Duart-Castells et al., 2019a, with minor modifications. Membranes were incubated overnight at 4°C with caspase-3 (1:1000, Transduction Laboratories, Lexington, USA), CB1 (1:1000, Frontiers Institute, South Africa), MBP (1:1000, Abcam, Cambridge, UK), NeuN (1:10000 Abcam, Cambridge, UK), NFκB (1:1000, Cell Signalling Technology, Inc) or TH (1:5000, Transduction Laboratories, Lexington, USA) primary antibodies. After

washing, membranes were incubated for 1 h at room temperature with their respective secondary peroxidase-conjugated anti-IgG antibody: donkey anti-rabbit, sheep anti-mouse or goat anti-rat (1:5000, GE Healthcare, USA). GAPDH (1:5000, Merck Millipore, USA) antibody was used as a control for loading.

Total RNA extraction and gene expression determination

RNA extraction and quantitative RT-PCR were performed using standard procedures. Briefly, hypothalamus was excised from five mice from each group. Total RNA was extracted from hypothalamus using TRIzol™ Reagent (Invitrogen, ref. 15596018), following the manufacturer's indications. cDNA was obtained using Transcriptor First Strand cDNA Synthesis Kit (Roche, ref. 04379012001), using 1 μg total RNA. The protocol was performed using Anchored Oligo (dT)18 Primers to obtain cDNA from all the mRNA. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Power SYBR Green PCR Master Mix adapted for LightCycler 480 (Applied Biosystems, ref.4367659), according to the manufacturer's indications in the LightCycler 480 Instrument II (Roche, ref. 05015243001). The sequences of the primers used for each gene were 5'- TGCAGACCGAGCAGAAGAAG (primer forward) and 5'- GACTCGTGCAGCCTTACACA (primer reverse) for *AgRP;* 5'- TATCTCTGCTCGTGTGTTTG (primer forward) and 5'-GTTCTGGGGGCGTTTTCTG (primer reverse) for *Npy;* and 5'- TATGCAGTCGCCCTTCCT (primer forward) and 5'-ACATCAATCAGGTGTGTCTGCT (primer reverse) for *Cpt1c.*

Data acquisition and statistical analysis

Data are expressed as mean ± SEM. Data from biochemical analysis (Western blot and [^{3}H]DA uptake experiments) were normalized with 100%

defined as the mean of the technical replicates in the control group (W-SD). In qPCR analysis, data were expressed as fold-change variations relative to W-SD.

Differences between groups were compared using two-way ANOVA with diet (SD or HFD) and maternal exposure (water or alcohol) as factors of variation. To analyze the food intake, cocaine-induced sensitization and cocaine-induced CPP, a three-way ANOVA was performed, with diet (SD or HFD), mother's exposure (water or alcohol) and time (day or week), as factors of variations. The α error probability was set at 0.05. Significant differences ($P < 0.05$) were analyzed using the Bonferroni post-hoc test for multiple comparison measures only if F achieved the necessary level of statistical significance ($P < 0.05$) and no significant variance in homogeneity was observed. Statistic calculations were performed using GraphPad Prism 8.0 software.

RESULTS

Maternal alcohol consumption

Two-way ANOVA with repeated measures analysis of water and alcohol volumes of consumption during DID testing showed a significant effect of day [$F_{23,1173} = 27.693$; $P < 0.001$] and alcohol [$F_{1,51} = 66.795$; $P < 0.001$], with interaction between factors [$F_{23,1173} = 10.482$; $P < 0.001$] (n=26-27/group) (Figure 2A). Bonferroni post-hoc comparisons revealed a significant increase in water consumption on day 4 compared with day 1 ($P < 0.001$), day 2 ($P < 0.001$) and day 3 ($P < 0.001$), on day 8 compared with day 5 ($P < 0.05$), day 6 ($P < 0.05$) and day 7 ($P < 0.01$), on day 16 compared with day

14 ($P < 0.05$) and on day 24 compared with day 21 ($P < 0.05$), day 22 ($P < 0.01$) and day 23 ($P < 0.05$). Furthermore, a significant increase in alcohol consumption on day 4 compared with day 1 ($P < 0.01$) and day 2 ($P < 0.05$), on day 8 compared with day 5 ($P < 0.05$) and on day 24 compared with day 22 ($P < 0.01$) was found. Additionally, one-way ANOVA with repeated measures analysis of alcohol intake showed a significant effect of day [$F_{23,575} = 7.389$; $P < 0.001$] (Figure 2B). Bonferroni post-hoc comparisons revealed a significant increase in alcohol intake on day 4 compared with day 1 ($P < 0.01$) and day 2 ($P < 0.05$), on day 8 compared with day 5 ($P < 0.05$) and on day 24 compared with day 22 ($P < 0.01$).

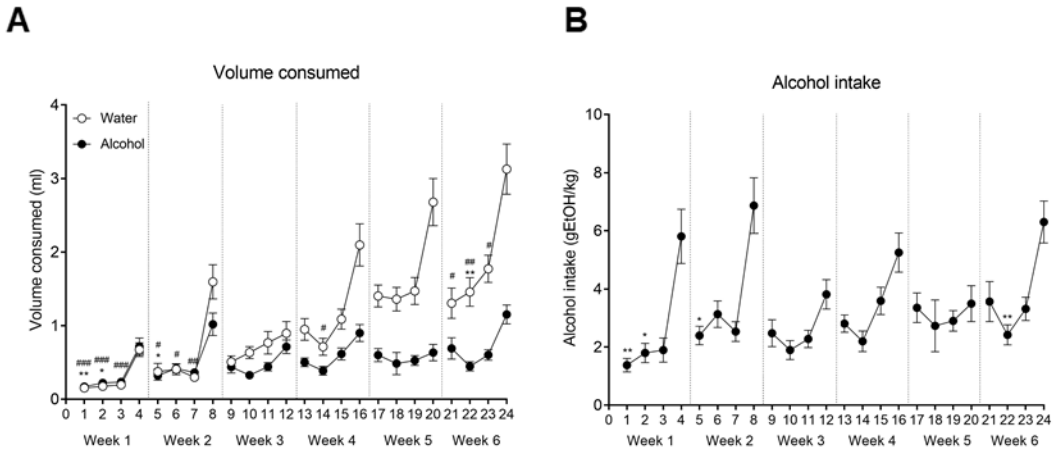

Figure 2. *Maternal alcohol drinking. (A) Volume of water or alcohol consumed and (B) Alcohol intake (g ethanol (EtOH)/kg), throughout the gestation (week 1-3) and lactation (week 4-6) periods during the DID test. Alcohol was available for two hours per day on days 1-3 and four hours on day 4 of the series. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01 day 4 of each week compared with the 3 previous days in the alcohol-exposed group. #P < 0.05, ##P < 0.01 and ###P < 0.001 day 4 of each week compared with the 3 previous days in the water-exposed group.*

Body weight and food intake

Initially, the body weight after weaning was not affected by alcohol exposure [$F_{3,85}$=1.241, n.s.]. From that moment on, all groups of animals gained weight over time across the 8-week exposure period. Two-way ANOVA of the weight gain revealed a significant effect of the alcohol factor [$F_{1,100}$=14.96; $P < 0.001$] but not of the diet [$F_{1,100}$=0.451; $P > 0.05$] (n=25-27/group). Accordingly, no significant differences in weight gain between the groups fed with HFD *vs.* those fed with SD were observed by the end of exposure. Bonferroni post-hoc multiple comparisons revealed that the weight gain of the A-HFD group was significantly lower than that of W-HFD. Conversely, no differences were observed in weight gain between animals fed with SD, whether exposed to water or alcohol (Figure 3A).

Weekly food intake, calculated either as an average of grams/day or Kcal/day, was also measured. Three-way ANOVA *(alcohol x diet x week)* with repeated measures of grams/day ingested yielded a significant effect of diet [$F_{1,100}$=305.5; $P < 0.001$] and time [$F_{7,651}$=68.85; $P < 0.001$], with interaction between *alcohol x diet* [$F_{1,100}$=6.034; $P < 0.05$] and *time x diet* [$F_{7,651}$=62.20; $P < 0.01$] (Figure 3B). Subsequent Bonferroni post-hoc tests revealed significant differences in food intake between SD and HFD groups in mice exposed to alcohol ($P < 0.001$). Furthermore, significant differences between SD and HFD levels of food intake were found on weeks 1, 2, 3, 6, 7 and 8 ($P < 0.001$; in all cases) (n=25-27/group).

Regarding the weekly food intake in Kcal/day, three-way ANOVA analysis *(alcohol x diet x week)* with repeated measures showed a significant effect of *time* [$F_{7,651}$=74.44; $P < 0.001$] and *diet* [$F_{1,100}$=27.86; $P < 0.001$], with significant interactions between *time x diet* [$F_{7,651}$=68.85; $P < 0.001$] and *alcohol x diet*

[$F_{1,100}$=6.36; P < 0.05] (Figure 3C). Bonferroni post-hoc comparisons indicated that, from the 2nd week of diet consumption until the 6th, the HFD-groups (water and alcohol) significantly ingested more Kcal/day than SD-fed mice (P < 0.001; in all cases). Nevertheless, from week 6 until the end, these groups reduced their Kcal intake until being equated to that of the SD-fed groups.

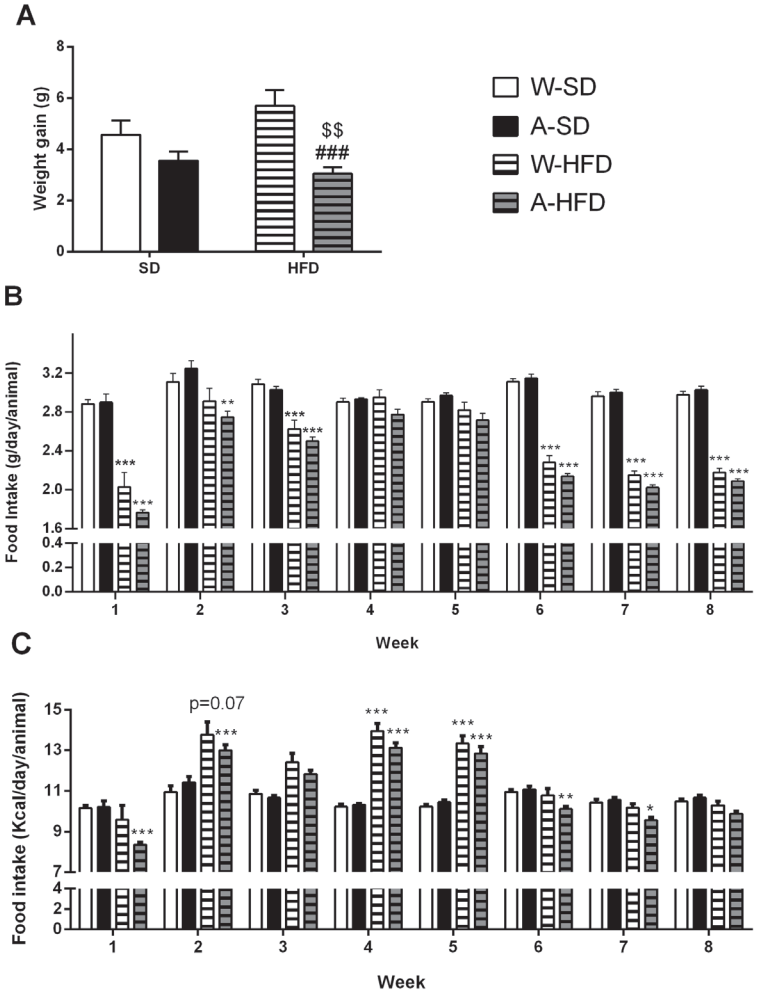

Figure 3. *(A) Body weight gain after 8 weeks of feeding. It was calculated for each animal as the difference of weight between the last and the first week of diet exposure, expressed in grams.*

(B) *Mean daily food intake measured in grams and (C) Kcal, of mice exposed to water (W) or alcohol (A) during the gestation and lactation periods and exposed to a standard diet (SD) or a high fat diet (HFD) for 8 weeks. Results are expressed as mean ± SEM. ###P < 0.001 vs W-HFD; $$P < 0.01 vs W-SD; *P < 0.05, ** P < 0.01, ***P < 0.001 vs the corresponding group (water or alcohol) fed with SD.*

In accordance with the significant interaction found (*alcohol x diet*), maternal exposure to alcohol did not modify the food and Kcal intake when female offspring were fed with SD but it did when fed with HFD.

Glucose and Triglyceride blood levels

Two-way ANOVA analysis of triglycerides blood levels showed a significant effect of the diet [$F_{1,19}$=11.275; P < 0.01] (n=25-27/group). Thus, HFD exposure significantly increased triglycerides blood levels in comparison to SD (P < 0.05 for both groups, alcohol and water). By contrast, neither the diet nor the maternal alcohol exposure altered the glucose blood levels by the end of exposure.

Cocaine-induced locomotor sensitization

Three-way ANOVA analysis (*alcohol x diet x day)* with repeated measures showed a significant effect of the *diet* [$F_{1,34}$=4.71; P < 0.05] and *time* [$F_{1,34}$=94.15; P < 0.001] (n=9-10/group). As shown in Figure 4B, a first administration of cocaine (8 mg/kg i.p., day -2) elicited a similar hyperlocomotion in all groups of animals, but an increased response in the W-HFD group compared to the W-SD (P < 0.05). Furthermore, all groups of animals developed behavioural sensitization to cocaine as the locomotor response induced by the drug (8 mg/kg i.p.) on day 15 (challenge day, 10 days

after treatment) was in all cases considerably higher than on day -2, before starting the treatment ($P < 0.001$; in all cases).

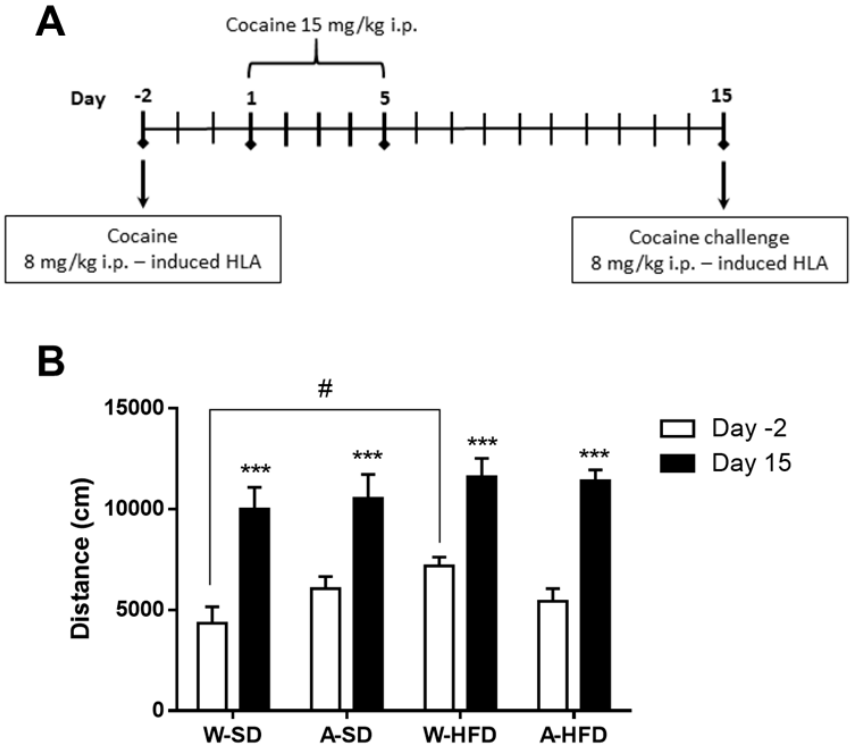

Figure 4. *(A) Drug administration protocol. Animals first received a single administration of cocaine 8 mg/kg i.p. (day -2). After three days, they were given a 5-days treatment of cocaine 15 mg/kg i.p. (days 1-5). Ten days later (day 15), all mice were challenged with cocaine 8 mg/kg i.p. The cocaine-induced horizontal locomotor activity (HLA) was registered on days -2 and 15, immediately after the drug injection. (B) Cocaine locomotor sensitization. Effect of cocaine (8 mg/kg i.p.) on the HLA: the white bars represent the HLA of mice after the first cocaine injection given on day -2. The black bars represent the distance travelled on day 15. Results are expressed as mean ± SEM of the distance travelled (cm) in 30 min. ***P < 0.001 vs day -2; # P < 0.05 significant differences between W-SD and W-HFD on day -2.*

Basal locomotor activity and cocaine-induced CPP

Another batch of female offspring were subjected to the CPP paradigm after the 8-week feeding period. Additionally, the locomotor activity of each group of animals was measured during the preconditioning test to determine any possible effect of these variables on basal locomotion.

Firstly, two-way ANOVA of the results did not show any significant effect of the diet nor alcohol exposure on the distance travelled by the animals.

Regarding the CPP experiments, three-way ANOVA analysis (*alcohol x diet x day)* revealed a significant effect of cocaine treatment [$F_{1,78}$=93.314; $P < 0.001$] (n=10-12/group), indicating that the repeated administration of cocaine (15 mg/kg, i.p.) produced a preference for the cocaine-paired compartment in all groups. However, no effect of alcohol [$F_{1,78}$=0.913; $P > 0.05$] or the diet [$F_{1,78}$=0.496; $P > 0.05$] were found. Interactions between factors were not statistically significant either.

Molecular determinations

Another batch of animals was sacrificed after the period of exposure to the diet for molecular determination purposes. In order to explain the differences observed in the food intake in the A-HFD group, the expression of genes involved in the ghrelin signalling pathway was determined in the hypothalamus. In parallel, as an attempt to explain the results observed from the cocaine-induced sensitization experiments, [^{3}H]DA uptake in striatal synaptosomes and the expression of tyrosine hydroxylase (TH) and cannabinoid 1 receptor (CB1) in VS were evaluated. Finally, the expression of proteins involved in neuronal and myelin damage were determined in the

PFC in view of previous studies using male offpsring from mice subjected to the DID procedure (Cantacorps et al., 2019): nuclear nuclei (NeuN), nuclear factor (NFκB). caspase-3 and myelin basic protein (MBP).

Effects on genes involved in the ghrelin signalling pathway

We analyzed by qPCR the mRNA levels of ghrelin-related genes that promote food intake: neuropeptide Y (NPY) and Agouti-related protein (AgRP), and no significative changes were observed. We also analyzed the mRNA levels CPT1C as a mediator of the ghrelin signalling but no significative changes were observed either. However, in the latter case, it should be mentioned that the variable alcohol almost reached statistical significance ($P = 0.055$), in such a way that both alcohol-exposed groups (SD and HFD) showed a decreased expression of this gene.

Effects on [^{3}H]DA uptake

Striatal synaptosome suspensions were prepared to perform [^{3}H]DA uptake experiments. Two-way ANOVA yielded a significant effect of diet [$F_{1,29}=16.28$; $P < 0.001$] (n=7-9/group). Accordingly, mice exposed to HFD presented lower DA uptake activity than those fed with SD ($P < 0.01$ and $P < 0.05$ *vs.* the water and alcohol-matching groups, respectively) (Figure 5).

Effects on TH and CB1 receptor expression in ventral striatum

As shown in Figure 6A, the expression of TH was not affected neither by alcohol nor by the diet. By contrast, two-way ANOVA of the results of CB1 expression yielded a significant effect of the diet [$F_{1,16}=6.576$; $P < 0.05$] and alcohol [$F_{1,16}=21.14$; $P < 0.001$] with interaction between both factors

[$F_{1,16}$=10.27; P < 0.01] (Figure 6B). Bonferroni post-hoc multiple comparisons showed a significant increase in CB1 receptor expression in W-HFD mice compared to W-SD (P < 0.01). However, such overexpression was not present in animals fed with HFD and exposed to alcohol (n=5/group).

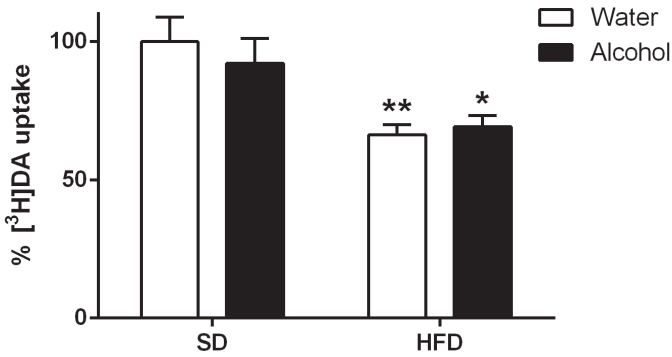

Figure 5. *[³H]DA uptake in striatal synaptosomes of mice exposed to water (W) or alcohol (A) during the gestation and lactation periods, after 8 weeks of feeding with a standard diet (SD) or a high fat diet (HFD). Data are expressed as a percentage of control uptake relative to W-SD (mean ± SEM). *P < 0.05 and **P < 0.01 vs the corresponding SD-group (water or alcohol)*

Neuronal and myelin damage within the prefrontal cortex

In our study, using female offspring, no differences were observed in NeuN or NFκB/p65 expression (Figure 6C and 6D). Regarding caspase-3, a protein that plays a key role in cell apoptosis, two-way ANOVA showed a significant interaction *diet x alcohol* [$F_{1,16}$=8.8; P < 0.01] (Figure 6E). Thus, alcohol exposure seemed to differentially affect the expression of caspase-3 depending on the ingested diet. Finally, two-way ANOVA of the results of MBP expression revealed a significant effect of alcohol [$F_{1,15}$=5.038; P < 0.05] (Figure 6F). Therefore, alcohol exposure significantly decreased MBP protein levels, mainly in animals fed with SD (n=4-5/group).

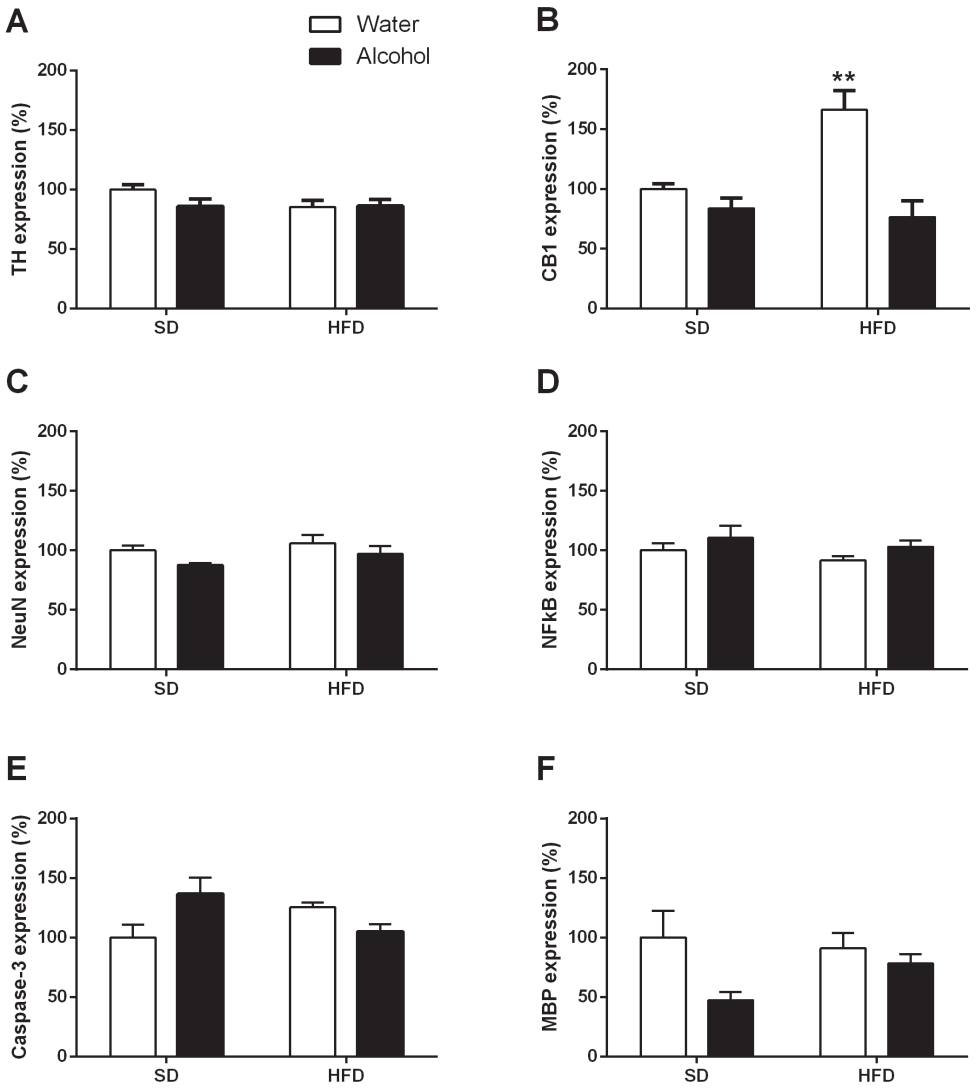

Figure 6. *Effects on (A) TH and (B) CB1 protein expression in ventral striatum and (C) NeuN, (D) NFκB, (E) caspase-3 and (F) MBP protein in the prefrontal cortex induced by PLAE and the subsequent exposure to a standard diet (SD) or a high fat diet (HFD) for 8 weeks. Results are expressed as mean ± SEM. **P < 0.01 vs W-SD.*

DISCUSSION

The present study aimed to assess the interaction between alcohol exposure during gestation and lactation and a high fat diet during the female offspring'adolescence on some nutrition-related parameters as well as on the response to cocaine.

In previous studies using an animal model of maternal binge-like alcohol drinking we found out that the increase in maternal body weight during the prenatal and lactational DID test was not affected by alcohol consumption (Cantacorps et al., 2017). Similarly, in our study, there was no contribution of PLAE to offspring's body weight either. In agreement with the results garnered from other studies (Hicks et al., 2016, Sims et al., 2013, Hwang et al., 2010), we did not observe any significant weight gain in mice fed with a HFD with respect to the SD group. When food intake was measured across the feeding period, we found that the HFD induced a metabolic reprogramming in such a way that, from week 6 until the end of the exposure, HFD-fed mice reduced the grams of ingested food, thus equaling their Kcal/day intake to those of the SD-group. Importantly, the equal intake of Kcal is in line with the non-significant differences observed in body weight between W-HFD and W-SD at the end of the experiment. In addition, previous studies have described that male C57Bl/6 mice on HFD are more susceptible to weight gain than females (Gelineau et al., 2017; Yang et al., 2014). Particularly, Hwang et al., (2010) reported that the HFD-induced weight gains appeared earlier in C57Bl/6 males (46-days old) than in females (129-days old) and were much greater in males. Overall, we can reasonably deduce that, in our model, female offspring were probably in a pre-obesity state and thus, they needed a longer exposure period to the HFD (more than 16-20 weeks) (Wang and Liao, 2013) to develop an obesity status.

Additionally, PLAE affected how C57Bl/6 responded to the HFD in such a way that, during almost the whole feeding-period, A-HFD mice ate significantly less grams of food, and thus, less Kcal, than the W-HFD, consistent with their lower weight gain. Indeed, the same results have been observed by Amos-Kroohs et al. (2018). Importantly, such lower weight gain observed in the A-HFD cannot be attributed to an increased basal locomotor activity of these animals since no differences were observed in their locomotion. However, regarding the molecular determinations and, more concretely, the effects of PLAE and a HFD on ghrelin-related genes, although no changes were observed in NPY or AgRP mRNA expression, an apparent decrease of the CPTC1 expression in A-HFD mice should be noted. Hypothalamic CPT1C mediates the central effects of leptin and ghrelin on feeding behaviour (Ramírez et al., 2013, Gao et al., 2011). More specifically, besides other mechanisms, ghrelin induces food intake through regulation of hypothalamic CPT1C, so the orexigenic action of ghrelin is totally blunted in CPT1C knockout mice despite having the canonical ghrelin signalling pathway activated (Ramírez et al., 2013). Accordingly, aside from the metabolic reprogramming induced by HFD, which caused an overall decrease in food intake, PLAE seemed to additionally induce a decrease in CPTC1 expression, a feature that might be involved in the lower weight gain observed in such group of animals, A-HFD.

TG and glucose blood levels were also assessed. The HFD significantly increased TG blood levels, but not those of glucose. Nonetheless, this lack of effect of HFD on carbohydrate metabolism should not be surprising as it has already been described that female C57Bl/6 mice are particularly resistant to the physiological changes caused by HFD as they exhibit a better glucose tolerance than males (Gelineau et al., 2017). Importantly, no additional effects of PLAE were evidenced on TG and glucose blood levels.

FASD is associated with a higher risk of later developing drug abuse. Our results showed that PLAE and/or a HFD did not modify CPP acquisition, thus the rewarding effects of cocaine were not altered. Consistent with our findings, Blanco-Gandía et al. (2017) suggested that consumption of a HFD during adolescence by male OF1 mice induces neurobiochemical changes that increased sensitivity to cocaine but only when fat is withdrawn, acting as an alternative reward. Nevertheless, in previous studies using the same DID procedure (Cantacorps et al., 2019), we found out that PLAE enhanced the preference for the cocaine-paired compartment in the CPP test. In this sense, the present study only differs from the previous one in that we tested females instead of males. Accordingly, we can reasonably attribute such discrepancy to sex-dependent differences; thereby PLAE enhances the rewarding properties of cocaine in males but not in females. No effects of the HFD were observed either.

The psychostimulant and sensitizing effects of cocaine were also tested using the horizontal locomotor activity test. Locomotion induced by an acute dose of cocaine was assayed as an indicative of its psychostimulant effect. In agreement with Collins et al., (2015), in our study the W-HFD group showed an enhanced locomotor response to the first administration of cocaine, as compared to W-SD. Data in rats indicate that the consumption of fat and, perhaps, the resulting hormonal changes, markedly alter dopamine systems (Baladi et al., 2010). In the same line, we observed an increased sensitivity to the psychostimulant effect of cocaine in animals fed with HFD, which at the same time, did not present any overweight. This is on chief importance since it provides evidence that consuming a HFD during early development enhances the psychostimulant effect of cocaine, and thus, might favor and increase the probability of repeating and perpetuating the use of the substance, ultimately leading to its abuse. Accordingly, we

can suggest that a HFD might increase vulnerability to cocaine abuse in water-exposed animals, so PLAE seemed to prevent such enhanced locomotor response to the drug. Locomotor sensitization (behavioural sensitization) to psychostimulants is the process whereby repeated exposure to drugs results in a progressive and enduring increase in the motor stimulant response to the drug (Kalivas et al., 1993). In this case, the hyperlocomotion induced by the cocaine challenge 10 days after repeated exposure the drug was the same for all groups. One possibility, which remains highly speculative, is that a longer feeding-period, and thus a more severe metabolic reprogramming might be needed to provoke long-lasting changes in cocaine effects. Even so, considering that the W-HFD group presented an enhanced response to the first administration of cocaine, we cannot rule out the possibility that the rate at which each experimental group developed locomotor sensitization was not the same, and thus, could be influenced by PLAE or, most probably, by a HFD. Therefore, further studies are needed to completely clarify this issue.

When assessing [^{3}H]DA uptake in striatal synaptosomes we found out that the HFD reduced the overall [^{3}H]DA uptake in both, A-HFD and W-HFD groups. However, no effect of PLAE was found. Our data provide further evidence for diet-induced alterations in DA uptake and thus, implicate diet in the regulation of DA function, which can lead to functional modifications in DA signalling (Cone et al., 2013; Morris et al., 2011; Speed et al., 2011; South and Huang, 2008a). By decreasing DA uptake, HFD consumption prolongs the duration of phasic DA release and could promote adaptations such as downregulation of DA receptors, a feature of both human and rodent models of obesity (Johnson and Kenny, 2010; Wang et al., 2009). Cone et al. (2013) described that prolonged HFD appears to reduce the rate of DA uptake via DAT likely by interfering with DAT trafficking

or perhaps maturation, but not by decreasing DAT gene expression or DAT mRNA stability. Therefore, diet-related decreases in membrane DAT could precede and contribute to the onset of DA receptors downregulation, obesity and compulsive eating behaviour that develop over the course of HFD consumption (Johnson and Kenny, 2010).

Cocaine mediates an increase of the motor activity by increasing DA signalling via the mesostriatal pathway through both DAT- dependent and independent mechanisms (Gardner and Ashby, 2000). In this sense, considering the results from the DA uptake and cocaine-induced locomotion experiments, it seems reasonable that the W-HFD group, which presented a lower DA uptake, elicited an enhanced psychostimulant response, so the few available functional transporters might have been blocked by cocaine, leading to high levels of the neurotransmitter at the synapse and thus, to a major psychostimulant effect. Nevertheless, A-HFD also showed reduced DA uptake but presented a similar locomotor response to that of the control group, W-SD, after the first administration of cocaine. In this regard, we can suggest that most probably alcohol might increase DA transmission by means of modifying DAT-independent mechanisms, that is mobilizing reserve DA vesicles, attenuating GABA transmission onto dopaminergic neurons within the VTA or oscillating the firing rate of DA cell bodies (Venton et al., 2006; Steffensen et al., 2008; Shi et al., 2004).

In addition to DA uptake, we assessed other two proteins related to dopaminergic neurotransmission in the ventral striatum: TH and CB1 receptor. In our model, the expression of the rate-limiting enzyme in DA synthesis, TH, was not affected neither by alcohol nor by the diet. By contrast, feeding animals with a HFD led to an increase in CB1 receptors but only in water-exposed animals, so PLAE prevented such overexpression.

This increment in CB1 receptors has been previously described as an early "defensive" stage during the first weeks of HFD consumption, which results in a long-lasting "failure" stage when obesity appears (South and Huang et al., 2008b). The CB1 receptor system plays an important role in regulating the positive reinforcing properties of alcohol, therefore, such increment observed in CB1 receptor density might be involved in the increased vulnerability to alcohol abuse (Hungund, 2003).

Recent studies have demonstrated that alcohol intake activates the innate immune system in the central nervous system, leading to neuroinflammation and contributing to brain damage and behavioural dysfunctions (Montesinos et al., 2016). In this context, we have already reported that PLAE increases pro-inflammatory markers, alters the expression of myelin proteins, and induces glial activation and neuron cell damage in the PFC of male C57Bl/6 mice (PND 70) (Cantacorps et al., 2017). In the present study, effects of PLAE were evident in caspase-3 and MBP protein expression, in such a way that alcohol exposure during gestation and lactation periods induced an increase in caspase-3 but a decrease in MBP. Regarding NeuN expression, although a significant decrease was found in A-SD mice compared to W-SD (t_7=2.557, $P < 0.05$), such difference was blunted until being non-significant when considering also the variable diet in the analysis. Therefore, we could confirm that the effects of PLAE on NeuN, caspase-3 and MBP expression were the same in both sexes albeit female offspring seemed more resistant to such effects than males. Furthermore, all the alterations induced by PLAE were blunted in HFD groups, thereby a short exposure to a fatty diet seems to counteract the deleterious effects of prenatal and lactational alcohol exposure.

In summary, the current study evaluated the impact of alcohol exposure during gestation and lactation periods and consuming a HFD

during adolescence on the effects of cocaine as well as on nutrition-related parameters. In our model, feeding young female C57Bl/6 mice with a HFD for 8 weeks increased their TG blood levels but did not induce an overweight compared to those fed with SD. Moreover, PLAE affected how females responded to the fatty diet as they consumed less amount of food than water-exposed offspring, consistent with their lower gain weight. HFD reduced DA uptake and, only in non-alcohol-exposed animals, increased the psychostimulant effect of cocaine. Surprisingly, PLAE attenuated body weight gain and prevented the enhanced psychostimulant response to cocaine and the overexpression of CB1 receptors induced by a HFD. By contrast, the biomarkers altered in the PFC by PLAE, such as NeuN, caspase-3 or MBP, were mitigated by a HFD-based feeding. Therefore, in female offspring, several effects triggered by one of these factors, PLAE or a HFD, were blunted by the other, noting some interactions between them.

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