UNIVERSITAT DE BARCELONA

Contribution to the study of the mechanisms of action and neuropsychopharmacological effects of MDMA and new β-ketoamphetamines

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ANDRÉS CIUDAD ROBERTS

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ABSTRACT

Drugs of abuse are a matter of great concern, as their use is widespread in all stratums of society, and can entail both acute and long-term negative consequences. For this reason, our research group is devoted to investigating the mechanisms underlying the action of amphetamine derivatives, as this is a family of drugs that is widely used, especially among adolescents and young adults.

The present doctoral thesis is divided into two blocks, each of which focuses on a separate research line, based on different antecedents and with different working hypotheses.

Block 1: MDMA and its interaction with nicotinic receptors

Previously, our group had described that methylenedioxymethamphetamine (MDMA) is a ligand for two of the main nicotinic acetylcholine receptor (nAChRs) subtypes, namely α4β2 and α7. Furthermore, after exposure to MDMA, receptor density has been found to be increased in PC-12 cells. Given that α4β2 and α7 nAChRs play an important role in reward, movement and memory processes, these findings warranted further research on the *in-vivo* implications they could entail.

For this reason, we sought to study whether α4β2 nAChRs were implicated in the sensitizing and conditioning effects of MDMA. Furthermore, we determined whether α4β2 nAChR up-regulation takes place in-vivo. Through autoradiography studies, we mapped the specific brain areas in which up-regulation takes place, and postulated an underlying mechanism for this process. Finally, we determined the involvement of α4β2 nAChR up-regulation in MDMA-induced sensitization and conditioning.

In summary, α4β2 nAChRs were found to be involved in the sensitizing and conditioning properties of MDMA. Furthermore, α4β2 nAChR up-regulation was confirmed in-vivo, a phenomenon that was found to have positive effects on sensitization and conditioning to MDMA.

Block 2: β-Ketoamphetamines and their interaction with ethanol and other psychostimulants

Recently, a new family of amphetamine derivatives, named synthetic cathinones, has broken into the illegal market, mephedrone and methylenedioxypyrovalerone (MDPV) being the most popular. Our research group has contributed to the characterization of the molecular mechanism of these compounds, as well as their pharmacokinetics and neurotoxic potential.

These compounds are commonly used concomitantly with ethanol, which is known to enhance the effects elicited by other psychostimulants, such as MDMA and cocaine. For this reason, we sought to explore, in depth, the consequences of the simultaneous administration of each of these two cathinone derivatives (i.e. mephedrone and MDPV) in combination with ethanol, focusing on the effects on locomotor activity, drug conditioning, neuroplasticity, neurotoxicity, and pharmacokinetics.

Furthermore, given that MDPV shares mechanism of action with cocaine, we performed preliminary assays investigating the potential interrelation between these two psychostimulants.

In summary, ethanol was found to enhance the psychostimulant and conditioning effects of mephedrone. In this sense, a unique role was found for D3 receptors and BDNF in the mediation of conditioning to mephedrone. Furthermore, the combination with ethanol was also shown to increase the sings of neuronal damage associated to the administration of mephedrone.

An opposite effect was revealed for MDPV: ethanol co-administration caused a reduction in locomotor activity and drug conditioning. Finally, MDPV was found to cause sensitization by itself and cross-sensitization with cocaine.

“How long will this last, this delicious feeling of being alive, of having penetrated the veil which hides beauty and the wonders of celestial vistas? It doesn't matter, as there can be nothing but gratitude for even a glimpse of what exists for those who can become open to it.”

Alexander Shulguin

To everyone in my life struggling with addiction,

for whom I have nothing but love.

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ABBREVIATIONS

5-HT:	Serotonin
ANA-12:	N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide
BBB:	Blood-brain barrier
BDNF:	Brain-derived neurotrophic factor
BrdU:	5-bromo-2'-deoxyuridine
cAMP:	Cyclic adenosine monophosphate
cDNA:	Cyclic deoxyribonucleic acid
COMT:	Catechol-O-methyltransferase
CPP:	Conditioned place preference
CPu:	Caudate-putamen
CREB:	cAMP response element-binding protein
CYP:	Cytochrome P-450
D3R:	D3 dopamine receptor
DA:	Dopamine
DAT:	Dopamine transporter
DHβE:	Dihydro-β-erythroidine
DNA:	Deoxyribonucleic acid
EtOH:	Ethanol
GABA:	γ-aminobutyric acid
Gpx:	Glutathione peroxidase
Hal:	Haloperidol
HHA:	3,4-dihydroxyamphetamine
HHMA:	3, 4-dihydroxymethamphetamine
HMA:	4-hydroxy-3-methoxyamphetamine
HMMA:	4-hydroxy-3-methoxymethamphetamine
HPβCD:	2-hydroxypropyl-β-cyclodextrin
Ket:	Ketanserin
LTD:	Long-term depression
LTP:	Long-term potentiation
MDA:	Malondialdehyde
MDMA:	3,4-N-methylenedioxymethamphetamine
MDPV:	Methylenedioxypyrovalerone
Mephedrone:	4-methylmethcathinone
Meph:	4-methylmethcathinone
METH:	Methamphetamine
MeO:	Methoxy
Methylone:	3,4-methylenedioxymethylcathinone
MLA:	Methyllycaconitine
mPFCx:	Medial prefrontal cortex
MWM:	Morris water maze

NAc:	Nucleus accumbens
nAChR:	Nicotinic acetylcholine receptor
NET:	Norepinephrine transporter
NMDA:	N-methyl-D-aspartate
PCA:	Principal component analysis
p-CREB:	Phospho-CREB
PK:	Protein kinase
PT:	Post-treatment
PV:	Pirovalerone
qPCR:	Quantitative polymerase chain reaction
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
SB-277011A:	N-{trans-4-[2-(6-cyano-3,4-dihydroisoquinolin-2(1H)-yl)ethyl]cyclohexyl}quinoline-4-carboxamide
SERT:	Serotonin transporter
SN:	Substantia nigra
Str:	Striatum
TH:	Tyrosine hydroxylase
TpH:	Tryptophan hydroxylase
TrkB:	Tropomyosin receptor kinase B
VMAT:	Vesicular monoamine transporter
VTA:	Ventral tegmental areaINDEX

DISCLOSURE

The present doctoral thesis is an original manuscript. The “methods and results” chapter is structured in 6 separate scientific articles. Part of this content (articles 1, 2 and 3) has already been published in scientific journals, whereas articles 4 and 6 are currently in press. However, article 5 is a report on results of a project that still is object of ongoing research. Therefore, although it has been included and structured as a full article, it may be significantly modified prior to being sent for peer review. Furthermore, entire fragments of this article have been included in the summary and discussion section of the doctoral thesis, as, on the date of its final edition, they constitute the most updated version and interpretation of a work that is susceptible to frequent additions and modifications as new results are obtained.

INTRODUCTION

The consumption patterns for drugs of abuse are rapidly changing; in this sense, in the past decades, there has been a significant increase in the use of designer drugs, which are generally associated to recreational and social settings. They are commonly taken sporadically, especially by adolescents and young adults, as part of their natural propensity to experiment and search for new experiences.

Amphetamines are family of compounds with potent psychostimulant properties; they act by increasing dopaminergic and/or serotonergic activity and, among them, methylenedioxymethamphetamine (MDMA), amphetamine and methamphetamine are used most widely.

Nicotinic acetylcholine receptors (nAChRs) are the main target for the neurotransmitter acetylcholine, as well as for other well-known ligands, such as the psychostimulant nicotine. In this sense, the α4β2 subtype plays an important role in the mediation of reinforcement and movement (Tapper et al., 2004). Previously, our research group demonstrated, in vitro, that MDMA is a ligand for this receptor type; furthermore, we evidenced significant α4β2 nAChR up-regulation after exposure to MDMA, which yielded an increase in receptor functionality (Garcia-Ratés et al., 2007, 2010).

This finding suggested that the interaction between α4β2 nAChRs and MDMA could play an important role in the mediation of the psychostimulant and rewarding properties of MDMA, through functional up-regulation of α4β2 nAChRs.

For this reason, it became of great interest to explore whether our *in vitro* findings could be replicated *in vivo*; furthermore, we sought to elucidate the potential implications of this phenomenon on the behavioral effects of MDMA (i.e. psychostimulant, sensitizing and conditioning properties).

During the turn of the decade, due to the scarcity and low purity of classical psychostimulants, a new family of amphetamine derivatives broke into the market under the name of synthetic cathinones. They did so taking advantage of a legal loophole whereby, by performing slight chemical modifications on popular banned amphetamines, new compounds with similar psychoactive properties were obtained, with no explicit legislation regarding their sale, purchase or use. Consequently, these new drugs of abuse became easy to obtain, especially through the Internet, head shops and in gas stations, and were commonly sold as bath salts or plant food, under a disclaimer stating that they were “not for human consumption” (Winstock et al., 2011). In this sense, the most widely used synthetic cathinones are 4-methylmethcathinone (mephedrone), and methylenedioxypyrovalerone (MDPV).

Prior to this doctoral thesis, our research group had been devoted to the characterization of the pharmacodynamic, pharmacokinetic and neurotoxic properties of synthetic cathinones, as well as their behavioral effects. In this regard, it is now known that mephedrone is a dopamine and serotonin releaser, while MDPV is a dopamine transporter blocker, making them both potent psychostimulants with strong rewarding properties (Aarde et al., 2013; Karlsson et al., 2014; López-Arnau et al., 2012).

Polysubstance abuse is an extremely prevalent phenomenon which has been described for virtually all popular drugs of abuse, including, but not restricted to, ethanol, cannabis, tobacco, MDMA, cocaine and opiates (Midanik et al., 2007; Pape et al, 2009; Piasecki et al., 2011). Ethanol is the most ubiquitous drug of abuse, it is used by people of all ages and stratums of society. In this sense, synthetic cathinones are commonly used concomitantly with ethanol (Winstock et al., 2011; Elliott and Evans, 2014), a combination which is generally perceived as innocuous (O'Neill and McElrath, 2012).

Ethanol significantly increases the psychostimulant and conditioning properties of MDMA (Ben Hamida et al., 2006; Jones et al., 2010) and exacerbates some of its deleterious effects (Izco et al., 2007; Hernández-Rabaza et al., 2010; Ros-Simó et al., 2012). Considering these antecedents, and given their similarities with MDMA, we hypothesized that similar phenomena could be taking place for synthetic cathinones. Accordingly, we sought to explore, in depth, the behavioral and neurochemical consequences that arise from the concomitant administration of ethanol with mephedrone or MDPV.

Furthermore, cocaine is another psychostimulant that is widely co-abused with other substances. Given that MDPV shares mechanism of action with cocaine, preliminary assays investigating the potential interrelation between these two psychostimulants were necessary

Taking into consideration all the above, we outlined the following objectives:

OBJECTIVES

The present thesis was structured in two main chapters

1) Study of MDMA and its interaction with nicotinic acetylcholine receptors

- To determine the role of α4β2 nAChRs and their differential regulation in the psychostimulant, sensitizing and conditioning properties of MDMA.
- To map the differential regulation of nAChRs elicited by a protracted MDMA treatment throughout the brain.
- To postulate the mechanism underlying α4β2 nAChR up-regulation by MDMA.

2) Study of β-ketoamphetamines and their interaction with ethanol and other psychostimulants

Characterization of the effects elicited by mephedrone and its interaction with ethanol.

- To determine the psychostimulant and conditioning properties of mephedrone and their modulation by ethanol.
- To postulate the role of changes in synaptic plasticity-related genes associated to the alluded conditioning treatment.
- To elucidate the neurotoxic consequences of a binge treatment with the combination of mephedrone and ethanol, and their effects on behavior.

Characterization of the effects elicited by methylenedioxypyrovalerone (MDPV) and its interaction with ethanol and cocaine.

- To characterize the psychostimulant and conditioning properties of MDPV and their modulation by ethanol.
- To study the effects of ethanol on MDPV pharmacokinetics.
- To determine the sensitizing properties of MDPV and cross-sensitization of MDPV with cocaine, as well as the potential role of changes in synaptic plasticity-related proteins in these processes.

THEORETICAL FRAMEWORK

Brief introduction to drugs of abuse

The World Health Organization defines a drug as a chemical substance that has known biological effects on humans or animals, foods being excluded from this definition. In this sense, humans have always searched for psychoactive drugs (natural or synthetic) with the capability of altering the central nervous system, giving rise to new subjective perceptions of reality, as well as changes in mood and normal human interaction.

Psychoactive drugs can have many different effects, ranging from stimulation to psychedelia or sedation, among others, and can have potential for abuse, due to the "pleasurable" nature of the effects they elicit. Currently, the list of existing psychoactive drugs is very extensive. Thus, the present theoretical introduction will focus exclusively on those compounds that are relevant to this doctoral thesis.

Ethanol

Ethanol, the psychoactive compound contained in alcoholic beverages, is probably the most ancient psychoactive currently known. Its use is believed to date back to the Neolithic era, approximately 10.000 BC. Numerous archeological findings and historical records from that date on indicate that the use of alcohol was widespread throughout many cultures and regions, Babylon, China and Egypt being the oldest and most notorious examples (Gately, 2009). Furthermore, there are also records of alcohol production and consumption among pre-colonial American civilizations, which stands out, given their complete dissociation from European and Asian cultures.

The use of alcohol is entirely embedded in all developed societies, and, although some attempts have been made at illegalizing it, the most patent of which is American prohibition from 1920 to 1933, its production, distribution and use are permitted in most countries.

Ethanol is a central nervous system depressant. At low doses it causes euphoria and talkativeness; it is therefore used primarily as a socializing drug. As blood ethanol concentration rises, users exhibit increasingly dangerous symptoms associated with the depressant effects of ethanol, such as slurred speech, poor judgment, ataxia, respiratory problems, and even death.

Ethanol is highly addictive; there is particular susceptibility towards its abuse, due to its legal status and perception as an innocuous socializing drug, leading

consumers to adopt use patterns which are characterized by excessive frequency and/or binge-like intake.

Mechanism of action

The effects of ethanol on the brain are numerous and extremely complex, due to its ability to cross biological membranes and to interact with multiple molecular targets (i.e. ligand-gated ion channels). One of the main mechanisms implicated in the effects of ethanol is the increase in GABA function through the activation of $GABA_A$ receptors. In this sense, ethanol can increase locomotion through GABA activation in the substantia nigra pars reticulata (Arizzi-LaFrance et al., 2006). Paradoxically, ethanol decreases GABA function in the ventral tegmental area, which leads to disinhibition of dopaminergic neurons, thus increasing the firing rate of dopamine into the nucleus accumbens. This activation of the mesolimbic pathway is therefore believed to be responsible for the reinforcing properties of ethanol (Xiao et al., 2007). Additionally, it also induces endogenous opioid release in the nucleus accumbens, and directly increases dopamine release in other areas of mesocortical pathways (see Siggins et al., 2005 for a review).

Acute ethanol also acts as an inhibitor of glutamate neurotransmission in different brain areas such as the hippocampus, cerebellum, cerebral cortex, NAc, amygdala and VTA in a concentration-dependent manner (Hoffman, 2003).

Pharmacokinetics

Ethanol follows order zero kinetics; thus, the clearance rate remains constant, regardless of blood concentration. The metabolism of ethanol consists of 2 reactions: ethanol is firstly oxidized into acetaldehyde; this reaction can take place through 3 different routes, involving distinct enzymes:

- Alcohol dehydrogenase (cytosol)
- Multiple cytochromes (microsomes)
- Catalase (peroxisomes)

The resulting acetaldehyde is further oxidized by acetaldehyde dehydrogenase in the mitochondria, generating acetate, which is innocuous. This metabolite is liberated into the blood circulation and subsequently excreted (Zakhari et al., 2006).

Neurotoxicity

Chronic ethanol use is known to cause serious cognitive impairment, accounting for 10% of all existing dementias. Animal studies have reported that heavy ethanol consumption can cause hippocampal cell deficiencies, loss of cholinergic neurons in the basal forebrain and pathological cellular changes in other areas, such as the

cerebral cortex and hypothalamus (reviewed by Brust, 2010). These effects are most prevalent after repeated binge-like use.

It is hypothesized that ethanol-induced neurotoxicity is mediated through several mechanisms, mainly glutamate excitotoxicity, oxidative stress and thiamine (vitamin B1) deficiencies (Gotz et al., 2001).

Cocaine:

Cocaine is an alkaloid that can be found in the leaf of the *Erythroxylum coca* (coca plant). 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. It had a wide range of uses, the most notorious of which were as a stimulant, a hunger suppressant, and a remedy to relieve pain. In 1860, Albert Niemann isolated cocaine for the first time, a method which he published as his PhD dissertation, titled *On a New Organic Base in the Coca Leaves.*

Following, its use for many purposes became widespread in all stratums of Western society, reaching the point where it became the stimulant additive in the ubiquitously known beverage Coca-Cola®; nonetheless, it was eventually replaced by caffeine. This phenomenon, although anecdotal, is a good reflection of the social impact it caused.

In 1914, cocaine became a controlled substance in the United States, due to its high abuse potential, and it is not until the mid XX century when its use increased substantially. Nonetheless, obtaining it was relatively expensive, which served as a significant limiting factor for its use. This restriction disappeared in the 1980s, with the rise of crack cocaine. Crack is the freebase form of cocaine; it has the distinct property of being stable when vaporized, generating inhalable smoke. The pulmonary route of administration allows users to obtain a more intense high with less amount of cocaine, despite the duration of the effect being substantially shorter. These properties make crack a notably cheaper and more addictive substance than its chlorhydrate homologue; this circumstance caused cocaine use and the number of cocaine addicts to rise dramatically, unleashing a full-blown epidemic, especially in the United States.

Mechanism of action

Currently, cocaine is used almost uniquely as a stimulant. Users report increased alertness, energy and well-being. This effect is mediated by a strong increase in extracellular monoamines, which cannot be pumped back into the terminal due to the blockade of their transporter protein, thus accumulating in the synaptic cleft.

This phenomenon holds true for DAT, SERT and NET, although the psychostimulant effect of cocaine is mostly attributed to an increase in dopamine.

Furthermore, cocaine causes the blockade of sodium channels, thus interfering with the transmission of action potentials; although this has little relevance as regards to the psychostimulant properties of cocaine, it does mediate its anesthetic effects, as well as some of its undesired effects, such as cardiac arrhythmia.

Pharmacokinetics

The half-life of cocaine is subject to the administered dose, with values averaging 60 minutes. Furthermore, cocaine fits into a first order pharmacokinetic model.

It is metabolized in the liver, mostly through hydrolytic ester cleavage, generating benzoylecgonine, ecgonine methyl-ester and ecgonine. Additional metabolites include norcocaine, p-hydroxycocaine, m-hydroxycocaine, p-hydroxybenzoylecgonine (pOHBE), and m-hydroxybenzoylecgonine (Kolbrich et al., 2006). Interestingly, when used concomitantly with alcohol, cocaine conjugates with ethanol molecules, forming the unique metabolite cocaethylene, which has been reported to possess higher cardiotoxicity than cocaine (Wilson et al., 2001).

Neurotoxicity

Although cocaine is known to exert strong cardiovascular toxic effects, the matter of its neurotoxic potential is controversial. There are many discrepancies in the extensive literature on this issue, which contrast significantly with the good agreement there is on the strong neurotoxic potential of amphetamines. Thus, cocaine is generally believed to have little to no neurotoxic effects (Benmansour et al., 1992); nonetheless, some studies have found that continuous cocaine exposure can cause persistent changes in acetylcholine (ACh) and GABA receptors, as well as in markers for dopaminergic function, pointing to the existence of damage in the structures of dopaminergic neurons (reviewed by Ellison et al., 1996).

Amphetamines

Amphetamines are psychostimulant substances; they cause an enhancement in alertness, energy and self-confidence, which are accompanied by an increase in the sense of euphoria and wellbeing, as well as a decrease in appetite (Green et al., 2003). Their use can trigger severe undesired effects, which range from cardiovascular complications to psychotic reactions, hallucinations and paranoia. Furthermore, if used frequently, most amphetamines have high abuse liability and can cause tolerance (Hoffman and Lefkowitz, 1996).

Amphetamines, as suggested by their generic name (Alpha-MethylPHenEThylAMINE), are comprised of a phenyl ring connected to an amino group by a two-carbon side-chain with a methyl group on carbon-1 of the side chain (Fig. 1). Amphetamine, Methamphetamine and methylenedioxymethamphetamine (MDMA) are the most popular substances of this group among users.

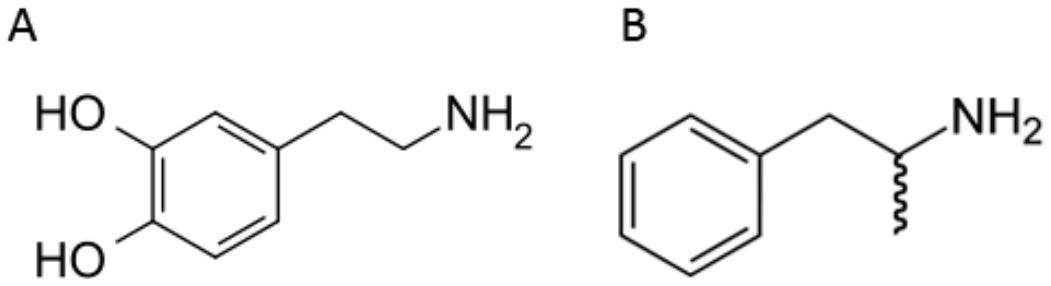

Figure 1: Chemical structure of dopamine (A) and amphetamine (B)

Generic mechanism of action of amphetamines:

Synaptic terminals are endowed with vesicles that store reserves of neurotransmitters, which, in physiological conditions, are released through exocytosis into the synaptic cleft at a controlled rate.

Amphetamines are, with few exceptions, psychostimulants of the releaser type. They increase extracellular neurotransmission by promoting the release of neurotransmitters found in presynaptic vesicles. Depending on their specific structure, these compounds can evoke an increase in dopamine, norepinephrine and serotonin, at different ratios and to different degrees. Nonetheless, the main psychostimulant and reinforcing effects of amphetamines are generally attributed to the release of dopamine (Gulley and Zahniser, 2003; Kuczenski et al., 1995; Sulzer et al., 2005).

Amphetamines enter the synaptic terminal through monoamine transporters, mainly the dopamine, norepinephrine and serotonin transporters (abbreviated DAT, NET and SERT, respectively), where they act as substrates (Liang and Rutledge, 1982; Zaczek et al., 1991). This high affinity for monoamine transporters is explained by the high homology between amphetamines and catecholamines, such as dopamine or norepinephrine.

Once they enter the synaptic terminal, amphetamines are capable of massively releasing neurotransmitters, which are contained in vesicles. Due to the high concentrations of cytoplasmic neurotransmitters, there is a shift in the gradient (i.e. there is a higher concentration of free monoamines inside the terminal), which, in

turn, causes their release into the synaptic cleft by reverse transport, mediated by the monoamine transporters mentioned above (Leviel, 2001) (Fig. 2).

There are two main hypotheses as to how vesicular content in released into the cytoplasm.

Weak base Hypothesis:

All sympathomimetic compounds are weak bases with amine moieties that are capable of accepting protons with pKs in the range of ~ 8 to 10. Thus, they can be protonated in acidic organelles including catecholamine vesicles (Sulzer and Rayport, 1990): once charged, they become less membrane-permeable and accumulate in the acidic structure.

The acidic pH gradient in secretory vesicles provides the energy to accumulate neurotransmitters against their concentration gradient. Secretory vesicles are acidic; vesicles maintain a pH of 5.0 – 5.7, depending on conditions (Markov et al., 2008) that provide the energy to accumulate monoamine transmitters.

Weak base compounds that are sufficiently membrane-permeable to enter secretory vesicles bind free protons, alkalinize the existing vesicular acidic pH gradient and thus decrease the energy that drives the accumulation of neurotransmitters (Markov et al., 2008; Sulzer and Rayport, 1990).

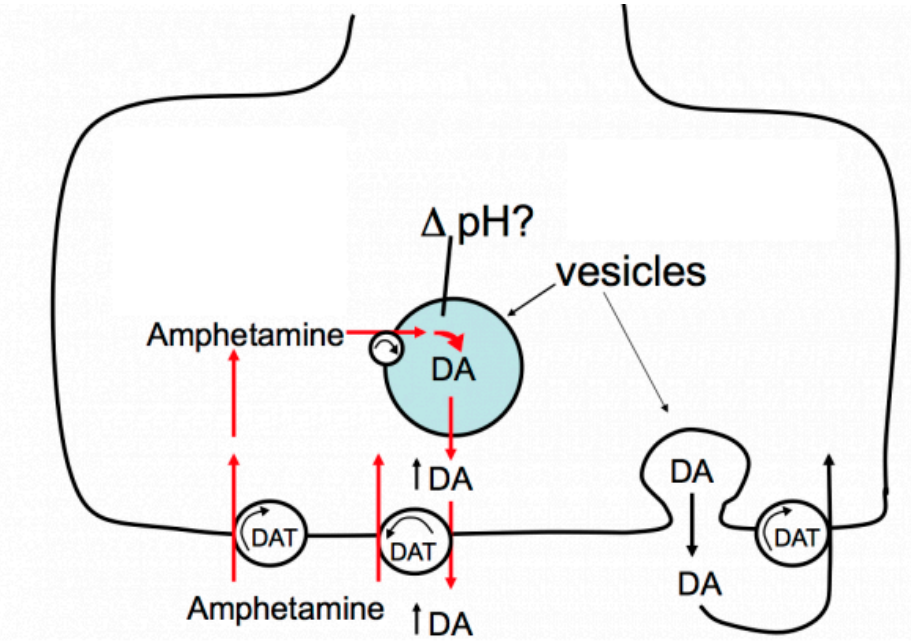

Figure 2: General mechanism of action of amphetamines.

Several studies have tested the weak base hypothesis by comparing effects on vesicular pH and catecholamine redistribution. Interestingly, there is not a direct correlation between vesicular pH and neurotransmitter release (Floor and Meng, 1996). Furthermore, (S+)-amphetamine stereoisomer is several-fold more effective at blocking uptake than its (R−)isomer (Peter et al., 1994); these phenomena cannot be explained uniquely by the weak base hypothesis. Thus, data points to the existence of a complementary mechanism of action in the mediation of vesicular monoamine release.

Substrate hypothesis:

It has been demonstrated that amphetamine can bind to the vesicular monoamine transporter (VMAT), the protein whereby monoamines are taken into the vesicle for storage (Erickson et al., 1996). This would allow reuptake blockade, which, in turn, would cause a gradual increase in cytosolic monoamines due to leakage across the permeable vesicular membrane (Schonn et al., 2003). Additionally, amphetamine acts as a substrate, thus entering the vesicle through the transporter (Partilla et al., 2006); this would allow the release of intravesicular monoamines in the process (amphetamine/monoamine exchange). In agreement with this hypothesis is the observation initially made on isomer-driven preferential effect of amphetamine, as the (S+)-isomer exhibits preferential binding to the transporter (Peter et al., 1994).

Classic amphetamine derivatives: 3,4-methilendioxymethamphetamine (MDMA)

There are numerous amphetamine derivatives, which derive from the same parent structure. Out of all of these compounds, 3,4-methilendioxymethamphetamine, also known as MDMA or Ecstasy, has gained the most popularity. It is a psychoactive drug with stimulant properties, which was first synthetized by Merk pharmaceuticals in 1912; no use was found for it until 1976, when Alexander Shulgin, chemist and pharmacologist, first described its mind-altering effects on humans. (Benzenhöfer and Passie, 2010).

MDMA structurally differs from amphetamine in a considerable manner. As depicted in Fig. 3, MDMA contains a methylendioxy group bound to positions 3 and 4 of the aromatic ring of the compound methamphetamine, which, in turn, results from the methylation of the primary amine of amphetamine.

Mechanism of action

MDMA is characterized by its empathogenic properties, providing a sense of emotional openness and affection towards others. These properties are a result of an increment in the levels of mostly serotonin in the neuronal synapse, together with other neurotransmitters (dopamine and norepinephrine) in lower proportions (Green et al., 1995).

As described above for most amphetamine derivatives, this serotonin increase is mediated by a massive release from presynaptic vesicles, which runs in parallel with an inhibition of its reuptake through the serotonin transporter by direct competition with the substrate. The same mechanism applies for the release of the other monoamines, albeit to a lesser degree (White et al., 1996). MDMA also inhibits tyrosine hydroxylase, the limiting enzyme in the *de novo* synthesis of serotonin (Che et al., 1995). Furthermore, MDMA is a partial agonist on post-synaptic serotonin$_{2A}$ (5-HT$_{2A}$) receptors, which endows it with light psychedelic properties. This is a shared characteristic with mescaline, with which it possesses a strong structural similarity (Fig. 3). How the activation of serotonin$_{2A}$ receptors leads to psychedelia is still unknown, but it likely somehow involves excitation of neurons in the prefrontal cortex.

Pharmacokinetics

In Sprague-Dawley rats, after a single 10mg/kg intravenous dose, its half-life was 1.7h, with a distribution volume of approximately 7 L/Kg. It undergoes stereo-selective metabolism, favoring clearance of (S)-MDMA over (R)-MDMA, and is has been shown to possess non-linear pharmacokinetics (Mechan et al., 2006). In rats,

its main metabolic route is that of N-demethylation, giving rise to 3.4-methylendioxyamphetamine (MDA), which is psychoactive. MDA can be found independently in the black market, as users have reported it to provide a slightly less empathogenic and more psychostimulant and psychedelic high to that of MDMA. Other metabolites that have been isolated in rats are 3-Hydroxy-4-methoxymethamphetamine, 3, 4-dihydroxymethamphetamine (HHMA), 4-hydroxy-3-methoxyphenylacetone, 3,4-methylenedioxyphenylacetone (Lim and Foltz, 1988).

In humans, MDMA is easily absorbed through the gastrointestinal tract, reaching its plasmatic concentration peak 2 h post-administration (Farré et al., 2004). Nonetheless, some of the data on oral pharmacokinetics differ, due to the pharmaceutical form in which the compound is administered.

In humans, there are two preferred routes through which MDMA is metabolized. O-demethylation is the main route, which is regulated by a great number of cytochrome P450 isoforms, thus giving rise to HHMA. In addition, N-dealkylation takes place as a secondary route, generating MDA, which can, in turn, suffer O-demethylation, converting it into 3,4-dihydroxyamphetamine (HHA). Both HHMA and HHA are O-methylated into HMMA and HMA in a reaction that is regulated by catechol-O-methyltransferase (COMT), or form glucurono/sulfate conjugates (de la Torre et al. 2004). Furthermore, fractions of HHMA and HHA can suffer auto-oxidation, generating the corresponding ortho-quinones, which can be successively conjugated, forming glutathione adducts.

Emerging amphetamine derivatives: Synthetic cathinones

Synthetic cathinones form a family of drugs that has recently thrived in the illegal market, a phenomenon that has been facilitated by clandestine Internet sites, which permitted their sale and distribution, taking advantage of the lack of legislation on these activities for this type of compounds (Winstock et al., 2011). Nonetheless, cathinone derivatives have existed for approximately a century. Cathinone and its metabolite cathine are responsible for the sympathomimetic amphetamine-like effects caused by the chewing of khat (*Catha edulis*) leaves, a plant which grows primarily in East African countries such as Somalia, Ethiopia, Uganda and Kenya, as well as in Yemen in the Middle East, where its use is deeply embedded as part of the local culture. There, approximately 50% of the adult population is believed to consume khat daily (90% in male adults).

Cathinone is the reference structure from which a great number of compounds have derived. It is closely related to amphetamine, from which it is differentiated by the addition of a ketone group on the β position of the aliphatic chain.

4-methylmethcathinone (Mephedrone, Meph), 3,4-methylenedioxy-N-methylcathinone (methylone), methylenedioxypyrovalerone (MDPV) and bupropion are the most widely used cathinone derivatives. Bupropion is a commercialized compound, prescribed mostly for depression and smoking cessation, although it has other off-label uses. By contrast, the former three compounds are consumed primarily for recreational purposes. Fig. 3 depicts their chemical structure and that of cathinone.

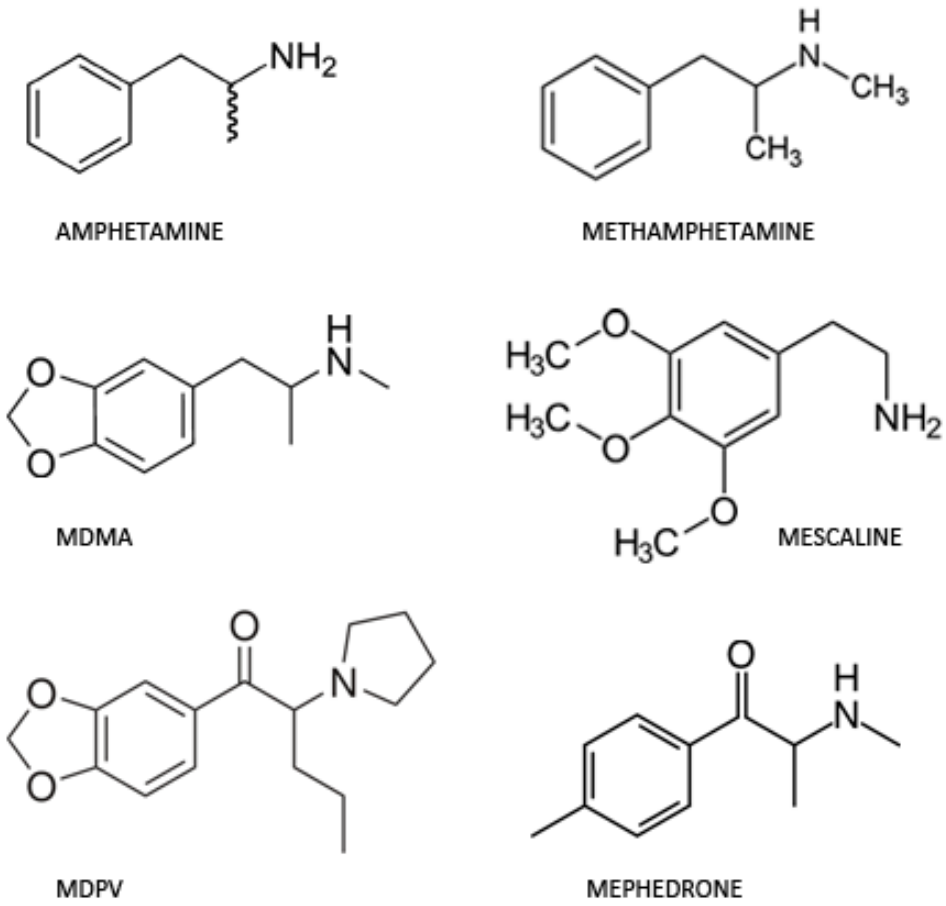

Figure 3: Structure of the main amphetamine and cathinone derivatives

4-methylmethcathinone (Mephedrone)

Mephedrone is a psychostimulant and empathogenic substance, classified under the phenethylamine and β-keto-amphetamine families. It is closely structurally related to methcathinone and methamphetamine. Its effects have been compared to those of cocaine, amphetamine and MDMA (Winstock et al., 2011). These effects include:

- Intense stimulation and alertness; euphoria
- Empathy, closeness towards others and sociability
- Intensification of sensory experiences
- Sexual arousal
- Perceptual distortions

Users described it, in terms of the subjective experience in produces, as a combination between the psychostimulant and empathogenic effects of cocaine and MDMA, respectively. Typical unwanted effects for mephedrone include loss of appetite, xerostomy, bruxism, tremors, tachycardia, temperature changes, agitation and irritability (James et al. 2011; Wood et al. 2009). Furthermore, mephedrone has been shown to elicit positive conditioning in rats and mice (Lisek et al., 2012; Karlsson et al 2014) and it has been reported that it could have comparable abuse potential to that of cocaine or MDMA (McElrath and O'Neill, 2011).

Mechanism of action

Until recently, little had been known as to the mechanism of action of cathinones. In 1999, Cozzi et al. published a comparative study on the ability to bind to monoamine transporters of MDMA, methamphetamine and their respective cathinone derivatives methylone and methcathinone. Nonetheless, mephedrone remained as an unresearched drug until the early 2010s.

Mephedrone is a substrate for both DAT and SERT with high affinity (López-Arnau et al., 2012), where it acts as a blocker (Simmler et al., 2012); it is internalized into the terminal, where it interacts with VMAT (López-Arnau et al., 2012), presumably promoting the release of vesicular dopamine and serotonin into the cytoplasm through the mechanisms discussed in the “generic mechanism of action of amphetamines” section (i.e. weak base and substrate hypotheses). Subsequently, monoamines are released into the synaptic cleft, via reverse transport. This mechanism resembles that of most amphetamine derivatives, as could be expected, due to the structural similarities. It is noteworthy to point out that mephedrone possesses a unique DAT/SERT blockade and dopamine/serotonin release profile, wherein the proportion of the degrees at which these phenomena take place is close to the unity (Simmler et al., 2012; Kehr et al., 2011). This translates into the characterization of mephedrone as a “nearly equally” dopaminergic and serotonergic drug, which explains the similarities in subjective effects to both cocaine and MDMA alluded to above.

Pharmacokinetics

Mephedrone has a short half-life (25 min when administered intravenously), and presents low bioavailability, due to an extensive first-step effect (i.e. a large percentage of the compound is metabolized before reaching the bloodstream) (Martínez-Clemente et al., 2013). These properties account for users’ preference for the intranasal over the oral route of administration as well as their tendency to re-dose frequently.

Mephedrone has non-linear pharmacokinetics. With increasing oral doses, the bioavailability becomes higher, the half-life longer and total and hepatic clearance lower. This can be explained by a saturation of the hepatic function.

Furthermore, mephedrone presents a 20% protein binding and a low brain/plasma concentration ratio (1.85) when compared to other amphetamine derivatives (Chu et al., 1996), reflecting higher difficulty crossing from the bloodstream into the brain.

Mephedrone is N-demethylated, yielding the corresponding methcathinone metabolite. Mephedrone also undergoes different oxidative reactions including aliphatic and aromatic hydroxylation, leading to the corresponding 3'-hydroxy-methylmethcathinone or hydroxyl-4-methylmethcathinone metabolites. Finally, mephedrone can also suffer an allylic hydroxylation, generating 4-hydroxymethylmethcathinone, which can, in turn, be further metabolized into 4-carboxymethylmethcathinone, through oxidation.

Neurotoxicity

Neurotoxicity of cathinone derivatives is a controversial matter. Angoa-Perez et al. (2012) and den Hollander et al. (2013) reported no damage by mephedrone to dopamine or serotonin systems when administered to mice, while more recent reports have shown the appearance of neurotoxicity when using a dosing schedule which better agreed with mephedrone pharmacokinetics and exploring cerebral areas others than striatum (Martínez-Clemente et al., 2014; Lopez-Arnau et al., 2015). In these studies, 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 exposition. Furthermore, changes in oxidative stress markers point to the possibility that these changes could be due to increases in the presence of free radicals. This has been found to have deleterious consequences on memory, as measured by the Morris water maze test (López-Arnau et al., 2015).

Methylenedioxypyrovalerone (MDPV)

MDPV is a psychostimulant substance, classified under the phenethylamine and β-keto-amphetamine families. Its effects have been compared to those of cocaine and amphetamine, causing effects that include euphoria and increased alertness. MDPV is a notably more potent psychostimulant than cocaine, as the threshold dose that

causes hyperlocomotion in rats is 0.3mg/kg, which contrast with that for cocaine (10mg/kg) (Baumann et al., 2013).

Due to its potency, MDPV in highly dangerous, making it the most commonly found cathinone in blood and urine of patients admitted to the emergency room in the United States. In this sense, it has been reported to cause agitation, psychosis, tachycardia, and even death (Borek and Holstege, 2012; Spiller et al, 2011).

Mechanism of action

MDPV is a catecholamine transporter blocker, mainly on DAT, thus eliciting the accumulation of dopamine and norepinephrine in the synaptic cleft. Unlike most phenethylamines, it does not act as substrate, which keeps it from entering the terminal and causing massive neurotransmitter release through reverse transport.

This mechanism of action is shared by the alkaloid cocaine and few amphetamine derivatives, bupropion (cathinone family) and methylphenidate being the most widely known examples. Nonetheless, MDPV is 10-fold more potent than cocaine in its ability to increase extracellular dopamine, as the lowest effective dose of MDPV is 0.1mg/kg, compared with 1.0 mg/kg for cocaine (Baumann et al., 2013). Furthermore, it is significantly more efficacious, resulting in a higher activity peak in hyperlocomotion assays.

Pharmacokinetics

To date, information on this matter is scarce. It is known that MDPV shows linear kinetics, with a relatively short half-life (≅75 minutes) (Anizan et al., 2014; Novellas et al., 2015). Furthermore, both studies described that MDPV undergoes phase I metabolism, consisting of a demethylation of the methylendioxy group to form the corresponding diol (3,4-catechol-PV), followed by a O-methylation, generating the hydroxylated metabolite (4-OH-3-MeO-PV or 3-OH-4-MeO-PV); these metabolites can be subsequently hydroxylated on the aromatic ring, forming 4,?-OH-3-MeO-PV or 3,?-OH-4-MeO-PV (the postition at which hydroxylation takes place has not been determined).

Neurotoxicity:

There are nearly no reports on MDPV-induced neurotoxicity. Ádam et al. (2014) demonstrated that MDPV does not affect apoptosis in the adult rat brain. Furthermore, a recent study showed that MDPV attenuates methamphetamine-induced signs of neurotoxicity (Anneken et al., 2015). This phenomenon could be 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 (ROS). This is backed by additional findings showing that MDPV dose-

dependently blocks methamphetamine-induced dopamine release (Simmler et al., 2013). Taken together, it can be hypothesized that MDPV possesses limited neurotoxic potential.

Nicotine and nicotinic acetylcholine receptors

Nicotine is an alkaloid naturally found in plants of the solanaceae family, such as *Nicotiana tabacum, Nicotiania rustica, Duboisia hopwoodii* and *Asclepias syriaca*. Its pharmacology is unique, as compared to any other drug of abuse, due to its activity both as a stimulant and a sedative. This phenomenon is referred to as the Nesbitt paradox, and it is dependent on the dose: as nicotine blood concentration increases, its pharmacological profile shifts from stimulant to increasingly relaxant.

When a person inhales smoke from a cigarette, nicotine is distilled from the tobacco and is carried in smoke particles into the lungs, where it is absorbed rapidly into the pulmonary venous circulation. It then enters the arterial circulation and moves quickly to the brain. Nicotine diffuses readily into brain tissue, where it binds to nicotinic acetylcholine receptors (nAChRs).

nAChRs are membrane proteins with a molecular mass of 290 kDa, formed by 5 symmetrically located subunits, constituting a central ion channel. The subunits for nAChRs are codified by 17 genes; of these, vertebrates express 9 type α subunits (α2- α10), and 3 type β subunits (β2- β4). The combination of different subunits gives rise to receptors with distinct pharmacological and kinetic properties. In the central nervous system, nAChRs are mostly located in presynaptic neurons, where they modulate the release of neurotransmitters such as dopamine, serotonin, GABA, norepinephrine, acetylcholine and glutamate. (Gotti et al., 2007; Taly et al., 2009)

The atomic structure of nAChRs has been well characterized (Unwin et al., 2005). Each subunit is formed by an extracellular domain with an amino terminal group, a transmembrane domain, consisting of 4 segments (TM1 – TM4) and a variable cytoplasmic domain. The binding site for ligands is located in the interphase between a α and a β subunit. This holds true for all heteromeric receptors; the ligand-binding site in homomeric nAChRs is located between any of the subunits.

There are 6 different receptor types. The most abundant ones in the mammalian central nervous system are α4β2 and α7 nAChRs. The α4β2 receptor is a heteromer formed by two different subunits, whose activation causes the entrance of sodium through the channel. α7 nicotinic receptors are homo-oligomers, formed by five subunits. Its activation causes the entrance of calcium into the cytoplasm and,

consequently, the activation of calcium-dependent enzymes such as protein kinase C (PKC) and nitric oxide synthase, whose overactivation is linked to neurotoxic processes (reviewed by Escubedo et al., 2009).

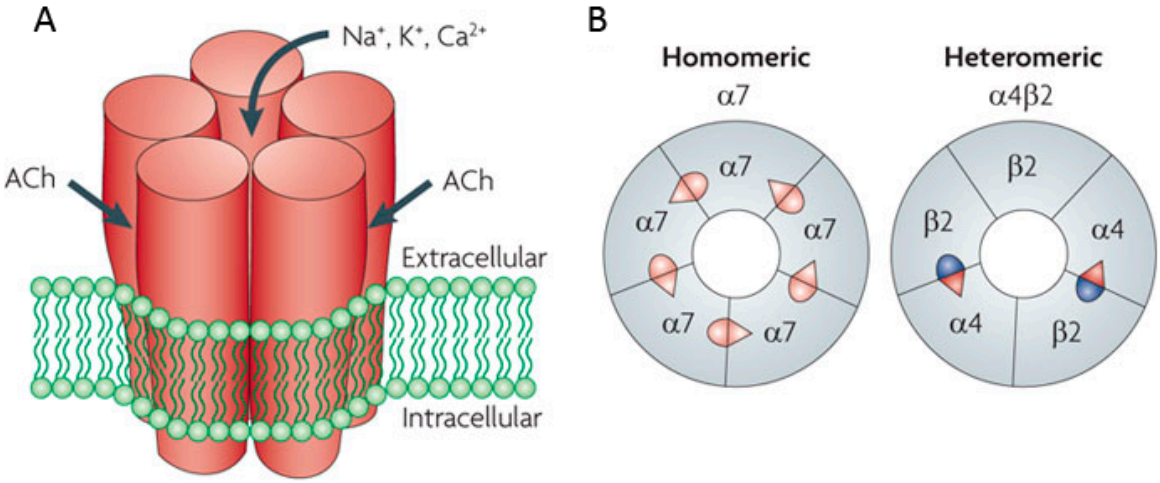

Figure 4: Molecular structure of α4β2 and α7 nAChRs, and their respective ligand binding sites (Changeux, 2010)

α4β2 nAChRs are predominant in the human brain and are believed to play a crucial role in mediating the reinforcing effects of nicotine, as well as nicotine dependence (Tapper et al., 2004). The homomeric α7 nAChR is thought to be involved in rapid synaptic transmission and may play a role in learning and sensory gating (Levin et al., 1999), which explains why patients with schizophrenia and attention deficit disorders have a strong tendency to resort to nicotine for the purpose of ameliorating their lack of focus.

The mesostriatal dopamine pathway is a major brain target for nicotinic agonists and has two principal components: the ventral mesolimbic pathway, which has cell bodies in the ventral tegmental area (VTA), and terminals in the nucleus accumbens (NAc) and tuberculum olfactorium; and the dorsal nigrostriatal pathway, which has cell bodies in the substantia nigra (SN) and terminals in the caudate-putamen (CPu). nAChRs in the dopaminergic neurons of the mesostriatal pathway play an important role in controlling locomotion and the development of some long-lasting adaptations associated with nicotine abuse. Behavioral and functional studies in rats have shown that α4β2 nAChRs in the dopaminergic neurons of the VTA are necessary for the rewarding effects of nicotine (Maskos et al., 2005). Moreover, in

the nigrosriatal pathway, this receptor type has been shown to play a crucial role in mediating locomotion (Avale et al., 2008).

A particular feature of some nAChR subtypes is that, after chronic nicotine exposure, they undergo up-regulation, changes in stoichiometry and an increase in their functional state (functional up-regulation) (reviewed by Gaimarri et al., 2007). nAChR up-regulation has been hypothesized to enhance addiction to nicotine by increasing the pleasant effects of the drug (Govind et al., 2009).

Such up-regulation occurs at a post-translational level and mainly two mechanisms have been proposed to explain it.

- **Chaperone- like maturation enhancing effect.** The endoplasmic reticulum (ER) is converted into a nicotine-favored state, where it becomes both more capable to export and less susceptible to misfold/degradate nascent $\alpha4\beta2$ than other states within the ER (Srinivasan et al., 2011).
- **Stabilization of the high-affinity state of the receptors.** Nicotine exposure slowly stabilizes $\alpha4\beta2$ receptors in the plasma membrane into a high-affinity state that is more easily activated (Vallejo et al., 2005).

Effect of amphetamines of nicotinic receptors

It has been reported that amphetamine derivatives bind to nicotinic receptors with moderate affinity and cause their up-regulation in-vitro. MDMA acts as a $\alpha7$ partial agonist and $\alpha4\beta2$ antagonist. Sustained activation of $\alpha7$ nAChR by MDMA has been postulated to participate in MDMA-induced neurotoxicity (Garcia-Ratés et al., 2007, 2010; Pubill et al., 2011)

The release of dopamine modulated by nAChRs is thought to be involved in the reinforcing action of many addictive drugs. The development of sensitization is linked to the addictive potential of drugs; nAChR antagonists attenuate amphetamine-induced behavioral effects, including locomotor sensitization (Schoffelmeer et al. 2002) and the discriminative effects of the drug (Desai et al., 2010). Conversely, acute nicotine administration facilitates the development of sensitized locomotor activity in response to amphetamines (Birrell et al., 1998). This effect seems to be attributable to $\alpha4\beta2$, but not $\alpha7$ nAChRs, as dihydro-β-erythroidine (DHβE, an αβ heteromeric nAChR antagonist) but not methyllycaconitine (a α7 nicotinic receptor antagonist) blocks amphetamine-stimulated locomotion (Kim et al., 2012)

The neurobiology of addiction: reward, reinforcement and sensitization

A rewarding stimulus is defined as a stimulus that is considered likeable and thus is worthy of being desired and pursued (Berridge and Robinson, 2003). Rewards (both natural and exogenous) trigger two important biological processes:

- Assignment of a **hedonic value**. This is defined as how much the reward is "pleasurable" or "liked".
- Assignment of an **incentive salience**, which is defined as a motivational value or "wanting" or a given rewarding stimulus. (Kelley and Berridge, 2002).

This distinction is important, since rewarding stimuli modulate behavior through an increase in dopamine in the nucleus accumbens (NAc). In this sense, dopamine is not a mediator of the hedonic state elicited by a rewarding stimulus (Cannon & Palmiter 2003). Rather, it is hypothesized that dopamine acts as a mediator in the development of incentive salience of rewarding stimuli. Therefore, an animal without dopamine is capable of perceiving the characteristic hedonic effects of a given stimulus, but it lacks the mechanisms that drive it towards its obtention (i.e. it cannot act on its preferences).

Positive reinforcement is defined as an increase in the frequency in which an individual works towards obtaining a particular rewarding goal, and it is the underlying phenomenon that gives rise to **addiction** (Berridge and Robinson, 1998). The incentive salience of a rewarding stimulus underlies its reinforcing properties; for this reason, drugs of abuse that elicit higher increases in synaptic dopamine tend to be more reinforcing, and thus, potentially more addictive.

Therefore, drugs that have mild or no hedonic value can be highly reinforcing (e.g. nicotine); similarly, drugs that have strong hedonic value can be non-reinforcing (e.g. many hallucinogenic compounds).

Drug-induced **sensitization** is a phenomenon wherein subjects exhibit an increase in the behavioral response to a particular substance (Kalivas & Stewart 1991). Cocaine and amphetamine both elicit robust behavioral sensitization, although it is observed in a great number of drugs of abuse (Segal et al., 1992). Sensitization occurs mainly when drug intake is intermittent (e.g. once a day). An archetypal example is the gradual increase in locomotor activity produced by daily injections of cocaine or amphetamine at a fixed dose. Furthermore, this phenomenon is long-lasting; in this sense, it has been described that a single dose of amphetamine is capable of

eliciting a sensitized behavioral response over a year after the last amphetamine administration (Paulson et al. 1991).

Sensitization is also context-dependent. In this sense, an animal which has been administered daily with a sensitizing compound in a particular test chamber will exhibit a higher behavioral response when given a drug challenge (i.e. additional dose of the tested compound) in the same test chamber, as opposed to receiving the challenge in its home cage. Furthermore, environmental cues alone can be sufficient to elicit a sensitized behavioral response (Anagnostaras and Robinson 1996).

Given these two main properties (endurance and context-dependence), sensitization has been postulated as a pivotal mechanism underlying addiction (Robinson & Berridge 1993). It is believed that, similarly to the sensitizing effect of intermittent doses on locomotor responses, the neuronal circuits that mediate incentive salience can become sensitized to drug-related cues. Therefore, repeated exposure to drug-related cues together with a reinforcing drug can potentiate this association. This results in increased incentive salience when re-exposed to solely the drug-related cues (e.g. syringes or other drug paraphernalia), resulting in strong desire to use (i.e. craving).

To summarize, reward, reinforcement, behavioral sensitization and the interrelation between these phenomena constitute complementary underlying mechanisms in the mediation of addiction.

Cellular and molecular mechanism of addiction: Neuroplasticity

As discussed extensively above, the state of addiction is characterized by three main traits:

- Compulsive drug intake triggered by repeated dopamine release
- Strong behavioral impulses (desire to use) triggered by drug-related cues
- Persistence over time of drug-induced changes in neural function and high risk of relapse

These phenomena, must be ultimately explained by cellular and molecular mechanisms of addiction. In this sense, drug induced signals, such as dopamine release, can be converted into long-term cellular modifications, through multiple

forms of neural plasticity, such as changes in intrinsic and global excitability of individual neurons (Nestler and Aghajanian, 1997; Zhang and Linden, 2003).

Neuroplasticity is defined as the ability of the brain to change and adapt over time, a phenomenon that underlies the formation of all memories (Barco et al., 2006). Plasticity also occurs as a result of triggering reward- and stress-related centers of the brain, with the purpose of discerning between advantageous and harmful stimuli in the future, thus being a basic contributor to animal survival. Drugs of abuse have the ability of altering this function by inducing strong and persistent drug reward-related memories that can eventually develop into full-blown addiction (Hyman et al., 2006).

Long-term potentiation (LTP) and Long-term depression (LTD)

The most ubiquitously known mechanisms mediating neuroplasticity are long-term potentiation (LTP) and long-term depression (LTD) (Malenka and Bear, 2004). These two phenomena are defined as the changes in synaptic transmission efficiency in response to stimuli, and are essential to the formation of new memories, which can be reward-related and triggered by drug use (Hyman et al., 2006). Thus, LTP and LTD play a central role in the development of addiction. Furthermore, changes in gene expression are known to be essential to the development of LTP (Stanton and Sarvey, 1984)

Transcription factors and plasticity

Gene expression is controlled by proteins named transcription factors. Upon stimulation, these proteins can trigger a series of actions, including nuclear entry, changes in nuclear stability and increases in co-factor and DNA binding, which, in turn, can cause gene up- and down-regulation. In this sense, and in the context of neuroplasticity, cAMP response element-binding protein (CREB) and ΔFos are the transcription factors that have been studied most extensively.

cAMP response element-binding protein (CREB)

CREB binds to what is known as the cAMP response element (CRE) in numerous gene promoters, which includes growth factors, enzymes, structural proteins and other transcription factors (Lonze and Ginty, 2002). The activity and stability of CREB varies due to chemical modifications, among which it is believed that the phosphorylation at Serine-133 by several signaling pathways plays a pivotal role (Mayr and Montminy, 2001; Lonze and Ginty, 2002).

Memory-developing stimuli increase CREB phosphorylation at Serine-133; this phenomenon subsequently mediates the decrease in the LTP-generating threshold (Barco et al., 2002), and enhances glutamatergic N-methyl-D-aspartate (NMDA)

receptor function (Marie et al., 2005). Thus, CREB function is believed to be central to the development of long-term memories.

Brain regions directly targeted by drugs of abuse undergo notable changes in CREB function upon acute and chronic drug exposure (Carlezon et al., 2005).

It is believed that CREB function induction is a feedback measure aimed at compensating for the abnormally increased neuronal activation in the nucleus accumbens, leading to a decrease in baseline dopaminergic function which, in turn, leads to tolerance, dependence and withdrawal-related dysphoria, three clear symptoms of addiction (Carlezon et al., 2005).

A Role for ΔFosB

The Fos transcription factor family is comprised mainly by cFos, FosB and ΔFosB, the activation of which is rapidly induced after acute stimulation. Conversely, after prolonged stimulation, the induction of Fos-type transcription factors becomes less intense, with the exception of ΔFosB. ΔFosB results from a splice variant of the FosB gene; it has the characteristic property of being unusually stable, thus accumulating after protracted treatments (McClung et a., 2004).

ΔFosB is believed to play an important role in the induction and maintenance of LTP, as it increases after a great variety of stimuli, including persistent drug use. This phenomenon occurs in specific regions, such as the nucleus accumbens (McClung and Nestler, 2003), where an increase in ΔFosB is related with drug-addiction-related phenotype (Kelz et al, 1999). It is therefore believed that the increase in ΔFosB leads to the induction of long-term transcriptional changes related to neuronal plasticity.

Polysubstance abuse and adolescence

Polysubstance abuse is an extremely prevalent phenomenon which has been described for virtually all popular drugs of abuse, including but not restricted to alcohol, cannabis, tobacco, MDMA, cocaine and opiates (Pape et al, 2009). There are many explanations as to why users tend to consume more than one substance. Often, the characteristic circumstances of social encounters play an important role, most importantly easy availability, social pressure and impaired judgment due to initial exposure to a first drug of abuse (e.g. alcohol). Furthermore, users are driven by the expectation of generating a “unique” subjective experience through the interaction of several pharmacological mechanisms.

Polysubstance use can be classified as simultaneous or concurrent. The former type implies the use of two or more substances at the same exact moment; in the latter type, the use of the respective substances fits in the same temporal frame (e.g. the same week), albeit it is not simultaneous. In this regard, although simultaneous polysubstance use is intuitively perceived as more dangerous, both types of polyabuse can entail notably harmful neurochemical and behavioral consequences.

Furthermore, polysubstance uses are known to constitute a great majority of first use experiences for most drugs, that is, people tend to try a new drug for the first time when disinhibited by the effects of a known drug. The most common example of this phenomenon is the use of alcohol (the most widely consumed drug), which causes inebriation and, in turn, leads to higher risk-taking, including experimenting with other drugs such as MDMA or cocaine. Even after having already experimented with these drugs, users will initially withhold from using them in successive sessions due to the knowledge of the risks their consumption entails or to social stigma; again, users will only overcome this psychological barrier when their judgment becomes impaired by the intake of alcohol. Thus, many circumstances lead to polysubstance use, especially in the context of initial experimentation with new substances (Olthuis et al., 2013).

In this sense, first time experiences with drugs of abuse are closely linked to young age, when risk assessment and impulsivity controlling capacities are not entirely developed (Cassey et al., 2008). Consumption of drugs of abuse at earlier ages, such as adolescence, is especially worrisome because this stage is crucial in brain maturation and will determine the social outcome of an individual (Steinberg, 2005).

Substance use during adolescence has been associated with alterations in brain structure, function, and neurocognition, as well as to an increased likelihood of drug abuse in adulthood (reviewed by Squeglia et al., 2009). Currently, most drug use during adolescence occurs in leisure environments, such as dance clubs and parties, leading to a preference for use of psychostimulants (i.e. cocaine and amphetamine derivatives), alcohol and tobacco, both of which are omnipresent due to their legal drug status (Winstock et al., 2011).

Literature in the field of drugs of abuse is very extensive, making it one of the most researched branches of neuroscience, due to the magnitude of the public health issues that arise from both acute and chronic drug use. Despite this, preclinical studies on the neurochemical and behavioral consequences of polysubstance use are notably scarce.

Taken together, it is clear that polysubstance use warrants more attention, due to the existence of potential interrelations between the pharmacological mechanisms of the respective consumed substances, which can give rise to unexpected harmful effects, both in terms of neurotoxicity as well as abuse potential associated to each individual substance. Furthermore, polysubstance use becomes a matter of the utmost concern especially in young adults, due to their characteristic propensity towards these types of use patterns, as well as their vulnerability to the neurochemical and behavioral effects that they entail.

METHODS AND RESULTS

Article 1: Heteromeric nicotinic receptors are involved in the sensitization and addictive properties of MDMA in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*

A particular feature of nAChRs is that chronic exposure to nicotine and other nicotinic ligands induce a higher level of epibatidine binding (up-regulation) that can lead to an increase in receptor function (functional up-regulation). Therefore, the up-regulation of heteromeric nAChR could, via dopamine release, explain the reinforcing effect of nicotine on the mesolimbic system mediating nicotine addiction.

Previous results from our group have demonstrated that α7 and α4β2 nAChR are a pharmacological target for MDMA. Furthermore, exposure to MDMA has been shown to up-regulate these receptor types.

This work was structured in two separate sections. The fist one focuses on investigating the role of nAChR on behavioral sensitization through locomotor activity assays, while the second one is devoted to elucidating their involvement in the modulation of the rewarding properties of MDMA by means of the conditioned place preference (CPP) paradigm.

Behavioral sensitization: When MDMA (5 mg/kg) was administered daily for 10 consecutive days, there was an increase in the hyperlocomotion induced by the drug on day 10 with respect to that measured on day 1 (early behavioral sensitization). Furthermore, behavioral sensitization was found to be highest after a 2 week-period following the discontinuation of MDMA treatment (a challenge dose of MDMA showed a stronger behavioral response than on day 10), demonstrating that the treatment schedule of MDMA used in this study induces not only early but also delayed sensitization.

DHβE (α4β2 nAChR antagonist) (1mg/kg) and varenicline (partial α4β2 nAChR agonist and full α7 nAChR agonist) (0.3mg/kg) were co-administered with MDMA in order to investigate the involvement of heteromeric nAChRs in its effects. nAChR ligands effectively attenuated acute MDMA-induced hyperlocomotion. Conversely, after the 10-day sensitizing treatment, locomotor activity reached equivalent values in all groups, suggesting that short-term sensitization was enhanced in animals treated with α4β2 nAChR ligands with respect to the control group (the ratio between locomotor activity on day 10 and day 1 is higher in the former groups,

albeit values on day 10 are equivalent across all groups). Finally, long-term sensitization is significantly attenuated in groups treated with α4β2 nAChR ligands, as only the MDMA-treated group showed significantly increased hyperlocomotion on day 25 with respect to day 10.

In parallel, α4β2 and α7 nAChR density was measured on days 10 and 25 in the striatum and the cortex. Interestingly, a good correlation was found between α4β2 nAChR levels in the cortex and the development of short- and long-term sensitization; while on day 10, all drug-treated groups showed an up-regulation ranging 25%, on day 25, this increase was only maintained in the MDMA-treated group. No changes were found for α7. All the above evidences a clear role of α4β2 nAChRs in MDMA-induced hyperlocomotion and behavioral sensitization, possibly mediated by differential regulation in the cortex.

Conditioned place preference: The role of α4β2 nAChRs in the development of MDMA-induced conditioned place preference (CPP) was also assessed. Both α4β2 nAChR ligands (2mg/kg varenicline and 2mg/kg DHβE) effectively blocked CPP induced by a moderate dose of MDMA (10mg/kg). Furthermore, a second CPP experiment was performed, where animals were pre-treated with saline or nicotine (2mg/kg) b.i.d. for 14 days, which has been shown to cause a robust α4β2 nAChR up-regulation (Dougherty et al., 2008). Subsequently, animals underwent the CPP protocol for a sub-threshold dose of MDMA (3mg/kg). Only nicotine-pretreated animals showed positive preference. Taken together, current results point to a clear involvement of α4β2 nAChRs in the mediation of MDMA-induced rewarding effects.

Translated to a clinical context, we show nAChR as a potential target for reducing MDMA's sensitizing and rewarding effects. Furthermore, we also postulate that nAChR up-regulation induced by chronic consumption of nicotine could potentiate the ability of other drugs, such as MDMA, to cause addiction due to an enhancement of nAChR-mediated rewarding effects.

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Heteromeric nicotinic receptors are involved in the sensitization and addictive properties of MDMA in mice

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ABSTRACT

We have investigated the effect of nicotinic receptor ligands in the behavioral sensitization (hyperlocomotion) and rewarding properties (conditioned place preference paradigm, CPP) of 3,4-methylenedioxy-methamphetamine (MDMA) in mice. Each animal received intraperitoneal pretreatment with either saline, dihydro-β-erythroidine (DHβE, 1 mg/kg) or varenicline (VAR, 0.3 mg/kg), 15 min prior to subcutaneous saline or MDMA (5 mg/kg), for 10 consecutive days. On day 1, both DHβE and VAR inhibited the MDMA-induced hyperlocomotion. After 10 days of treatment, MDMA induced a hyperlocomotion that was not reduced (rather enhanced) in antagonist-pretreated animals. This early hyperlocomotion was accompanied by a significant increase in heteromeric nicotinic receptors in cortex that was not blocked by DHβE or VAR. Behavioral sensitization to MDMA was highest 2 weeks after the discontinuation of MDMA treatment. This additional increase in sensitivity was prevented in animals pretreated with DHβE or VAR. At this time, MDMA-treated mice showed a significant increase in heteromeric receptors in cortex that was prevented by DHβE and VAR. An involvement of α7 nicotinic receptors in this effect is ruled out.

MDMA (10 mg/kg) induced positive CPP that was abolished by DHβE (2 mg/kg) and VAR (2 mg/kg). Moreover, chronic nicotine pretreatment (2 mg/kg, ip, b.i.d., for 14 days) caused MDMA, administered at a low dose (3 mg/kg), to induce CPP, which would otherwise not occur. Finally, present results point out that heteromeric nicotinic receptors are involved in locomotor sensitization and addictive potential induced by MDMA. Thus, varenicline might be a useful drug to treat both tobacco and MDMA abuse at once.

1. Introduction

MDMA is a synthetic drug that has properties of both stimulants and hallucinogens. Compared to other amphetamine derivatives, MDMA triggers a larger increase in serotonin and a smaller increase in dopamine release (Johnson et al., 1986). The behavioral and neurochemical adaptations related to chronic MDMA treatment are largely unknown. For instance, an increase in the functionality of cortical 5-HT_{2A} and a decrease in striatal D_2 receptors in mice treated with MDMA have been described (Varela et al., 2011). Many drugs of abuse, at low doses, can increase motor behavior producing heightened locomotion and exploration (Wise and Bozarth, 1987) and, after repeated administration, behavioral sensitization can arise from various neuroadaptations in multiple brain nuclei. This is not only the result of distinct molecular targets for the drugs, but may also include a differential involvement of learned associations. It is postulated that the relatively more robust pharmacological capacity of amphetamine derivatives to release dopamine may induce a form of sensitization that is more dependent on adaptations in mesoaccumbens dopamine transmission in comparison to cocaine and morphine sensitization (Vanderschuren and Kalivas, 2000).

There is evidence that acetylcholine plays an important role in the hyperlocomotor activity induced by psychostimulants (Williams and Adinoff, 2008). Dihydro-β-erythroidine (DHβE), a high-affinity competitive antagonist of α4 subunit-containing nAChR (nicotinic acetylcholine receptor) inhibits the induction of locomotor sensitization to d-amphetamine (Karler et al., 1996; Schoffelmeer et al., 2002). Moreover, knockout mice lacking the β2 nAChR subunit do not self-administer nicotine (Picciotto et al., 1999) and show less cocaine-conditioned place preference than wild-type mice (Zachariou et al., 2001). All of these results indicate that heteromeric α4β2 nAChR subtypes appear to play an essential role in nicotine dependence (Govind et al., 2009); in this regard, an activation of α4β2 nAChR is strongly associated with dopamine release in the nucleus accumbens (NAcc) (Champtiaux et al., 2003) and with drug-seeking behavior (Balfour et al., 2000; Picciotto et al., 1999). A particular feature of nAChR is

Abbreviations: AUC, area under the curve; CPP, conditioned place preference; DHβE, dihydro-β-erythroidine; MDMA, 3,4-methylenedioxy-methamphetamine; MLA, methyllycaconitine; NAcc, nucleus accumbens; nAChR, nicotinic acetylcholine receptors; VAR, varenicline; VTA, ventral tegmental area.

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that chronic exposure to nicotine and other nicotinic ligands induces a higher level of epibatidine binding (up-regulation) that can lead to an increase in receptor function (functional up-regulation) (reviewed by Gaimarri et al., 2007). Therefore, the up-regulation of heteromeric nAChR could, via dopamine release, explain the reinforcing effect of nicotine on the mesolimbic system mediating nicotine addiction (Balfour et al., 2000).

Studies examining the interactions between nAChR and psychostimulant drugs have focused primarily on d-amphetamine and cocaine but it is unclear whether such findings can be extended to other psychostimulants. Previous results from our group (for a review see Pubill et al., 2011) have demonstrated that nAChR are a pharmacological target for both methamphetamine and MDMA and are involved in some actions of these drugs of abuse such as analgesia or locomotor activity (Camarasa et al., 2009), tumor necrosis factor alpha suppression (Camarasa et al., 2010) and neurotoxicity (Chipana et al., 2008b; 2008c; Escubedo et al., 2009). We have described the direct and specific interaction of MDMA with α7 and α4β2 nAChR in mouse brain membranes and cultured PC12 cells (García-Ratés et al., 2007). The interaction with nAChR occurs at low micromolar concentrations that can be reached in the mammalian central nervous system after its administration (Chipana et al., 2008a). Also, similarly to nicotine, MDMA induces nAChR up-regulation in PC12 cells and in rat brain, where it also potentiates the regulatory effects of nicotine (García-Ratés et al., 2007; Pubill et al., 2013).

MDMA's interaction with nAChR might account for some clinical features of this drug such as fasciculation and muscle cramps, which occur especially in MDMA abusers after high-dose intake (Klingler et al., 2005). Moreover, tobacco is one of the most widely consumed drugs and MDMA abusers very often smoke (Scholey et al., 2004); thus, a pharmacodynamic interaction between nicotine and MDMA can be expected and could have several consequences that will be suggested at a later point in this text.

This study was undertaken to determine whether nAChR are involved in the behavioral sensitization and addictive potential of MDMA. DHβE (antagonist) and varenicline (partial α4β2 nAChR agonist and full α7 nAChR agonist; Mihalak et al., 2006; Rollema et al., 2007) were associated with MDMA in order to investigate the involvement of heteromeric nAChRs on its effects. Also, the effect of a chronic pretreatment with nicotine on MDMA addictive effects was investigated. We focused on the locomotor hyperactivity induced by MDMA as an indicator of its psychostimulant effect and on the conditioned place preference (CPP) paradigm to assess its addictive properties. Also, we investigated the changes in the density of homomeric and heteromeric nAChR in determined brain areas as a possible consequence of the treatment that could be related with the observed behavioral effects.

2. Material and methods

2.1. Animals and treatment groups

Data were collected from adult male Swiss CD-1 mice (Charles River, Barcelona, Spain) weighing 24 to 30 g at the beginning of the experiments (first drug administration). They were housed three per cage under standard laboratory conditions (21 ± 1 °C room temperature and a 12-h light/dark cycle from 8:00 am to 8:00 pm). Animals had free access to food (standard laboratory diet, PANLAB SL, Barcelona, Spain) and drinking water. All experimental procedures were conducted between 9:00 am and 5:00 pm and were in compliance with the guidelines of the European Community Council (86/609/EEC) and approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia. Efforts were made to minimize suffering and reduce the number of animals used.

In our experiments we administered MDMA at doses closely related to its recreational use in humans rather than at high doses that would lead to neurotoxic effects.

Mice were assigned randomly to one of six treatment groups: saline (saline i.p. + saline s.c.), MDMA (saline i.p. + MDMA s.c.), DHβE (DHβE i.p. + saline s.c.), DHβE + MDMA (DHβE i.p. + MDMA s.c.), VAR (saline i.p. + varenicline s.c.), VAR + MDMA (varenicline i.p. + MDMA s.c.). Doses and schedule are detailed below.

Prior to experimentation, all of the animals received two habituation sessions (48 and 24 h before testing) that were intended to reduce the novelty and stress associated with handling and injection.

2.2. Drugs

Drugs and reagents were obtained from the following sources: 3,4-methylenedioxymethamphetamine hydrochloride was provided by the National Health Laboratory (Barcelona, Spain). Varenicline was a gift from Pfizer Laboratories (New York, USA). Aprotinin, DHβE, methyllycaconitine (MLA), nicotine bitartrate dihydrate, phenylmethylsulfonyl fluoride and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]epibatidine was from PerkinElmer (Boston, MA, USA), while [^{3}H]MLA came from American Radiolabeled Chemicals (St. Louis, MO, USA). Drugs were dissolved in saline (NaCl 0.9%). All other reagents were of analytical grade.

2.3. Locomotor activity

This test was used to assess the psychostimulant effects of MDMA along the treatment and its modulation by nicotinic drugs.

2.3.1. Drug treatment

According to its treatment group allocation, each animal received pretreatment with either saline (5 ml/kg), DHβE (1 mg/kg) or varenicline (0.3 mg/kg), given intraperitoneally, 15 min prior to saline or MDMA (5 mg/kg), given subcutaneously, for 10 consecutive days. These doses were chosen based on previous reports (Camarasa et al., 2009; Kim et al., 2011). We administered MDMA at a 5 mg/kg dose because, although it is relatively low, it induces robust behavioral activation (Ball et al., 2009). Once the 10-day repeated treatment phase was completed, all of the animals remained in their home cages for a 14-day drug-free period (days 11–24). On day 25, all of the mice were accordingly challenged with either a dose of saline or DHβE or varenicline plus saline or MDMA to assess for conditioned hyperactivity. Locomotor activity was measured on days 1, 10 and 25. To evaluate the development of behavioral sensitization we compared data from day 1 vs day 10 or day 25 of the same group.

2.3.2. Measurement

On the different testing days and immediately after the i.p. injection (saline or MDMA), the mice were placed in a Plexiglas cage. This cage constituted the activity box that was placed inside a frame system of two sets of 16 infrared photocells (LE8811, PANLAB SL, Barcelona, Spain) mounted according to the x, y axis coordinates and 1.5 cm above the wire mesh floor. The registration of horizontal locomotor activity then began. Occlusions of the photo beams were recorded and sent to a computerized system (SedaCom32, PANLAB SL, Barcelona, Spain). The interruption counts (beam breaks), in a 10-min block, were used as a measure of horizontal locomotor activity. The locomotor activity of each mouse was monitored over 180 min. All experiments were conducted between 9:00 am and 3:00 pm. Results are expressed as cumulative breaks per mouse for 180 min or as AUC (area under the curve), which was measured as the total changes from baseline at each recording interval over the total measuring time.

2.4. Radioligand binding experiments

2.4.1. Tissue sample preparation

Six hours after the challenge with MDMA on day 10 or on day 25, 5–6 animals per group were killed by cervical dislocation, then decapitated and the brains rapidly removed from the skull. Cortex, striata and hippocampus were quickly dissected out, frozen on dry ice and stored at −80 °C until use. When required, tissue samples were thawed and homogenized at 4 °C in 10 volumes of buffer consisting of 5 mM Tris–HCl, 320 mM sucrose and protease inhibitors (aprotinin 4.5 mg/ml, 0.1 mM PMSF and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogenizer. The homogenates were centrifuged at 15,000 × *g* for 30 min at 4 °C. The pellets were resuspended in fresh buffer and incubated at 37 °C for 10 min to remove endogenous neurotransmitters. The protein samples were subsequently re-centrifuged and washed two additional times. The final pellets (crude membrane preparations) were resuspended in 50 mM Tris–HCl buffer plus protease inhibitors and stored at −80 °C until later 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.

2.4.2. [^{3}H]Epibatidine binding

[^{3}H]Epibatidine binding was used to label heteromeric nAChR, which in CNS are mainly α4β2. Binding of [^{3}H]epibatidine to brain membranes from cortex and striatum was measured as described previously (Chipana et al., 2008b). Briefly, experiments were carried out in glass tubes containing 1 nM [^{3}H]epibatidine (55.5 Ci/mmol)–at this concentration primarily α4β2 receptors are labeled (Avila et al., 2003)–and incubation was carried out for 3 h at 25 °C. The incubation buffer was 50 mM Tris–HCl plus protease inhibitors. Non-specific binding was determined in the presence of 300 μM nicotine. Binding was terminated by filtration, and data were treated as described below.

2.4.3. [^{3}H]MLA binding

[^{3}H]MLA binding was used to quantify homomeric α7 nAChR. Binding of [^{3}H]MLA to brain hippocampal membranes was measured as described by Davies et al. (1999). Briefly, 0.25 ml of membranes (containing 200 μg of brain membranes) was incubated in borosilicate glass tubes with 2 nM [^{3}H]MLA (60 Ci/mmol), in a final volume of 0.5 ml for 2 h at 4 °C. The incubation buffer consisted of 50 mM Tris–HCl, 120 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.1% bovine serum albumin. Non-specific binding was determined from tubes containing 1 μM unlabeled MLA. Incubation was completed by rapid filtration under vacuum through Whatman GF/B glass fiber filters (Whatman Intl. Ltd., Maidstone, UK) pre-soaked in 0.5% polyethyleneimine. Tubes and filters were washed rapidly 3 times with 4 ml ice-cold 50 mM Tris–HCl and the radioactivity trapped was measured by liquid scintillation spectrometry. Specific binding was calculated as the difference between the radioactivity measured in the absence (total binding) and in the presence (non-specific binding) of the excess of non-labeled ligand, and expressed as the percentage of that obtained from saline-treated mice.

2.5. Conditioned place preference (CPP) paradigm

The place conditioning protocol used was non-biased (Robledo et al., 2004). The apparatus was composed of three distinct compartments separated by manually operated doors. The central compartment (corridor) measured 27 × 10 × 25 cm (w × d × h) and served as a thoroughfare between the two pairing sides. The pairing compartments are 20 × 20 × 25 cm (w × d × h). One compartment had black and white checkered walls with a smooth and shiny floor. The other compartment had white and light blue painted walls and rough flooring. The light intensity within the conditioning chambers was 30 lx. CPP was performed in three phases: preconditioning, conditioning and test. During the pre-conditioning phase (day 1), naive or nicotine pre-treated mice 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 time spent in each compartment was recorded by computerized monitoring software (Smart Junior, PANLAB SL, Barcelona, Spain). During the conditioning phase (days 2, 4, 6 and 8), mice were treated with MDMA (3 and 10 mg/kg, s.c.), or saline, 20 min before being confined into one of the two conditioning compartments for 30 min. On days 3, 5, 7 and 9 of the conditioning phase, animals received saline and were confined to the opposite compartment. The animals were exposed to only one pairing per day and treatments were counterbalanced to assure that some animals received MDMA in the black and white compartment while others received MDMA in the white and light blue compartment.

Control animals received saline every day. For conditioning studies with DHβE or varenicline, these drugs or saline were administered intraperitoneally 15 min before MDMA, at doses previously described as effective in antagonizing nicotine-induced CPP (2 mg/kg) (Biala et al., 2010; Walters et al., 2006). The test phase (day 10) was conducted identically to the preconditioning phase; animals were drug-free and had free access to the three compartments for 20 min.

To investigate whether nicotine (administered in a previous chronic treatment) potentiates MDMA-induced CPP, nicotine was given intraperitoneally at a dose of 2 mg/kg (Dougherty et al., 2008) b.i.d. for 14 days. The day after, nicotine was withdrawn and preconditioning for CPP was started with MDMA at a dose of 3 mg/kg as above. A preference score was expressed in seconds and calculated for each animal as the difference between the times spent in the drug-paired compartment in the post-test minus the time spent in the pre-conditioning phase.

2.6. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were compared using two-tailed one-way analysis of variance (ANOVA). Significant ($p < 0.05$) differences were then analyzed by Tukey's post hoc test for multiple means comparisons, where appropriate. AUC values were calculated by nonlinear regression using GraphPAD Prism (GraphPAD software, San Diego, CA, USA). All statistic calculations were performed using Graph Pad Instat (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Effect of nAChR ligands on induction of behavioral sensitization to MDMA

Locomotor activity was used to measure behavioral sensitization to MDMA in the different treatment groups through time. On day 1 an acute challenge of MDMA (5 mg/kg) produced significantly greater locomotor activity than saline alone (total breaks (TB): 3423 ± 267 saline, 4870 ± 244 MDMA, $p < 0.001$). This psychostimulant effect was fully abolished by pretreatment with DHβE or varenicline ($F_{5,89} = 6.92$, $p < 0.001$, see Fig. 1, Table 1). DHβE and VAR control groups revealed the absence of effect of these drugs alone on locomotor activity.

Similarly, on day 10, one-way ANOVA showed a significant effect of treatment ($F_{5,89} = 23.04$, $p < 0.001$). Daily exposure to MDMA or DHβE + MDMA or varenicline + MDMA revealed sensitization, expressed as a significant increase in the psychostimulant effect of MDMA. The inhibitory effect of DHβE and varenicline observed in the acute challenge of MDMA on day 1 was not present after 10 consecutive days of treatment. Day10/day1 ratio of total breaks ($F_{2,41} = 175.92$, $p < 0.001$; 136.32 ± 3.24% MDMA, 169.23 ± 3.10% DHβE + MDMA and 225.29 ± 2.59% VAR + MDMA) revealed that these drugs

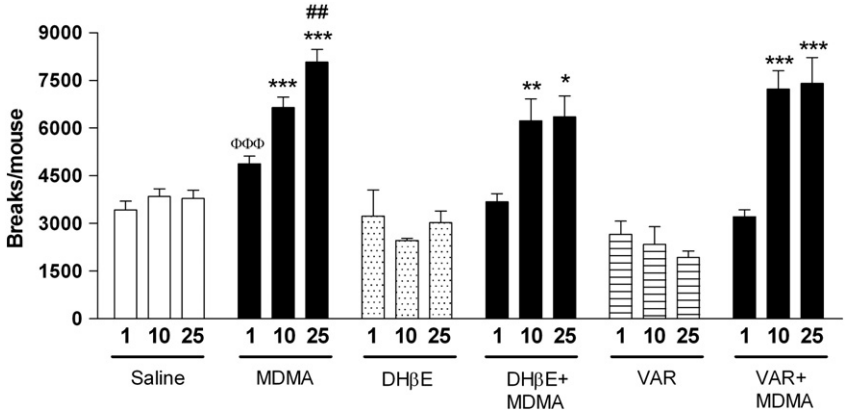

Fig. 1. Cumulative breaks after 180 min for the effect of saline, DHβE (1 mg/kg), or varenicline (VAR) (0.3 mg/kg) on saline/MDMA (5 mg/kg)-induced hyperlocomotion. Locomotor activity was measured on day 1 (acute challenge), day 10 (after a daily dose for ten days) and day 25 (acute challenge of saline, DHβE or varenicline plus saline or MDMA after 14-day withdrawal). Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different from day 1 of the same treated group. $^{\#\#}p < 0.01$ significantly different from day 10 of the same treated group. $^{\Phi\Phi\Phi}p < 0.001$ significantly different from saline day 1.

enhanced rather than attenuated this early sensitization. As on day 1 the animals treated with DHβE/VAR alone denoted the absence of effect of these antagonists on locomotor activity on day 10.

Behavioral sensitization was monitored up to 2 weeks after the discontinuation of MDMA treatment. Analysis of results on day 25 to assess conditioned hyperactivity showed an overall significant difference among treated groups ($F_{5,74} = 37.25$, $p < 0.001$, see Fig. 1, Table 1). A challenge dose of MDMA induced a stronger behavioral response than that administered on day 10 (day 25: 8075 ± 404; day 10: 6639 ± 332; $p < 0.01$). DHβE- or varenicline-pretreated mice showed a response on day 25 that did not differ from that on day 10 (see Fig. 1). These results were assessed when analyzing day 25/day 10 ratio of total breaks ($F_{2,23} = 7.12$, $p < 0.01$: 118.12 ± 2.49% MDMA, 105.81 ± 3.02% DHβE + MDMA $p < 0.01$ vs MDMA and 108.00 ± 2.86% VAR + MDMA $p < 0.05$ vs MDMA). Differences between total breaks on day 25 and total breaks on day 10, confirm the results ($F_{2,23} = 29.15$ $p < 0.001$; 1436 ± 163 MDMA, 128 ± 12 DHβE + MDMA, varenicline + MDMA = 193 ± 18).

Table 1
Effect of DHβE (1 mg/kg) and varenicline (VAR) (0.3 mg/kg) on MDMA (5 mg/kg)-induced locomotor sensitization in mice. Locomotor activity was measured on day 1 (acute challenge), day 10 (after a daily dose for ten days) and day 25 (acute challenge of saline, DHβE or varenicline plus saline or MDMA after 14-day withdrawal). Results are expressed as mean ± S.E.M. of the total area under the curve (AUC) over a period of 180 min (left column) and the time during which a significant hyperlocomotion was present (right column).

Drug	Locomotor activity	
	Total AUC	Hyperlocomotion for (min)
Day 1		
Saline	71,192 ± 6915	60
MDMA	114,874 ± 16034[a]	150
DHβE + MDMA	86,100 ± 6782[b]	90
VAR + MDMA	77,246 ± 4932[b]	60
DHβE	61,718 ± 8959	60
VAR	44,405 ± 5329	60
Day 10		
Saline	79,914 ± 8790	60
MDMA	161,774 ± 22363[c]	150
DHβE + MDMA	147,198 ± 19630[c]	120
VAR + MDMA	197,120 ± 11987[d]	120
DHβE	47,325 ± 1819	30
VAR	47,097 ± 6898	60
Day 25		
Saline	78,143 ± 8768	60
MDMA	190,550 ± 20777[d]	150
DHβE + MDMA	156,582 ± 18953[a]	90
VAR + MDMA	211,860 ± 22595[c]	90
DHβE	58,315 ± 6665	60
VAR	39,740 ± 3902	60

[a] 0.05 vs saline.
[b] $p < 0.01$ vs MDMA.
[c] 0.01 vs saline.
[d] $p < 0.001$ vs saline.

3.2. Effect of nAChR ligands on the density of nicotinic receptor subtypes in different mouse brain areas

Due to the effects observed in locomotor activity experiments, the density of nAChR was measured in several brain areas of the same animals in order to establish a possible relationship between such effects and changes in receptor populations. 5 animals of each treatment group were killed on day 10 after treatment and locomotor activity measurement, while the rest were kept to obtain the results on day 25.

Treatment with MDMA, DHβE or varenicline for 10 days induced a significant increase in [^{3}H]epibatidine binding in cortex, compared with those receiving saline alone ($F_{5,34} = 2.908$, $p < 0.05$). DHβE also induced such an increase in the striatum. In this area, MDMA did not modify [^{3}H]epibatidine binding and did not alter the increase in heteromeric nAChR expression induced by DHβE. Moreover, pretreatment with varenicline significantly reduced [^{3}H]epibatidine binding in mouse striatum; this was not altered by MDMA ($F_{5,29} = 27.231$, $p < 0.001$) (Fig. 2B).

After the 14-day drug-free period, the mice treated previously with MDMA (but not those pretreated only with DHβE or varenicline alone), showed a significant increase in heteromeric nAChR density in cortex and striatum. The cortical increase in [^{3}H]epibatidine binding was not present in animals which received pretreatment with DHβE or varenicline ($F_{3,21} = 18.936$, $p < 0.001$) (Fig. 3A). Only pretreatment with DHβE prevented the up-regulation induced by MDMA in striatum ($F_{3,23} = 3.376$, $p < 0.05$) (Fig. 3B).

When analyzing the density of homomeric α7 nAChR in hippocampus, where they are more highly expressed, no differences in receptor densities, measured as [^{3}H]MLA binding, were found in MDMA-treated mice (Fig. 4).

3.3. Effect of nAChR ligands on the acquisition of MDMA-induced CPP

The CPP paradigm was used to study the effect of the different treatments on the addictive/rewarding properties of MDMA.

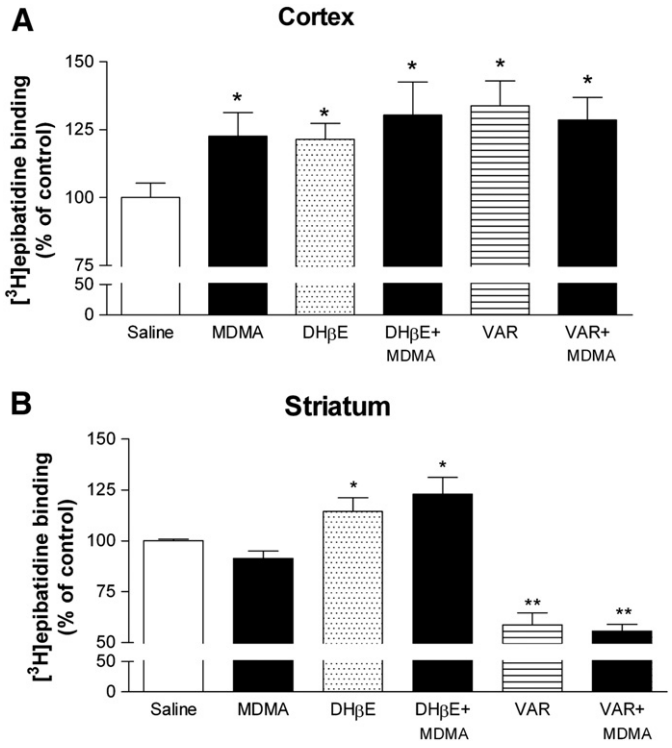

Fig. 2. Effect of treatment with saline, DHβE (1 mg/kg), or varenicline (VAR) (0.3 mg/kg) plus saline or MDMA (5 mg/kg) during 10 consecutive days on α4β2 nAChR density (measured as [^{3}H]epibatidine binding) in mouse cortex (panel A) or striatum (panel B). Data are expressed as mean ± SEM from the values obtained from 5–6 animals per group. $^{*}p < 0.05$ and $^{**}p < 0.01$, significantly different from saline-treated group.

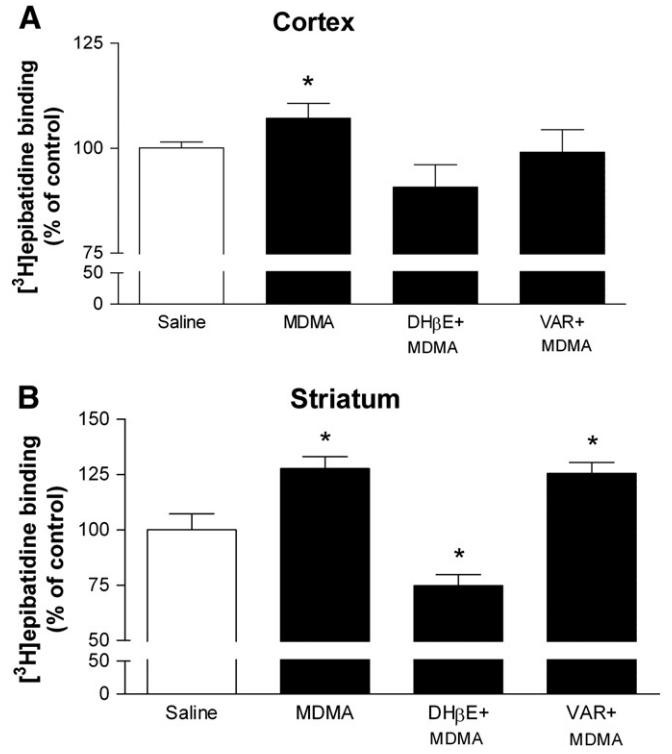

Fig. 3. Effect of a 14 day withdrawal after a 10 consecutive day treatment with saline, DHβE (1 mg/kg), or varenicline (VAR) (0.3 mg/kg) plus saline or MDMA (5 mg/kg) on α4β2 nAChR density (measured as [^{3}H]epibatidine binding) in mouse cortex (panel A) or striatum (panel B). On day 25, mice were killed 6 h after receiving the assigned treatment and their brains were used for this experiment. Data are expressed as mean ± SEM from the values obtained from 5–6 animals per group. $^{*}p < 0.05$ significantly different from saline-treated group.

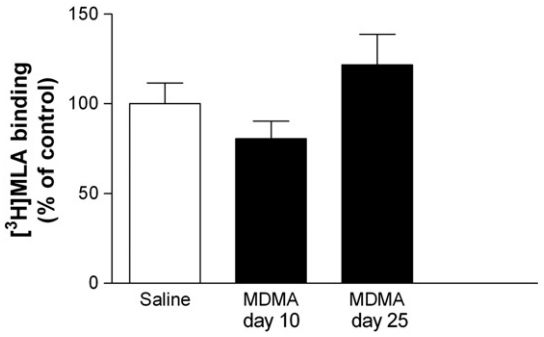

Fig. 4. Effect of MDMA (5 mg/kg) alone for 10 consecutive days (day 10) or after a 14 day withdrawal period (day 25) on α7 nAChR density (measured as [^{3}H]MLA binding) in mouse hippocampus. Data are expressed as mean ± SEM from the values obtained from 5–6 animals per group.

Throughout all experiments, a within-subject comparison revealed that mice had no bias. Time (in seconds) spent in both compartments during pre-conditioning were 367.58 ± 56.70 and 326.05 ± 35.69, indicating a lack of preference for either side. This did not significantly change in the test session (309.12 ± 35.14 and 276.19 ± 28.73) when saline was paired with both compartments during the conditioning phase.

We first investigated the effect of varenicline and DHβE in the CPP induced by MDMA (10 mg/kg). On the test day (day 10, post-conditioning), one-way ANOVA revealed a significant effect of treatment ($F_{5,36} = 4.56$, $p < 0.01$). The ability of MDMA to produce a CPP was assessed while some mice were under the influence of DHβE or varenicline (2 mg/kg) treatment, administered 15 min before the MDMA dose. Both reduced MDMA's ability to produce a CPP, fully blocking MDMA's effects ($p < 0.05$ for varenicline and $p < 0.01$ for DHβE vs. MDMA-treated mice) (Fig. 5B). Neither DHβE nor varenicline alone had any effect on CPP.

During the pre-conditioning phase and test day we measured the distance and speed of travel in each of the two compartments. Results corresponding to the drug-paired compartment are shown in Table 2 and demonstrate that treatment with MDMA during the conditioning phase induces an increase in locomotor activity in the test day that is not present in animals pretreated with varenicline or DHβE. This increase in locomotor activity was not accompanied by an increase in speed and confirms a psychostimulant effect in these animals.

To explore the effect of a chronic nicotine treatment on the addictive behavior caused by a low dose of MDMA (3 mg/kg) which is not supposed to induce CPP when given alone (Robledo et al., 2004), we pretreated mice with nicotine at a dose of 2 mg/kg, given subcutaneously (b.i.d.) for 14 days. This treatment induced a significant

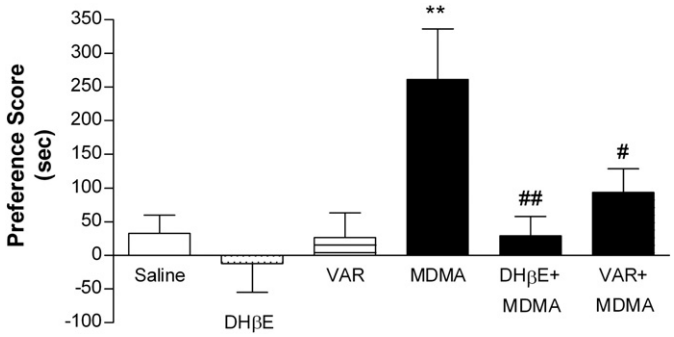

Fig. 5. Effect of DHβE (2 mg/kg) and varenicline (VAR) (2 mg/kg) alone and on MDMA (10 mg/kg)-induced conditioned place preference. The *x-axis* represents the treatment group and the *y-axis* represents the preference score (test day minus preconditioning day) in seconds. $^{**}p < 0.01$, significantly different from saline-treated group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, significantly different from the corresponding value of MDMA-treated group.

Table 2
Distance traveled and the speed in the drug-paired compartment measured in the pre-conditioning day and in the test day (absence of drug treatment). Results are expressed as mean ± standard error of the mean from 8 different animals.

Drug treatment	Distance traveled (cm)		Speed (cm/s)	
	Pre-conditioning	Test	Pre-conditioning	Test
Saline	1112.23 ± 176.39	1120.03 ± 143.73	3.30 ± 1.19	2.90 ± 0.97
MDMA	1329.62 ± 51.62	2063.11 ± 55.46[a]	3.63 ± 0.23	3.04 ± 0.43
VAR + MDMA	1660.90 ± 178.57	1953.87 ± 154.16	4.14 ± 0.91	3.66 ± 0.66
DHβE + MDMA	1664.25 ± 61.64	1872.36 ± 151.60	3.62 ± 0.24	3.71 ± 0.13

[a] $p < 0.01$ vs. the corresponding value of the preconditioning day (paired Student's *t*-test).

increase in α4β2 nAChR density in the striatum (147.98 ± 13.13%, nicotine-treated vs 100.00 ± 10.56%, saline-treated, $p < 0.05$, Student's *t* test). This nicotine treatment schedule did not induce a significant CPP on its own (Dougherty et al., 2008) and, therefore, at the end of the nicotine treatment, animals did not show preference for either of the two compartments (445.85 ± 69.28 vs 551.02 ± 27.82). Repeated nicotine administration during the 14 days prior to pre-conditioning led to a decreased MDMA threshold for CPP. As reflected in Fig. 6, when animals were exposed to chronic nicotine pretreatment, they showed a positive preference score at a dose of MDMA (3 mg/kg) that proved to be ineffective when administered alone ($F_{2,23} = 5.808$, $p < 0.01$).

4. Discussion

This study examines the involvement of heteromeric nAChR in the behavioral sensitization as well as the addictive potential of MDMA in mice. The results indicate that an antagonism or a partial agonism on nAChR reduces the addiction, blocks the acute locomotor effects and changes the development of sensitization induced by MDMA. α4β2 nAChR appear to mediate these effects given that DHβE and varenicline, but not MLA (data not shown), antagonized the acute effects of MDMA. In fact, previous studies (Walters et al., 2006) have demonstrated that MLA at doses of 5 and 10 mg/kg (s.c), does not inhibit nicotine-induced CPP, ruling out an involvement of the α7 nAChR in this behavior.

The psychomotor stimulant effect of MDMA is considered subsequent to an extracellular increase in DA and 5-HT in the NAcc and VTA (Bankson and Cunningham, 2001). In a previous study we demonstrated the involvement of nicotinic receptor subtypes in the hyperlocomotion induced by methamphetamine (Camarasa et al., 2009). Here we report that the stimulant effects of an acute dose of MDMA are blocked by antagonists acting on α4β2 nAChR. Nicotinic agonists can differentially affect neurotransmitter release in a given brain region and the magnitude of such responses will largely be determined by the subtype selectivity of the agonist (Rao et al., 2003).

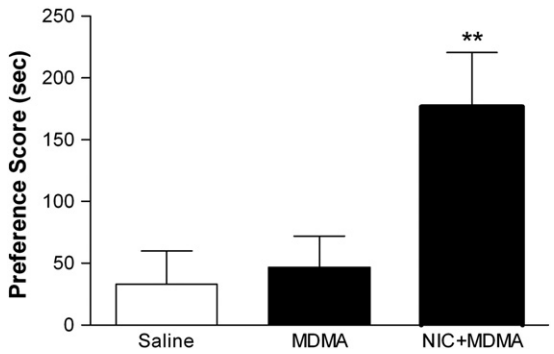

Fig. 6. Effect of a 14 day chronic nicotine pretreatment (2 mg/kg, b.i.d.) on the conditioned place preference assay on MDMA (3 mg/kg). Data are expressed as mean ± SEM. **$p < 0.01$, significantly different from saline- or MDMA-treated groups.

Nicotine activates nAChR localized in the dopaminergic nerve terminals in the nucleus accumbens and elicits a complex pattern of inhibitory–stimulatory effects on locomotion (Avale et al., 2008).

Although there are subtle differences between MDMA and other commonly abused amphetamines, a clear overlap in the behavioral pharmacology of MDMA and other amphetamine-like compounds can be found, especially in the induction of behavioral excitation. In rodents, this effect, called behavioral sensitization, persists many months after the last administration, thus mimicking long-term sensitivity to drugs observed in human addicts. Expression of this persistent drug-induced behavioral sensitization has been suggested to contribute to craving and high relapse rates in addicts (Robinson and Berridge, 2003). Studies of the neurobiological basis of behavioral sensitization have focused on the increased capacity of these drugs to release dopamine in the midbrain dopamine system (Cadoni et al., 2000) although multiple limbic-associated areas such as the prefrontal cortex provide the excitatory cortical innervation to the NAcc (Kita and Kitai, 1990). This dopaminergic system mediates locomotor stimulation as well as the ability of drugs to elicit craving and lead to abuse.

When MDMA was administered daily for 10 consecutive days, there was an increase in the hyperlocomotion induced by this drug on day 10 in respect to that measured on day 1 (early behavioral sensitization). These results are in agreement with those previously described in rats (Kalivas et al., 1998) demonstrating that repeated administration of MDMA over the course of ten days produces sensitization to the behavioral stimulant effects of MDMA. Furthermore, the behavioral sensitization in mice was found to be highest after a 2 week-period following the discontinuation of MDMA treatment, (a challenge dose of MDMA showed a stronger behavioral response than on day 10) demonstrating that the treatment schedule of MDMA used in this study induces not only an early but also a delayed sensitization that can be modulated by drugs acting on α4β2 nAChR.

Neither DHβE nor varenicline blocked but rather enhanced the development of early behavioral sensitization by MDMA, conversely to the inhibitory effect observed in the acute challenge (day 1). When comparing the ratios D10/D1 of the different groups, a potentiation was revealed for those treated with MDMA plus DHβE or varenicline. In other words, the groups receiving MDMA plus the nicotinic ligand showed a day-to-day greater increase in locomotion than the group receiving MDMA alone.

The increased delayed sensitization to MDMA was prevented when it was administered together with either the α4β2 nAChR antagonist (DHβE) or the partial agonist (varenicline). It is known that nAChR ligands regulate sensitization to stimulant drugs such as d-amphetamine and cocaine. For instance, DHβE, a high-affinity competitive antagonist of α4 subunit-containing nAChR, attenuates the induction of locomotor sensitization to d-amphetamine, cocaine, ephedrine and methylphenidate in mice and rats (Karler et al., 1996; Miller and Segert, 2005; Schoffelmeer et al., 2002; Wooters and Bardo, 2009). Additionally, the sensitizing effect of acute nicotine on amphetamine-stimulated behavior and dopamine efflux requires activation of β2 subunit-containing nAChRs (Kim et al., 2011).

Varenicline is an effective aid in smoking cessation. This drug, by acting on α4β2 nAChR, stimulates dopamine release when the basal

tone is depressed and simultaneously blocks the effects of a full agonist when simultaneously present. Partial agonists aim to provide a low-to-moderate level of dopamine stimulation to reduce craving and withdrawal symptoms. When varenicline is administered to nicotine-sensitized rats, it reduces the expression of nicotine sensitization (Zaniewska et al., 2008). Similarly, in our experiments, varenicline inhibited the increase in the delayed sensitization observed on day 25.

Due to the described dynamic plasticity of nAChR after treatment with nicotinic ligands, we assessed the density of heteromeric (mainly α4β2) and homomeric α7 receptors through radioligand binding studies. The results showed that early sensitization on day 10 was accompanied by changes in α4β2 nAChR density in certain brain areas. MDMA induced in the cortex, but not in the striatum, a significant increase in α4β2 nAChR that was not blocked by DHβE or varenicline. However, the results on day 25 correlate with the in vivo effects: although these animals had a 14-day drug-free period, the increased α4β2 nAChR density in the cortex and striatum was still present in the MDMA group, but not in the animals co-treated with DHβE. Varenicline appears to do the same in the cortex. From these results it can be deduced that the α4β2 nAChR subtype is involved in the early and delayed sensitization elicited by MDMA. If treatment leads to an increase in α4β2 nAChR subtype population in the cortex, the sensitization takes place. By contrast, when this up-regulation is prevented, sensitization is attenuated. The role of the cortex in sensitization is not an exception as it is known that the prefrontal cortex and the hippocampus exhibit converging projections to the NAcc and have functional reciprocal connections via indirect pathways (Day et al., 1991; Goto and Grace, 2008). Medial prefrontal neurons, including those projecting to the NAcc (McGinty and Grace, 2008), are also excited by conditioned stimuli (Laviolette, 2007; Ball et al., 2009) demonstrating that long-lasting locomotor sensitization to MDMA is accompanied by reorganization of synaptic connectivity, not only in NAcc, but also in the medial prefrontal cortex.

Effects derived from changes in α7 nAChR population can be ruled out from present binding studies. The difference between the effects of DHβE and varenicline can be explained by their different pharmacological profile.

Once the correlation between nAChR and behavioral sensitization to MDMA was demonstrated, we examined the effect of α4β2 nAChR ligands as well as that of a nicotine chronic treatment on the CPP score induced by MDMA. In this study we provide evidence that MDMA at a dose of 10 mg/kg, but not 3 mg/kg, causes positive CPP in mice. These results are in agreement with those of Salzmann et al. (2003) and Robledo et al. (2004). Bilsky et al. (1998) demonstrated that the CPP induced by MDMA was effectively blocked by the dopamine release inhibitor CGS10746B. These results and those of Vidal-Infer et al. (2012) demonstrate that, in mice, the dopaminergic system is involved in the acquisition and expression of MDMA-produced CPP. Moreover, results of the present study provide pharmacological evidence of the involvement of the α4-containing nAChR in the CPP induced by MDMA, as this effect was antagonized by DHβE and varenicline.

Acute nicotine challenge induces behavioral sensitization to amphetamines (Birrell and Balfour, 1998; Jutkiewicz et al., 2008) and consequently can enhance its addictive potential. In this study we used a chronic nicotine treatment in order to increase the density of α4β2 nAChR (Dougherty et al., 2008). It is important to note that nicotine treatment took place previously and this drug was not present during the CPP experiments with MDMA, avoiding any interaction on the test day. Abstinence signs of nicotine are dose-dependent and appear at doses equal to or higher than 6.3–8 mg/kg/day (Gould et al., 2012; Isola et al., 1999) and not at 6 mg/kg/day or lower (Damaj et al., 2003), as in our experiments. These signs last for a maximum of 3–4 days (Zhang et al., 2012) and are supplemented with deficits in contextual learning (Gould et al., 2012). In the present study, sustained exposure to nicotine significantly increased MDMA rewarding in the CPP paradigm. While MDMA at a low dose (3 mg/kg) did not induce CPP on its own, this dose of MDMA showed a very significant preference score in nicotine-pretreated mice.

As in the behavioral sensitization experiments, this increase in the CPP score caused by MDMA runs parallel to an increase in α4β2 nAChR density induced by nicotine, pointing to an up-regulation of these receptors as an additional factor in MDMA's reinforcing effect. The up-regulated nAChR could mediate enhanced synaptic transmission when stimulated by local and brief releases of ACh at synapses. Stimulation of dopamine neurons in the VTA via the α4β2 nAChR leads to an increase of dopamine in the NAcc that plays a crucial role in drug reward as measured by CPP (Di Chiara and Imperato, 1998). Consequently, the modulation of dopamine release by means of α4β2 nAChR activation could result in a modification of the CPP induced by MDMA. Although animals were not under the effect of nicotine when tested in the CPP paradigm, and despite the very low dosage of this stimulant administered during the pretreatment phase, we cannot rule out an influence of nicotine withdrawal in the first days of the conditioning phase.

The influence of chronic nicotine treatment on MDMA effects extends not only to CPP but also to its hyperlocomotion properties. In previous studies (Camarasa et al., 2009) we have described that nicotine, when administered in a chronic low-dose schedule, significantly potentiates the methamphetamine-induced increase in locomotor activity and rearing. These results suggest that up-regulation of nAChR leads to a very significant potentiation of the increase in locomotor activity induced by this drug. Similar results were obtained for MDMA-induced hyperlocomotion using the same nicotine pretreatment than in the study with methamphetamine (a 30% potentiation, unpublished results).

A great number of MDMA consumers also smoke concomitantly (Scholey et al., 2004). In view of results obtained in the present paper it can be deduced that smoking can increase neuronal sensitization to MDMA and its addictive potential, making MDMA-users more susceptible to addiction. Although further research must be done on this subject, our results suggest that α4β2 nAChR are a potential target towards treating nicotine and MDMA polyabuse. Although DHβE is a useful pharmacological tool for preclinical studies on nAChR, it is not adequate for clinical use due to its toxicity: it can produce neuromuscular blockade, hypotension and has a very narrow dosage window (the i.p. DL50 in mice is 4.5 mg/kg, Megirian et al., 1955). Also DHβE, as a pure antagonist, can precipitate nicotine abstinence syndrome (Malin et al., 1998). Conversely varenicline, as a marketed drug for smoking cessation with a good security profile, should be taken into consideration as a possible candidate drug.

5. Conclusion

In summary, although it is well known that nAChR are a pharmacological target for understanding the neurotoxic effects of amphetamine derivatives (Chipana et al., 2008c), they are also involved in other behavioral effects of these drugs such as hyperlocomotion and addictive properties. This paper demonstrates the involvement of specific α4-containing nAChR subtypes by using specific modulators of these receptors. Our results point out that the effects induced by MDMA such as locomotor sensitization and addictive potential, both related with the release of dopamine, are modulated by DHβE and varenicline. Consequently, varenicline, a commercial drug used to treat tobacco addiction, could also be considered for treating MDMA abuse. Finally, these results may have clinical implications because MDMA abusers are often smokers; in this regard, varenicline would be the first useful drug to simultaneously treat both tobacco and MDMA abuse.

Contributors

JC and EE were responsible for the study concept and design. AC, JC and DP contributed to the acquisition of animal data. JC and DP

performed data analysis. EE interpreted the findings and provided critical revision of the manuscript.

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Previously, our research group had demonstrated that MDMA is capable of up-regulating α4β2 receptors in PC12 cells (García-Ratés et al., 2007) and *in vivo*, after both a neurotoxic and a long-term treatment schedule (Pubill et al., 2013; Ciudad Roberts et al., 2013). In the present study, we sought to map where MDMA produces this effect. Additionally, we aimed to determine whether this phenomenon is due to transcriptional changes or post-transcriptional protein modifications, as occurs after a protracted nicotine treatment (Kuryatov et al., 2005; Vallejo et al., 2005).

For this purpose, adolescent (200g) Sprague-Dawley rats were treated following a protracted schedule of MDMA, consisting of 10mg/kg b.i.d. for 10 days; control animals underwent the same treatment, and were administered saline throughout the entire protocol. The *in-vivo* treatment was run twice, as each biochemical assay required a different tissue pre-treatment.

In the first experiment, [^{3}H]epibatidine radioligand binding studies confirmed that gross brain areas undergo α4β2 nAChR up-regulation after exposure to treatment; furthermore, no changes were detected in Western blot of α4 subunit density, pointing to the hypothesis that, as occurs with nicotine, up-regulation is in fact due to post-translational modifications, rather than transcriptional changes.

The second experiment focused on mapping the exact regions in which α4β2 up-regulation takes place, by means of autoradiography binding of [^{125}I]epibatidine to brain slices.

In control animals, [^{125}I]epibatidine labeled heteromeric nAChRs in accordance with the established patterns in previous publications (i.e. Nguyen et al., 2003; Tribollet et al., 2004). Intermediate nAChR levels were found in the cortex, striatum, thalamic nuclei, geniculate nuclei and substantia nigra. Receptor density was most intense in the superior colliculi, medial habenula and interpeduncular nucleus, while the hippocampus and hypothalamus showed the lowest nAChR levels.

We demonstrate nAChR up-regulation in key areas involved in addiction, such as the ventral tegmental area, the nucleus accumbens and several areas of the cortex that are involved in sensory and motor functions (i.e. auditory, somatosensory,

motor), as well as in the olfactory tubercle, which is heavily interconnected with several affective, reward and motivation related centers of the brain. The nigrostriatal pathway was also affected by nAChR up-regulation, as a 33% increase was found in the substantia nigra as well as a 16% increase in the anterior caudate–putamen.

Interestingly, we found a good relationship between brain areas that showed a significant α4β2 up-regulation in our study and those areas with higher serotonin transporter (SERT) density (Battaglia et al., 1991), suggesting that there might be a regulatory interaction between SERT and nAChRs, in which the degree of the variation in nAChR density is dictated by the amount of MDMA which can be internalized in the synaptic terminal by SERT.

In sum, in this study we demonstrate that a protracted treatment with MDMA induces an up-regulation of heteromeric nAChRs in key areas of the rat brain involved in reward, motivation and learning, which could account, at least in part, for the reinforcing properties of this amphetamine derivative, as well as some neuropsychiatric disorders related to its chronic use.

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Protracted treatment with MDMA induces heteromeric nicotinic receptor up-regulation in the rat brain: An autoradiography study

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ABSTRACT

Previous studies indicate that 3,4-methylenedioxy-methamphetamine (MDMA, ecstasy) can induce a heteromeric nicotinic acetylcholine receptor (nAChR, mainly of α4β2 subtype) up-regulation. In this study we treated male Sprague–Dawley rats twice-daily for 10 days with either saline or MDMA (7 mg/kg) and sacrificed them the day after to perform [^{125}I]Epibatidine binding autoradiograms on serial coronal slices. MDMA induced significant increases in nAChR density in the substantia nigra, ventral tegmental area, nucleus accumbens, olfactory tubercle, anterior caudate–putamen, somatosensory, motor, auditory and retrosplenial cortex, laterodorsal thalamus nuclei, amygdala, postsubiculum and pontine nuclei. These increases ranged from 3% (retrosplenial cortex) to 30 and 34% (amygdala and substantia nigra). No increased α4 subunit immunoreactivity was found in up-regulated areas compared with saline-treated rats, suggesting a post-translational mechanism as occurs with nicotine. The heteromeric nAChR up-regulation in certain areas could account, at least in part, for the reinforcing, sensitizing and psychiatric disorders observed after long-term consumption of MDMA.

1. Introduction

3,4-Methylenedioxy-methamphetamine (MDMA, ecstasy) is a psychostimulant and entactogen amphetamine derivative used illicitly for recreational purposes. Chronic MDMA can induce serotonergic and, to a lesser extent, dopaminergic neurotoxicity in rodents and primates (reviewed by Capela et al., 2009). Such neurotoxicity can be a consequence of coordinated oxidative stress, metabolic compromise and inflammation (reviewed by Yamamoto and Raudensky, 2008) originating upon the interaction of MDMA with several targets such as monoamine transporters. Our research group reported a new target for MDMA involved in its neurotoxicity: the neuronal nicotinic acetylcholine receptors (nAChRs). MDMA behaves as a partial agonist on α7 nAChR, inducing prolonged Ca^{2+} entry, which is related with calpain/caspase 3 activation and cytotoxicity (Garcia-Rates et al., 2010) and as an antagonist on α4β2 nAChR. These effects could attenuate the reported protective effect of a full receptor activation (Mudo et al., 2007). In animal and in in vitro models, α7 nAChR antagonists have protective effects against MDMA-induced neurotoxicity and cognitive impairment (see Pubill et al., 2011 for a review).

nAChRs are a family of ligand-gated cation channels widely distributed in the nervous system, whose subunit composition and signaling effects depend on subtype and localization (Albuquerque et al., 2009; Gotti et al., 2007). They exert relevant effects on brain functions, involving fast synaptic transmission, cognitive enhancement, memory or reinforcement, and they are the main target of smoked nicotine. nAChRs are pentameric structures formed by the association of α and β subunits and can be either homomeric or heteromeric. Of these combinations, the most abundant are the heteromeric $(\alpha4)_2(\beta2)_3$ and homomeric α7 receptors. A particular feature of some nAChR subtypes is that, after chronic nicotine exposure, they undergo radioligand binding up-regulation, changes in stoichiometry and increase in their functional state (functional up-regulation) (reviewed by Gaimarri et al., 2007). Such up-regulation occurs at a post-translational level and several mechanisms have been proposed to explain it, including a chaperone-like maturation enhancing effect (Kuryatov et al., 2005; Lester et al., 2009; Sallette et al., 2005; Srinivasan et al., 2011) and stabilization of the high-affinity state of the receptors (Vallejo et al., 2005). Moreover, nAChR up-regulation could enhance addiction to nicotine by increasing the pleasant effects of the drug (Govind et al., 2009).

Besides its functional effects, we demonstrated that MDMA also induces in vitro up-regulation of both homomeric and heteromeric receptors on PC12 cells (Garcia-Rates et al., 2007), through a mechanism that seemed to mimic that of nicotine. Moreover, in recent in vivo studies (Ciudad-Roberts et al., 2013; Pubill et al., 2013), we have demonstrated that the classic neurotoxic treatment schedule of MDMA in rats (20 mg/kg b.i.d., 4 days) induces nAChR up-regulation in gross brain regions thus potentiating the up-regulation induced by nicotine; and that a

Abbreviations: 5-HT, serotonin; DA, dopamine; DAT, dopamine transporter; MDMA, 3,4-methylenedioxy-methamphetamine; nAChR, nicotinic acetylcholine receptor; SERT, serotonin transporter; VTA, ventral tegmental area.

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sensitization schedule of MDMA in mice (5 mg/kg/day for 10 days) leads to heteromeric nAChR up-regulation in the cortex. Changes in these receptors could have a role in drug addiction and explain some psychiatric effects of this drug, such as memory impairment and psychoses, among others in which nAChRs have been found to play a role (Levin and Rezvani, 2002; Martin et al., 2004; Ripoll et al., 2004).

Accordingly this study had two aims: first to assess whether a more protracted MDMA treatment but at a lower dose in rats could induce such up-regulation and, if so, to obtain through radioligand binding autoradiography a more precise mapping of the brain areas and nuclei that undergo this phenomenon.

2. Material and methods

2.1. Drugs and radioligands

Racemic MDMA hydrochloride was obtained from the National Health Laboratory, Barcelona, Spain. Its purity was assessed by spectral analysis. Nicotine bitartrate dihydrate and clomipramine were obtained from Sigma (St. Louis, MO, USA). [^{3}H]Epibatidine, [^{3}H]Paroxetine and [^{125}I]Epibatidine were obtained from Perkin Elmer (Boston, MA, USA). All buffer reagents were of analytical grade and purchased from several commercial sources.

2.2. Animals and treatment

The experimental protocols for the use of animals in this study follow the guidelines set out by the European Communities Council (86/609/EEC) and were supervised by the ethics committee of the University of Barcelona, which specifically approved this study. Efforts were made to minimize the suffering and reduce the number of animals used. Male Sprague–Dawley rats weighing 200–230 g (Harlan Ibérica, Barcelona, Spain) were used. They were housed at 21 °C ± 1 °C under a 12 h light/dark cycle with free access to food (standard laboratory diet, Panlab, Barcelona, Spain) and drinking water.

Treatment 1 was conducted in order to determine in several gross brain regions whether the proposed MDMA dosing schedule exerted nAChR up-regulation in a significant manner, using radioligand binding to tissue homogenates and Western blotting of lysates. 10 rats were used for this assay; 5 were administered saline (1 ml/kg) and 5 were administered MDMA (7 mg/kg in 1 ml/kg) subcutaneously twice a day within an interval of 7 h. The treatment lasted 10 days and the rats were killed the day after. Given the positive results of this preliminary study, the same treatment was repeated with 12 more rats (6 saline, 6 MDMA, treatment 2) of the same characteristics, following the same dosing schedule to obtain whole brains in order to perform the autoradiography experiments in slices and undertake a more accurate localization of the areas where up-regulation takes place.

2.3. Tissue processing

The rats were killed by decapitation under isoflurane anesthesia and the brains were removed rapidly from the skull. In the case of treatment 1, the frontal and parietal cortex, striatum, hippocampus, and a coronal slice delimited by the thickness of superior colliculi, after removal of the cortex and hippocampus, (from here on we will refer to this section as "colliculus slice") were quickly excised on a refrigerated surface, frozen on dry ice and stored at −80 °C until later use (Chipana et al., 2008). In the case of treatment 2, the whole brains were quickly frozen by short immersion in isopentane pre-cooled in dry ice, then stored at −80 °C until slicing for autoradiography experiments.

Samples for use in radioligand binding experiments or Western blot determination were thawed and homogenized in 10 volumes of buffer: 5 mM Tris–HCl, 320 mM sucrose and protease inhibitors (aprotinin 4.5 μg/μl, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate), pH 7.4, with a Polytron homogenizer. The homogenates were centrifuged at 15,000 ×*g* for 30 min at 4 °C. The resulting pellets were washed twice and the final pellets (crude membrane preparation) were resuspended in 50 mM Tris–HCl buffer. Protein concentration was determined using the BioRad Protein Reagent (Bio-Rad Labs. Inc., Hercules, CA, USA) according to the manufacturer's instructions and the samples were stored at −80 °C until later use.

For Western blotting, aliquots of tissue homogenates were centrifuged at 15,000 ×*g* for 30 min at 4 °C. The supernatants were discarded and the pellets were resuspended in an appropriate volume of ice-cold solubilization buffer consisting of 20 mM Tris–HCl pH 8, 137 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 4.5 μg/μl aprotinin and 0.1 mM phenylmethylsulfonyl fluoride. Proteins were solubilized by incubation for 2 h at 4 °C under gentle rotation. Thereafter, the samples were centrifuged at 15,000 ×*g* for 30 min at 4 °C and the supernatants were stored at −80 °C after determination of protein content using the BioRad Protein Reagent and bovine serum albumin standards prepared in the same dilution of solubilization buffer, in order to compensate for the reaction with the buffer detergent.

The samples for use in autoradiography experiments were processed as follows: the brains were coronally-sectioned (16 μm thickness) using a Leica CR 3050S cryostat (chamber temperature: −20 °C; sample temperature: −16 °C). Sections were thaw-mounted on Fisher Superfrost Plus microscope slides and immediately returned to the cryostat chamber until storage. Two consecutive sections were mounted on each slide. The slides containing the sections were stored in tightly sealed containers with Drierite bags at −80 °C until use. The sections were numbered according to the most approximate coordinates obtained from the Paxinos and Watson rat brain atlas (2005).

2.4. Radioligand binding assays

[^{3}H]Paroxetine binding was used to label serotonin (5-HT) transporters (SERTs) in order to assess whether MDMA dosage had caused any serotonergic alterations, including neurotoxic effects (Pubill et al., 2003). Binding was determined in membrane preparations from the parietal cortex, an area very sensitive and representative to the effects of MDMA on SERT. Binding was performed in glass tubes containing 0.1 nM [^{3}H]Paroxetine and 150 μg of membranes. Incubation was carried out at 25 °C for 2 h in a Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl and 5 mM KCl to a final volume of 1.6 ml. Clomipramine (100 μM) was used to determine non-specific binding.

[^{3}H]Epibatidine binding was used to label heteromeric (mainly α4β2) nAChR in order to determine whether MDMA had caused an up-regulation of these receptor types in the gross regions obtained from treatment 1. Concretely, binding was carried out using the preparations from the rat cortex, striatum and the "colliculus slice". These experiments were performed in glass tubes containing 1 nM [^{3}H] Epibatidine and 200 μg of brain membranes. Incubation was carried out at 25 °C for 2 h in Tris–HCl buffer (50 mM, pH 7.4). Nicotine (300 μM) was used to determine non-specific binding.

Bindings were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters (Whatman Intl. Ltd., Maidstone, U.K.) presoaked in 0.5% polyethyleneimine. Tubes and filters were washed rapidly 3 times with 4 ml ice-cold 50 mM Tris–HCl. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Specific binding was calculated as the difference between the radioactivities measured in the absence (total binding) and in the presence (non-specific binding) of the excess of non-labeled ligand.

2.5. Western blotting and immunodetection

A general Western blotting and immunodetection protocol was used to determine nAChR α4 subunit in the frontal cortex and colliculus slice extracts from treatment 1, which had shown a significant [^{3}H] Epibatidine binding up-regulation. For each sample, 30 μg of protein was mixed with sample buffer [0.5 M Tris–HCl, pH 6.8, 10% glycerol,

2% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue, final concentrations], boiled for 10 min, loaded onto a 10% acrylamide gel and separated by electrophoresis. Proteins were then transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA). Membranes were blocked 1 h at room temperature with 5% bovine serum albumin in Tris-buffered saline buffer plus 0.05% Tween 20 (TBS-T) and incubated overnight at 4 °C with a primary rabbit monoclonal antibody against nAChR α4 subunit (ab124832) purchased from Abcam (Cambridge, UK) and used at a 1:1000 dilution in TBS-T buffer plus 5% bovine serum albumin. After washing, the membranes were incubated with a peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK). Immunoreactive protein was visualized using a chemiluminescence-based detection kit (Immobilon Western, Millipore) and a BioRad ChemiDoc XRS gel documentation system. Apparent molecular weight bands corresponding to the target protein were 70 kDa. Scanned blots were analyzed using BioRad Quantity One software. Immunodetection of β-actin (mouse monoclonal anti β-actin antibody, Sigma, St. Louis, USA; dil.1:2500) served as a control of load uniformity for each lane and was used to normalize differences due to protein content. The α4 levels are expressed as a percentage of those obtained from saline-treated animals.

2.6. Autoradiography experiments

Slides containing the brain sections were removed from the −80 °C freezer and left to warm to room temperature. A hydrophobic barrier was drawn around every slice using an ImmEdge™ Pen (Vector Laboratories, Burlingame, CA, USA) to provide a heat-stable, hydrophobic barrier that kept reagents localized on tissue specimens and prevented mixing when multiple sections were mounted on the same slide.

The binding of [^{125}I]Epibatidine to brain slices was conducted as follows. After warming, the slides (containing two slices each) were placed on a flat surface and 0.5 ml of binding buffer (50 mM Tris–HCl buffer, pH 7.4; aprotinin 4.5 μg/μl; 0.1 mM phenylmethylsulfonyl fluoride) was distributed onto each sample during 15 min. Thereafter, buffer was aspirated and the samples were pre-incubated again for another 15 min in binding buffer.

In preliminary experiments, one section of each slide was incubated during 1 h in binding buffer containing 0.2 nM [^{125}I]Epibatidine and, in order to determine the non-specific binding, the adjacent section was incubated in [^{125}I]Epibatidine containing 300 μM nicotine. Under these conditions, non-specific binding in adjacent sections was not distinguishable from background and therefore total binding was identical to specific binding, as previously reported by other groups (Nguyen et al., 2003). Thus further sections were incubated only with [^{125}I] Epibatidine to obtain a larger number of sections to quantify. After incubation, the radioligand was aspirated and the samples were washed by immersing each slide in two trays cubets filled with ice-cold 50 mM Tris–HCl buffer during 5 min each. Samples were finally dipped in ice-cold bidistilled water to remove salts and quickly dried using a stream of cold dry air.

Once slides were completely dry, they were stuck onto a cardboard paper sheet using double-sided tape, together with [^{125}I] standards (American Radiolabeled Chemicals, St. Louis, MO, USA). Slides were then placed into an exposition cassette and covered with a plastic sheet and a phosphor plate (storage phosphor screen GP, Kodak, Rochester, NY, USA) on top of it. Expositions lasted 24–48 h depending on the signal intensity of the areas of interest and plates were scanned at maximum resolution using a phosphorimager (BioRad Personal Molecular Imager, Bio-Rad Labs. Inc., Hercules, CA, USA).

The autoradiography images were processed using BioRad Image Lab software, where each area or region of interest (ROI) was manually delineated as closely as possible to the actual area delimited by the Paxinos and Watson atlas (2005). The ROI shape was copied/pasted and fitted to the same area of similar slices. Different shapes were made for the left and right hemispheres in order to properly adjust to each area. The corresponding intensity and area values were exported to Microsoft Excel. The mean density count (counts/area) was calculated, the background was subtracted and the bound radioactivity was determined through 2nd order polynomial (quadratic) interpolation in the curve defined by radioactivity standards using GraphPad Prism 3.0 software. Values were normalized taking into account the radioactivity decay of the radioligand and the standards for each determination day. All the intensity values of the samples fell within the standard curve defined by the radioactivity standards, where the relationship between activity and intensity was practically linear. Data (mean ± SEM) are reported in normalized arbitrary units (AUs).

For each rat and brain area, at least 6 values were obtained from different slices and averaged. According to the Paxinos and Watson atlas (2005), the caudate–putamen was divided into anterior (plates 13–15) and posterior (plates 16–39) and data represent the measurements of the entire area at those levels. Also, thalamic nuclei were grouped into laterodorsal, medial, ventral and ventral pallidum. For visualization purposes, images were converted from grayscale to color spectrum using the Image Lab software.

Note that although a large number of regions (31) were assessed, this was not an exhaustive survey of binding in all brain regions, but a demonstration that MDMA can induce regional nAChR up-regulation.

2.7. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.) of the values obtained for each treatment group. Unpaired Student's *t*-test for two-sample (saline vs. MDMA) was used to analyze the statistical significance ($P < 0.05$) of the difference between the means of the two groups. Values of *t*-test and degrees of freedom (df) are also reported for each comparison.

3. Results

3.1. [^{3}H]Paroxetine binding

There was a significantly marked decrease (around 50%) in [^{3}H] Paroxetine binding in the parietal cortex from the rats treated with MDMA and killed the day after the last dose ($t = 2.88$, df = 8, $P = 0.02$), thus indicating a loss of serotonin transporters (Fig. 1).

3.2. [^{3}H]Epibatidine binding to homogenates

Significant increases ($P < 0.05$) in [^{3}H]Epibatidine binding were found in the frontal portion of the cortex (24%, $t = 3.42$, df = 7, $P = 0.014$) and the colliculus slice (28%, $t = 5.21$, df = 8, $P = 0.0008$, this

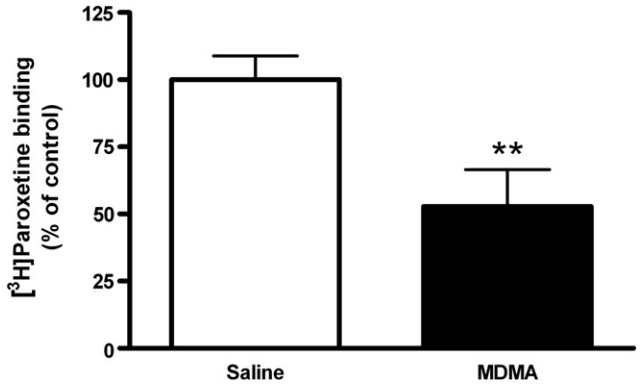

Fig. 1. Levels of serotonin transporters (SERTs), measured as [^{3}H]Paroxetine binding, in membranes from the parietal cortex of saline- and MDMA-treated rats. Male Sprague–Dawley rats were treated for 10 days b.i.d. with a dose of MDMA (7 mg/kg) or saline and were sacrificed the day after. Results are shown as mean ± SEM of the values from 6 animals per group. **P < 0.01 vs. saline group.

slice contains the colliculi, the geniculate nuclei, the substantia nigra (SN) and the ventral tegmental area (VTA)) originating from MDMA-treated rats (Fig. 2). No significant increases were found in the striatum ($t = 2.023$, df = 8, P = 0.08) or in the parietal cortex ($t = 2.24$, df = 7, P = 0.06) although a tendency towards an increase can be observed.

3.3. Western blot of α4 protein

In order to determine whether the increase in α4β2 nAChRs observed in treatment 1 was due to the increased protein synthesis or to post-translational modifications, α4 subunits were immunodetected in the lysates from the areas where significant increases in [^{3}H]Epibatidine binding were found, namely the frontal cortex and the colliculus slice. No significant changes were observed between the subunit levels of saline and MDMA-treated rats (Fig. 3) (frontal cortex: $t = 0.59$, df = 8, P = 0.57; colliculus slice: $t = 0.25$, df = 8, P = 0.80).

3.4. [^{125}I]Epibatidine autoradiography

[^{125}I]Epibatidine labeled heteromeric nAChRs in accordance with the established patterns in previous publications (i.e. Nguyen et al., 2003; Tribollet et al., 2004). Intermediate nAChR levels were found in the cortex, striatum, thalamic nuclei, geniculate nuclei and SN. Receptor density was most intense in the superior colliculi, medial habenula and interpeduncular nucleus, while the hippocampus and hypothalamus showed the lowest nAChR levels.

MDMA-treated animals showed a significant increase in nAChR density in the SN, VTA, nucleus accumbens, olfactory tubercle, anterior caudate–putamen, somatosensory, motor, auditory, and retrosplenial cortex, laterodorsal thalamic nuclei, amygdala, postsubiculum and pontine nuclei (Table 1). These increases (Table 1 and Fig. 4) ranged from 3% (retrosplenial cortex) to 30 and 34% (amygdala and SN). The rest of areas showed no significant difference between saline and MDMA-treated rats.

4. Discussion

In this study we have demonstrated, using the radioligand autoradiography technique, that a prolonged treatment with the psychostimulant drug MDMA induces up-regulation of heteromeric nAChRs (which in the brain are mainly α4β2) in specific areas of the rat brain. This is in agreement with our previous reports showing this effect in cultured PC12 cells (Garcia-Rates et al., 2007), in gross brain areas after the classical neurotoxic schedule (Pubill et al., 2013) or in mice after a sensitizing schedule

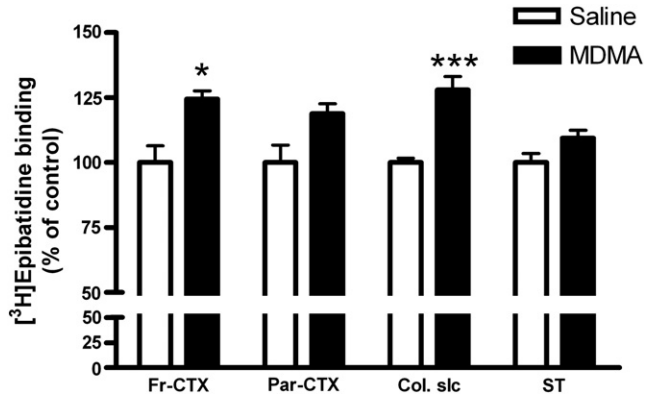

Fig. 2. Density of heteromeric nAChRs, measured as [^{3}H]Epibatidine binding, in membranes from gross areas of the brains from saline- and MDMA-treated rats. Rats were treated for 10 days b.i.d. with a dose of MDMA (7 mg/kg) or saline and were sacrificed on day 11. Binding was assessed in the frontal cortex (Fr-CTX), parietal cortex (Par-CTX), the coronal slice of mesencephalon defined by the thickness of superior colliculi (Col. slc) and the striatum (ST). Results are shown as mean ± SEM of the values from 6 animals per group. *P < 0.05 and ***P < 0.001 vs. the same area of saline group.

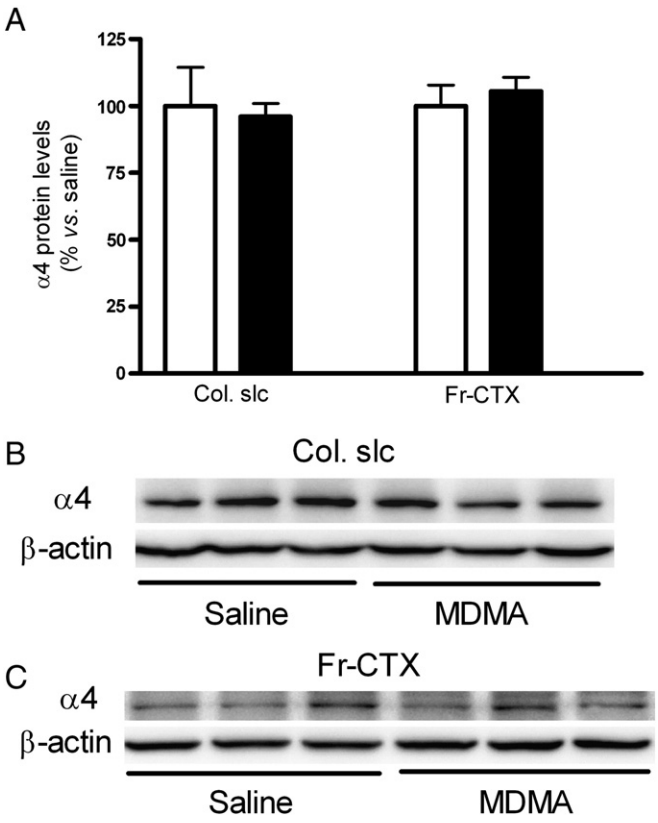

Fig. 3. Western blot analysis of α4 nAChR subunit in protein extracts of the colliculus slice (Col. slc) and frontal cortex (Fr-CTX) from rats treated for 10 days b.i.d. with a dose of MDMA (7 mg/kg) or saline and sacrificed the day after. Bar graph (panel A) shows an overall quantification expressed as percentage over control (6 animals per group, mean ± SEM), while a representative autoradiography from each area (panels B and C) is shown below. β-Actin levels were used to ensure gel-loading uniformity and normalize the protein values accordingly.

(Ciudad-Roberts et al., 2013). MDMA has affinity for α4β2 nAChRs (Chipana et al., 2008; Garcia-Rates et al., 2007) as occurs with several nicotinic ligands, either agonists or antagonists, that have been reported to induce nAChR up-regulation (Gopalakrishnan et al., 1997; Peng et al., 1994).

Binding to homogenates from gross brain areas is useful for screening purposes, but is not accurate enough to ascertain the changes in small areas that are involved in specific brain functions. Also, if the increase took place only in a small area that is part of a gross portion used for the assay, this increase would not be detected owing to the dilution effect (as we found, for example, in the different parts of the striatum). Thus, looking at the previous results, it was mandatory to carry out an autoradiography study in brain slices to determine nAChR levels in more defined areas.

In addition, we used lower MDMA doses than in the previous study (Pubill et al., 2013), during an extended treatment period at normal housing temperature in order to reduce serotonergic neurotoxicity that could hinder nAChR up-regulation (Gordon et al., 1991; Green et al., 2005). In this respect we must point out that regardless of the conditions of this treatment, we found a marked loss of paroxetine binding sites, which has been generally linked to serotonergic neurotoxicity. However, we must point out that in our treatment the rats were killed 24 h after the last dose, while most studies (i.e. Biezonski and Meyer, 2010; Broening et al., 1995; Malberg and Seiden, 1998; O'Shea et al., 1998) make the measurement after leaving a time of at least one week to allow the neurotoxic process to occur. We cannot assert whether the decrease in paroxetine binding is due to serotonergic terminal degeneration or to a regulatory process. In fact, a significant reduction in SERT gene expression, which could explain a reduction in SERT protein

Table 1
[^{125}I]Epibatidine binding to several brain areas of saline- and MDMA-treated rats.

Area	Saline	MDMA	*t*, df	P	Increase (%)
Frontal cortex	23.53 ± 0.98	23.69 ± 0.65	0.123, 9	n.s.	
Anterior olfactory nucleus	15.45 ± 1.03	14.64 ± 0.92	0.586, 8	n.s.	
Nucleus accumbens	23.69 ± 0.84	27.79 ± 1.19	2.815, 10	<0.05	17
Cingulate cortex	24.85 ± 0.78	25.88 ± 0.72	0.970, 10	n.s.	
Motor cortex	21.07 ± 0.32	23.07 ± 0.80	2.321, 8	<0.05	10
Somatosensory cortex	26.25 ± 0.44	28.86 ± 0.93	2.368, 9	<0.05	10
Insular cortex	16.57 ± 1.44	16.85 ± 0.93	0.163, 10	n.s.	
Olfactory tubercle	18.81 ± 0.89	22.62 ± 0.34	3.697, 9	<0.01	20
Retrosplenial cortex	31.10 ± 0.26	32.14 ± 0.27	2.739, 9	<0.05	3
Visual cortex	23.22 ± 0.60	24.83 ± 2.06	0.750, 10	n.s.	
Auditory cortex	25.79 ± 0.49	28.76 ± 1.06	2.371, 9	<0.05	12
Caudate–putamen					
Anterior	31.91 ± 1.73	37.03 ± 1.68	2.253, 10	<0.05	16
Posterior	21.62 ± 2.44	21.77 ± 0.72	0.059, 10	n.s.	
Hippocampus (CA1)	13.94 ± 0.68	14.92 ± 0.57	1.104, 10	n.s.	
Hippocampus (dentate gyrus)	19.32 ± 2.58	18.98 ± 1.42	0.115, 10	n.s.	
Medial habenula	83.57 ± 3.21	79.79 ± 3.73	0.768, 8	n.s.	
Ventral pallidum	57.99 ± 1.35	58.08 ± 1.46	0.045, 10	n.s.	
Laterodorsal thalamic nuclei	69.68 ± 1.93	77.33 ± 1.77	2.921, 10	<0.01	11
Ventral thalamic nuclei	66.08 ± 1.62	65.38 ± 0.62	0.403, 10	n.s.	
Medial thalamic nuclei	60.69 ± 1.41	61.08 ± 1.86	0.167, 10	n.s.	
Hypothalamus	23.22 ± 0.60	24.83 ± 2.06	0.815, 9	n.s.	
Amygdala	15.78 ± 1.27	20.48 ± 0.89	3.031, 10	<0.05	30
Dorsal lateral geniculate nuclei	57.96 ± 6.45	49.49 ± 3.09	1.184, 8	n.s.	
Superior colliculus, superficial gray layer	49.97 ± 4.12	52.13 ± 1.52	0.492, 8	n.s.	
Medial geniculate nuclei	35.3 ± 4.65	38.97 ± 1.09	0.768, 8	n.s.	
Substantia nigra	27.52 ± 3.59	36.81 ± 0.96	2.287, 9	<0.05	34
Ventral tegmental area	27.47 ± 1.69	33.15 ± 0.69	3.112, 10	<0.05	21
Pontine nuclei	19.37 ± 0.39	22.72 ± 1.12	2.825, 8	<0.05	17
Interpeduncular nucleus	81.51 ± 2.92	91.41 ± 4.78	1.767, 8	n.s.	
Postsubiculum	46.33 ± 1.8	52.4 ± 1.24	2.777, 8	<0.05	13
Cerebellum	10.45 ± 0.52	10.20 ± 0.49	0.350, 8	n.s.	

Semi-quantitative analysis of [^{125}I]Epibatidine binding to several areas of brain slices from rats treated with saline (Ctrl) or MDMA (7 mg/kg/day, b.i.d., 10 days). Data (mean ± SEM from 5 to 6 animals per group) are reported in normalized arbitrary units. P indicates the degree of statistical significance; n.s., non significant (P > 0.05); *t* is the Student's *t* value and df is the degrees of freedom.

irrespective of altered terminal integrity, has been reported after treatment with MDMA (Biezonski and Meyer, 2010). As will be discussed below, as a number of presynaptic nAChRs are localized on serotonergic terminals and there seems to be a positive correlation between their up-regulation and SERT, it can be suggested that the decrease in paroxetine binding in this study is most likely to be due to a regulatory process rather than to terminal destruction.

As most up-regulation studies carried out with nicotine use continuous dosing through prolonged constant infusion or osmotic minipumps (i.e. Nguyen et al., 2003) we chose a MDMA dosing schedule aimed to reach sufficient plasma levels during enough time to induce up-regulation with reduced neurotoxic potential, compared with our previous work in gross brain areas. This schedule, therefore, was not intended to model any human consumption pattern but to demonstrate and localize nAChR up-regulation by MDMA. Once this was demonstrated, further work using other schedules closer to recreational use patterns should be performed.

Phosphor imaging was used in order to obtain and quantify the images. Traditionally, in situ autoradiography has been performed using X-ray film, which provides the highest resolution. However, depending on the radioligand used, exposure times with films are much longer than those with phosphor plates and, due to the evolution and raise of the digital imaging systems, obtaining suitable and affordable X-ray films is becoming more and more difficult. As an alternative, quantitative phosphor imaging provides lower exposure time, economization due to the reutilization of phosphor screens, no waste of developing solutions, direct quantification on the scanned digital image and an optical resolution suitable for most quantitative purposes (Strome et al., 2005). This technique was used by another group for quantifying α7 nAChRs using [^{3}H]methyllycaconitine (Mugnaini et al., 2002) and, to our knowledge, the present study is the first to employ this technique to quantify heteromeric nAChRs using [^{125}I] Epibatidine.

A number of studies carried out with nicotine have suggested several mechanisms to explain nAChR up-regulation (see Introduction), and most agree on the fact that up-regulation occurs at a post-translational level. To assess this possibility with MDMA we analyzed by Western blot the α4 subunit levels in the same protein samples from brain portions that had showed increased epibatidine binding in homogenates and compared them with those from saline-treated rats. No significant differences were found between the two groups, indicating that up-regulation of binding takes place without increased protein levels, probably through post-translational modifications that increase the affinity or promote maturation of receptors towards a form capable of binding the ligand, similarly to what has been described for nicotine in the articles referenced above.

nAChRs have a predominant presynaptic localization, on the nerve endings, where they modulate the release of key neurotransmitters such as acetylcholine, dopamine (DA), GABA, glutamate and serotonin (reviewed by Marchi and Grilli, 2010), although they are also at the preterminal level and on different postsynaptic locations in the brain (Mameli-Engvall et al., 2006). NAChRs play a key role in addiction to nicotine (Govind et al., 2009). It has been described that the addictive effects of nicotine are produced through its interaction with nAChR in the mesolimbic pathway, especially those in the nucleus accumbens, leading to DA release that activates the reward circuitry. In fact, mice with deletion of the β2 gene do not self-administer nicotine after previous administration and do not show increased release of DA in the ventral tegmental area (Picciotto et al., 1999).

Although the mechanisms involved in the establishment of addiction are complex and still being investigated, up-regulation of nAChRs increasing the pleasant effects of the drug is an event that could feasibly play a role. In the present study, we demonstrate nAChR up-regulation in key areas involved in addiction, such as the VTA, the nucleus accumbens and several areas of the cortex that are involved in sensory and

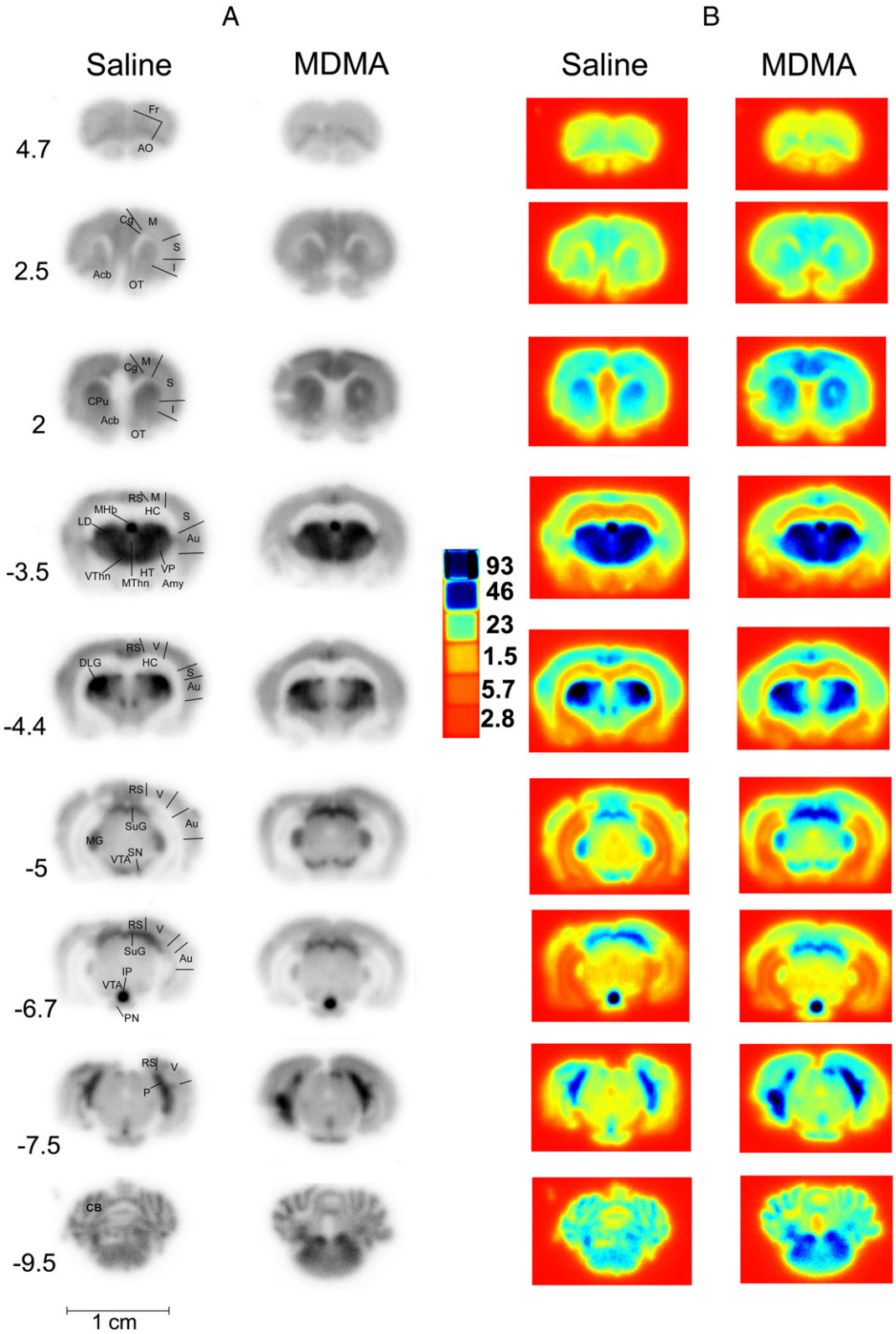

Fig. 4. Representative images of [^{125}I]Epibatidine binding to brain slices from rats treated for 10 days b.i.d. with saline or MDMA (7 mg/kg) and sacrificed the day after. All the labeled regions are illustrated. Numbers on the bottom left corner of photographs indicate the approximate distance of the sections from the coronal plane passing through bregma according to Paxinos and Watson (2005). Panel A shows the grayscale scanned images, while panel B shows the same images converted to color spectrum which allows improved visual appreciation of the receptor densities. Abbreviations used (in order of appearance): Fr, frontal cortex; AO, anterior olfactory nucleus; Acb, nucleus accumbens; Cg, cingulate cortex; M, motor cortex; S, somatosensory cortex; I, insular cortex; OT, olfactory tubercle; RS, retrosplenial cortex; V, visual cortex; Au, auditory cortex; HC, hippocampus; MHb, medial habenula; VP, ventral pallidum; LD, laterodorsal thalamic nuclei; VThn, ventral thalamic nuclei; MThn, medial thalamic nuclei; HT, hypothalamus; Amy, amygdala; DLG, dorsal lateral thalamic nuclei; SuG, superior colliculus, superficial gray layer; MG, medial geniculate nuclei; SN, substantia nigra; VTA, ventral tegmental area; PN, pontine nuclei, IP, interpeduncular nucleus; P, postsubiculum, CB, cerebellum.

motor functions (i.e. auditory, somatosensory, motor), as well as in the olfactory tubercle. Heteromeric nAChRs play a role in the hyperlocomotion induced by amphetamine derivatives (Camarasa et al., 2009; Ciudad-Roberts et al., 2013), thus an increase in nAChR in these areas could account for sensitization to the locomotor effects and the addictive properties of MDMA. In fact, blockade of nAChR containing the α4 subunit with dihydro-β-erythroidine or varenicline inhibits the hyperlocomotion induced by MDMA in mice, as well as an increased delayed sensitization (Ciudad-Roberts et al., 2013). Moreover, in the same study it was demonstrated that pretreatment with nicotine inducing nAChR up-regulation reduced the dose threshold for MDMA-conditioned place preference.

In the olfactory tubercle, an area that underwent a 20% increase, β2 subunit-containing nAChRs increase DA release (Grady et al., 2002). This area is a component of the ventral striatum, it is heavily interconnected with several affective, reward and motivation related centers of the brain, being the area that modulates behavior during certain physiological and mental states (Wesson and Wilson, 2011). The olfactory tubercle has also been shown to be especially involved in reward and addictive behaviors, so that rats have been shown to self-administer cocaine into this area more than the nucleus accumbens and ventral pallidum (Ikemoto, 2003). Also, the olfactory tubercle receives an important serotonergic innervation (Cumming et al., 1997). Therefore the up-regulation found in this area could also account for reinforcing effects after a chronic treatment.

The nigrostriatal pathway was also affected by nAChR up-regulation, as we found a 33% increase in the substantia nigra as well as a 16% increase in the anterior caudate–putamen. α4β2 nAChRs have been identified in soma and dendrites of SN, as well as in the dopaminergic terminals in the striatum (Jones et al., 2001). Dopaminergic neurones from the SN possess the ability to release DA not only from axon terminals in the striatum, but also from the soma and dendrites within SN (Cheramy et al., 1981). It has been suggested that serotonergic afferents to SN may evoke this dendritic dopamine release through a mechanism that is uncoupled from the impulse-dependent control of nerve terminal DA release (Cobb and Abercrombie, 2003).

Nicotine and nicotinic agonists increase DA release from mesolimbic and nigrostriatal neurones in vitro and in vivo (Wonnacott, 1997). In the striatum, an important percentage of β2 subunit-containing nAChRs is associated to dopaminergic buttons from the nigrostriatal pathway, and the rest may correspond to other neurotransmitter afferents such as serotonergic fibers coming from the dorsal raphe nucleus (Jones et al., 2001; Reuben and Clarke, 2000; Schwartz et al., 1984). As this pathway is involved in the control of movement, increased nAChRs in the striatal DA terminals could be involved in motor disorders or stereotypes.

Previous studies on nicotine-induced nAChR up-regulation indicate that chronic nicotine exposure differentially affects the number (up-regulation), subunit composition, stoichiometry and functional status of some nAChR subtypes, leaving others substantially unaffected (Gaimarri et al., 2007). In this respect, when comparing the results of the present study with those obtained from chronic nicotine-treated rats by Nguyen et al. (2003) in the same brain areas, we can find similarities regarding these aspects. For example, in both studies, the amygdala and the substantia nigra exhibit some of the highest up-regulation rates, while other areas with a high density of nAChR such as the interpeduncular nucleus or the medial habenula show no change in radioligand binding. The nucleus accumbens undergoes the highest up-regulation in the corpus striatum, while in the caudate–putamen it is more modest. Conversely, some areas within the cortex and the hippocampus that had shown robust nAChR up-regulation after nicotine administration were unaffected or showed a lower effect in our study. We have cited above that nAChR up-regulation by nicotine and MDMA is a mechanistically-complex process that implies the interaction of the ligand with intracellular immature forms of the receptor. Nicotine is known to penetrate the cell membrane (Whiteaker et al., 1998), which allows such an interaction to occur, while MDMA is known to use the monoamine transporters, mainly the SERT (Fitzgerald and Reid, 1990), to access the intracellular compartments. Thus the different abilities of each brain area to take up MDMA could explain the main differences found in comparison to nicotine.

Moreover, when looking at previous literature such as the article from Battaglia et al. (1991), who similarly quantified the levels of SERT in the brains from rats of the same strain, gender and age than ours, we found that the areas with increased epibatidine binding tend to correspond with areas having higher SERT levels in the control animals. In fact, it has been reported that a single acute in vivo exposure to nicotine significantly increases 5-HT uptake via SERT in prefrontocortical synaptosomes (Awtry and Werling, 2003), which indicates that there is a regulatory interaction between nAChR and SERT. In this respect we noticed that SERT density in the areas showing greatest nAChR up-regulation by MDMA was not affected (amygdala and VTA) or even increased (substantia nigra, nucleus accumbens) by MDMA in the study from Battaglia et al. (1991). On the other hand, areas from the cortex and hippocampus which are capable of showing a robust nAChR up-regulation by nicotine, suffer from a strong reduction of SERT after MDMA, as we also assessed in cortex homogenate in the present study; this could explain why these areas show a lower radioligand binding increase after treatment with this amphetamine derivative, as this would difficult drug internalization in the synaptic terminal, thus impeding nAChR up-regulation. Nonetheless, further investigation should be conducted to pursue this hypothesis.

The predominantly presynaptic localization and widespread distribution of nAChR in several brain circuits make it particularly difficult to functionally characterize the specific behavioral or brain roles affected by MDMA-induced up-regulation; nonetheless the present results can illustrate which functions could probably be altered and suggest a mechanism to explain, at least in part, the reinforcing properties of MDMA.

5. Conclusion

In this study we demonstrate that a protracted treatment with MDMA induces a heteromeric nAChR up-regulation in key areas of rat brain involved in motivation and learning, and sensory and movement control, which could account for reinforcing and some neuropsychiatric disorders related with chronic consumption of this drug.

Translated to a clinical context, we show nAChR as a potential target for reducing MDMA's reinforcing effects. Furthermore, we also postulate that nAChR up-regulation induced by chronic consumption of MDMA could potentiate the ability of other drugs to cause addiction due to an enhancement of nAChR-mediated reinforcing effects.

Disclosure statement

All authors declare no actual or potential conflict of interest including financial, personal or other relationships with other people or organizations that could inappropriately influence the present work. All authors reviewed the content and approved the final version.

Contributors

DP and EE were responsible for the study concept and design. AC and DP performed the experiments. EE and JC contributed to animal treatment. DP and JC performed the data analysis. DP wrote the manuscript draft and AC revised the language. JC and EE interpreted the findings and provided critical revision of the manuscript.

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Article 3: Alcohol enhances the psychostimulant and conditioning effects of mephedrone in adolescent mice; postulation of unique roles of D_3 receptors and BDNF in place preference acquisition. *British Journal of Pharmacology*

Mephedrone is the most commonly used compound of an increasingly popular family of designer drugs named cathinones. Given that it is mostly consumed concomitantly with ethanol, especially by young adults (Winstock et al., 2011), we sought to investigate the interrelation between these two substances in adolescent mice, focusing on the potential enhancement by ethanol of the psychostimulant and rewarding properties of mephedrone, measured as horizontal locomotor activity and conditioned place preference (CPP). Furthermore, we aimed to identify possible neuroplasticity-related transcriptional modifications that could underlie the development of mephedrone-induced CPP and its potentiation by ethanol.

For this purpose, the present work was structured in two sections:

- **Locomotor activity assays:** horizontal locomotor activity elicited by a range of acute doses of mephedrone and mephedrone+ethanol was assessed in order to identify a potential enhancement effect. The involvement of dopamine and serotonin in ethanol-induced potentiation was also tested by blocking serotonin2A and D2 receptors with ketanserin and haloperidol, respectively.
- **Conditioned place preference coupled to full genome microarray:** the rewarding properties of mephedrone (10 and 25 mg/kg) and mephedrone+ethanol (10 or 25 mg/kg + 0.75 g/kg) were tested by means of the CPP assay. Upon termination of the CPP protocol, a full genome microarray was run on the ventral striatum (containing the nucleus accumbens) of the various treatment groups in an attempt to identify transcriptional changes in neuroplasticity-related genes.

Locomotor activity experiments showed the highest potentiation effect for ethanol 1g/kg, when administered concomitantly with mephedrone 10mg/kg. The effect of ketanserin and haloperidol was assessed on this association. Ethanol-induced locomotor activity enhancement was blocked by haloperidol, but not ketanserin, pointing to a possible involvement of synaptic dopamine in the mediation of this potentiation effect.

In agreement with locomotor activity findings, CPP was also potentiated by the simultaneous administration of mephedrone (25 mg/kg) and ethanol (0.75 g/kg).

Microarray experiments revealed multiple differentially expressed genes. Of particular interest, are the increases in Drd3, Arpc5, Gpx6, Muted, Nfu1 and Syt10, which were all validated through q-PCR.

Interestingly, despite the significant potentiation in CPP, gene expression was not notably modified by the administration of ethanol (0.75 g/kg), when compared to saline- or mephedrone-treated animals. In this sense, principal component analysis revealed two clearly differentiated transcriptional profiles (A: saline and ethanol groups and B: mephedrone and mephedrone+ethanol groups). Accordingly, Arpc5 was the only synaptic plasticity-related gene whose expression was directly correlated with CPP score and enhanced when associating mephedrone and ethanol. Its product, ARPC5, plays an important role in maintaining the ARP2/3 complex nucleating capability, which is essential for actin remodeling at a pre- and post-synaptic level; thus, the involvement of this structural protein in the establishment of reward-associated memories warrants further research.

Additionally, due to the significant up-regulation in the Drd3 gene, the role of D3 dopamine receptors (D3R) in the establishment of CPP was also investigated. SB-277011A, a selective D3R antagonist, completely abolished CPP in all treatment groups; furthermore, Drd3 up-regulation was also prevented. As Drd3 differential expression can be regulated by BDNF, we also assessed whether mephedrone-induced CPP and Drd3 differential expression could be blocked by the administration of ANA-12, a selective TrkB (BDNF receptor) antagonist, as occurred for SB-277011A; this effect was confirmed. Taken together, results evidence a clear involvement of DR3s and their differential regulation by BDNF in the mediation of the rewarding effects of mephedrone.

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RESEARCH PAPER

Alcohol enhances the psychostimulant and conditioning effects of mephedrone in adolescent mice; postulation of unique roles of D_3 receptors and BDNF in place preference acquisition

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BACKGROUND AND PURPOSE
The psychostimulant mephedrone is often consumed in combination with alcohol (EtOH). This kind of drug consumption during adolescence is a matter of concern.

EXPERIMENTAL APPROACH
We studied, in adolescent CD-1 mice, whether EtOH could enhance the psychostimulant (locomotor acivity) and rewarding [conditioned place preference (CPP)] effects of mephedrone. We also determined the transcriptional changes associated with a conditioning treatment with these drugs.

KEY RESULTS
Mephedrone (10 mg·kg^{-1}) increased locomotor activity, which was further enhanced by 40% when combined with EtOH (1 g·kg^{-1}). This enhancement was blocked by haloperidol. Furthermore, mephedrone (25 mg·kg^{-1}) induced CPP, which increased by 70% when administered with a dose of EtOH that was not conditioning by itself (0.75 g·kg^{-1}). There was enhanced expression of the D_3 dopamine receptor mRNA (*Drd3*) and *Arpc5* in all drug-treated groups. The D_3 receptor antagonist SB-277011A and the BDNF receptor antagonist ANA-12 completely prevented CPP as well as the increases in *Drd3* in all groups. Accordingly, increased expression of BDNF mRNA in medial prefrontal cortex was detected at 2 and 4 h after mephedrone administration.

CONCLUSIONS AND IMPLICATIONS
If translated to humans, the enhancement of mephedrone effects by ethanol could result in increased abuse liability. D_3 receptors and BDNF play a key role in the establishment of CPP by mephedrone, although an accompanying increase in other synaptic plasticity-related genes may also be necessary.

Abbreviations
ANA-12, N-[2-[[(hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide; CPP, conditioned place preference; EtOH, ethanol; MDMA, 3,4-methylenedioxy-methamphetamine; NAc, nucleus accumbens; PCA, principal component analysis; qPCR, quantitative real-time PCR; SB-277011A, *N*-{*trans*-4-[2-(6-cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl]cyclohexyl}quinoline-4-carboxamide

Tables of Links

TARGETS	
GPCRs[a]	**Catalytic receptors**[b]
5-HT2A receptor	TrkB (BDNF receptor)
D2 receptor	
D3 receptor (Drd3)	

LIGANDS	
5-HT	Haloperidol
Alcohol (EtOH)	Ketanserin
BDNF	MDMA
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 (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Consumption of drugs of abuse at earlier ages, such as adolescence, is especially worrying because this stage is crucial in brain maturation and will determine the social outcome of an individual (Steinberg, 2005). Experimentation with alcohol and other drugs during adolescence is common; there is a low risk perception, as the regions of the brain that control impulses are still immature, as well as increased risk taking and novelty/sensation seeking behaviours attributable to transformations in prefrontal areas (Casey *et al.*, 2008; Chambers and Potenza, 2003; Spear, 2000).

Substance use during adolescence has been associated with alterations in brain structure, function and neurocognition (reviewed by Squeglia *et al.*, 2009), as well as to an increased likelihood of using drugs of abuse in adulthood (Izenwasser, 2005). Currently, most drug use during adolescence occurs in leisure environments, such as dance clubs and parties, leading to a preference for use of psychostimulants (i.e. cocaine and amphetamine derivatives such as mephedrone) and alcohol, which is omnipresent due to its legal drug status (Winstock *et al.*, 2011). Thus, the association of psychostimulants and alcohol is frequent. The consequences of these combinations in adolescent subjects need to be studied, because a potentiation of their effects may increase their abuse liability and subsequent negative effects.

Mephedrone (4-methylmethcathinone) is an increasingly consumed synthetic psychostimulant compound, which first appeared for sale on the Internet around 2007. It belongs to the β-ketoamphetamines group, also known as cathinones and is commonly taken orally or insufflated (Winstock *et al.*, 2011). Preclinical studies have shown that mephedrone stimulates the release of dopamine, 5-HT and noradrenaline and inhibits their re-uptake in the CNS (Kehr *et al.*, 2011; Baumann *et al.*, 2012; López-Arnau *et al.*, 2012; Martínez-Clemente *et al.*, 2012). These actions explain the psychostimulation and the effects on perceptions reported by human consumers (Schifano *et al.*, 2011). Experiments carried out in rats and mice demonstrate the psychostimulant (measured as hyperlocomotion) and reinforcing [measured as conditioned place preference, (CPP)] effects of mephedrone, which are indicative of its abuse liability (Lisek *et al.*, 2012; López-Arnau *et al.*, 2012).

Mephedrone is, after cannabis, 3,4-methylenedioxy-methamphetamine (MDMA) and cocaine, one of the most frequently used drugs (Brunt *et al.*, 2011; Winstock *et al.*, 2011). Re-dosing is common, and users state that the total dose taken during a single session usually ranges between 0.5 and 2.0 g (7.7–30.8 mg·kg^{-1} for a person weighing 65 kg) (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Annual Report). The most frequent mephedrone users are reported to be men between their late teen years and their twenties.

Mephedrone is also commonly combined with many other drugs, but mainly alcohol (EtOH) (Elliot and Evans, 2014) which, in turn, is the most consumed drug. In the UK, around 95% of cathinone consumers combine it with alcohol (Winstock *et al.*, 2011). Previous studies in rodents have shown that EtOH can effectively potentiate the rewarding effects of MDMA measured as CPP (Jones *et al.*, 2010), as well as its psychostimulant effect, measured as increased locomotor activity (Cassel *et al.*, 2004). Such potentiation appears to be elicited by a combination of both pharmacokinetic and pharmacodynamic interactions with EtOH. Given the resemblance between mephedrone's mechanism of action and that of MDMA (Green *et al.*, 2003; Baumann *et al.*, 2012; López-Arnau *et al.*, 2012), a similar profile should be expected when combined with alcohol.

The first aim of this work was to assess, in adolescent mice, whether ethanol enhances the psychostimulant (locomotor activity) and conditioning effects of mephedrone when administered concomitantly at doses mimicking human recreational use. Secondly, as conditioning implies long-term neuronal changes and EtOH potentiated mephedrone-induced CPP, we sought to determine major transcriptional modifications caused by these treatment patterns, focusing on those implicated in neuronal plasticity, which plays a crucial role in the acquisition of addiction. Using a functional genomics approach and after identifying potential candidates, we further explored the role of dopamine D_3 receptors in the acquisition and potentiation of CPP by mephedrone and EtOH.

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. Efforts were made to minimize suffering and reduce the number of animals used. 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 five to six per cage at 22 ± 1 °C under a 12 h light/dark cycle with free access to standard diet and drinking water.

Drugs

Pure racemic mephedrone was synthesized and characterized in house as described previously (López-Arnau *et al.*, 2012). Absolute ethanol was purchased from Scharlau (Barcelona, Spain). Ketanserin hydrochloride, haloperidol, *N*-{*trans*-4-[2-(6-cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl]cyclohexyl} quinoline-4-carboxamide (SB-277011A) and *N*-[2-[[(hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide (ANA-12) were purchased from Sigma-Aldrich. Ethanol was diluted in saline at different concentrations, never exceeding 10% (w v^{-1}) to avoid tissue irritation. Doses of mephedrone and alcohol were equivalent to those used for recreational purposes by humans. Common mephedrone doses in humans range between 100 and 200 mg (Measham *et al.*, 2010), which represent 1.4–2.8 mg·kg^{-1} for a 70 kg person. Therefore, a dose of 25 mg·kg^{-1} in mice corresponds to 2 mg·kg^{-1} in a human adult. This equivalent dose was calculated following the body surface area normalization method (Reagan-Shaw *et al.*, 2008; Martínez-Clemente *et al.*, 2014). Mephedrone solutions for injection were prepared in saline or ethanol/saline solutions immediately before s.c. administration at a volume of 10 mL·kg^{-1}. Ethanol doses (0.5–1 g·kg^{-1}) were in the same range used by other authors (Cassel *et al.*, 2004). Ketanserin was dissolved in saline, while haloperidol was prepared as a micro-suspension in carboxymethylcellulose-Tween 80 vehicle (0.5–0.1% w·v^{-1}). SB-277011A and ANA-12 were dissolved in 2-hydroxypropyl-β-cyclodextrin (25% w·v^{-1}) and DMSO (10% v·v^{-1}) respectively. All the antagonists were administered i.p. at a volume of 5 mL·kg^{-1}. Previous experiments demonstated that the i.p. injection of the three different vehicles used to dissolve the antagonists do not modify either locomotor activity or CPP and *Drd3* expression compared with the injection of saline (data not shown). For this reason, and to simplify the treatments' design and statistical analysis, all the animals, which did not receive any of the antagonists, were injected i.p. with saline previously to the assigned s.c. treatment.

The drug/molecular target nomenclature used in this word conforms to BJP's Concise Guide to Pharmacology (Alexander *et al.*, 2013a,b).

Locomotor activity measurement

Experiments were performed as previously described (López-Arnau *et al.*, 2012). The animals were administered substances s.c. and placed in the activity box that was later placed inside the frame system equipped with infrared photocells (LE8811, Panlab, Barcelona, Spain). Occlusions of the photo beams were recorded and sent to a computerized system (SedaCom32, Panlab). The interruption counts, over a 10-min block, were used as a measure of horizontal locomotor activity. Animals received mephedrone (10 or 25 mg·kg^{-1}; s.c.) alone or combined with ethanol (0.5 or 1 g·kg^{-1}; s.c.), 0.5 or 1 g·$kg^{-1}$of ethanol or saline and were immediately placed in the activity box. Because ethanol, at certain doses, can impair or enhance locomotion, it was administered at doses reported not to affect basal activity (Cassel *et al.*, 2004; Hodge *et al.*, 2004). Each treatment group consisted of six to eight mice that were only tested once in the apparatus after receiving a treatment. Locomotor activity was monitored for 150 min, although hyperlocomotion had already ceased at 120 min, and this time point was taken for calculations. When appropriate, locomotor activity was recorded after administering the 5-HT_{2A} and D_2 receptor antagonists ketanserin (1 mg·kg^{-1}) and haloperidol (0.25 mg·kg^{-1}), respectively given i.p. 15 min before the assigned treatment. These doses of antagonists were chosen as they neither affect basal locomotor activity nor completely abolish mephedrone-induced hyperlocomotion, according to the literature and previous experiments in our lab (Kelly *et al.*, 1998; López-Arnau *et al.*, 2012; Williams *et al.*, 2012).

Conditioned place preference test

We used the non-biased protocol and the same apparatus as described previously (Ciudad-Roberts *et al.*, 2013). The apparatus was composed of three distinct areas (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 pre-conditioning phase (day 1), mice were placed in the middle of the corridor and had free access to and were allowed to roam among the three compartments of the apparatus for 20 min. The time spent in each compartment was recorded by computerized monitoring software (Smart, Panlab, Barcelona, Spain).

The first CPP experiment was designed to assess the conditioning properties of two doses of mephedrone (10 and 25 mg·kg^{-1}) and their association with ethanol. During the conditioning phase (days 2, 4, 6 and 8), mice (n = 6–15 per group) were treated with mephedrone (10 or 25 mg·kg^{-1}), mephedrone + ethanol (10 or 25 mg·kg^{-1} + 0.75 g·kg^{-1}), ethanol (0.75 g·kg^{-1}) or saline, 20 min before being confined into one of the two conditioning compartments for 30 min. On days 3, 5, 7 and 9 of the conditioning phase, animals received saline and were confined to the opposite compartment. The animals were exposed to only one pairing per day, and treatments were counterbalanced to assure that drugs were equally administered in both compartments.

When associating mephedrone + ethanol, these drugs were administered s.c. in the same solution. The post-conditioning test (day 10) was conducted identically to the pre-conditioning phase. A preference score was expressed in s 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 pre-conditioning phase.

We intended to use a dose of ethanol that did not produce CPP on its own. An extensive review on CPP, compiled from many studies performed with ethanol, showed that doses of 0.5 and 1 g·kg^{-1} consistently followed these requirements (Tzschentke, 2007). Despite general agreement around this

fact, there is one report by Maurice *et al*. (2003) showing CPP with 1 g·kg^{-1} EtOH. Accordingly, the intermediate dose of 0.75 g·kg^{-1} was chosen for our experiments.

The second CPP experiment was conducted in an attempt to block drug-induced CPP acquisition, based on the significant up-regulation of dopamine D_3 receptor mRNA found in the animals from the initial CPP experiment (Results). Thus, animals were given SB-277011A (25 mg·kg^{-1}; i.p.), a selective D_3 receptor antagonist or saline, 15 min before the s.c. administration of mephedrone + ethanol (25 mg·kg^{-1} + 0.75 g·kg^{-1}), mephedrone (25 mg·kg^{-1}), ethanol (0.75 g·kg^{-1}) or saline. The rest of the protocol remained identical to that described previously. The SB-277011A dose was selected according to dose range used in numerous studies in the literature (Song *et al*., 2012; Vorel *et al*., 2002).

The third CPP experiment investigated the pathways involved in mephedrone-induced CPP and D_3 receptor expression. As brain-derived neurotrophic factor (BDNF) has been reported to participate in the up-regulation of *Drd3* induced by addictive drugs such as cocaine (Le Foll *et al*., 2005), the role of its pathway on the acquisition of CPP induced by mephedrone was also investigated. Accordingly, animals were given ANA-12 (0.5 mg·kg^{-1}; i.p.), a selective trkB (BDNF receptor) antagonist, or saline b.i.d. 2 days prior to initiating and throughout the entire CPP protocol 15 min before the s.c. administration of mephedrone + ethanol (25 mg·kg^{-1} + 0.75 g·kg^{-1}), mephedrone (25 mg·kg^{-1}), ethanol (0.75 g·kg^{-1}) or saline. Ethanol-treated groups were omitted from the experiment for simplicity. The ANA-12 dose was selected according to those used in multiple studies in the literature (Cazorla *et al*., 2011; Leggio *et al*., 2014). The rest of the protocol remained identical to that described previously.

Finally, we treated three groups of six mice with 25 mg·kg^{-1} (s.c.) of mephedrone acutely and killed them immediately ($t = 0$), 2 or 4 h after its administration. Q-PCR for BDNF mRNA was performed on samples from the medial prefrontal cortex, as described below.

Tissue processing and microarray experiments

For microarray experiments, only the mephedrone dose of 25 mg·kg^{-1} was assessed, as it was most likely to cause transcriptional modifications. Thus, we assessed four groups (six animals per group) as follows: mephedrone + ethanol (25 mg·kg^{-1} + 0.75 g·kg^{-1}), mephedrone (25 mg·kg^{-1}), ethanol (0.75 g·kg^{-1}) or saline.

Mice were killed by cervical dislocation 24 h after the post-conditioning test. Their brains were rapidly removed and ventral striata [comprising the nucleus accumbens (NAc)] were quickly dissected out, frozen on dry ice and stored at −80 °C. Total RNA was prepared using RNeasy Lipid Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA concentration and integrity were assessed. Total RNA for cDNA arrays came from triplicate pooled samples (two animals per pool). Gene expression was analysed by hybridization of 500 µg RNA to GeneChip Mouse Gene 1.0 ST Affymetrix microarrays, containing 28 869 transcripts and variants (Functional Genomics Unit, IDIBAPS, Barcelona, Spain).

Microarray data were uploaded to The Gene Expression Omnibus under the accession reference GSE58279.

Real-time PCR (qPCR)

Differentially regulated genes of interest from microarray experiments were confirmed by quantitative PCR (qPCR). Furthermore, *Drd3* mRNA levels from CPP experiments using the antagonists SB-277011A and ANA-12 were also determined.

Briefly, complementary DNA was synthesized in a total volume of 20 µL by mixing 2 µg of total RNA, 125 ng of random hexamers (Roche), in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 U RNasin (Invitrogen), 0.5 mM dNTPs (AppliChem), 200 U M-MLV reverse transcriptase (Invitrogen) and 50 mM Tris–HCl buffer, pH 8.3. The reaction mixture was incubated at 37 °C for 50 min. The cDNA product was used for subsequent real-time PCR amplification using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with 25 ng of the cDNA mixture and the assays-on-demand from Applied Biosystems Mm00432887_m1 for *Drd3*, Mm01350708_m1 for *Muted*, Mm00444512_m1 for *Syt10*, Mm00777068_m1 for *Nfu1*, Mm04208715_m1 for *Arpc5*, Mm00513979_m1 for *Gpx6*, Mm04230607_s1 for *BDNF* and Mm00607939_s1 for *Actb* as an endogenous control. Fold-changes in gene expression were calculated using the standard δδCt method (Livak and Schmittgen, 2001).

Statistical analysis

For locomotor activity, CPP and qPCR experiments, data are expressed as the mean ± SEM. Differences between groups were compared using ANOVA. The significance of the interaction between time and treatment in locomotor activity experiments was assessed by two-way ANOVA. Significant ($P < 0.05$) differences were then analysed by Tukey's *post hoc* test for multiple means comparisons, where appropriate. All statistical calculations were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA).

Microarray analysis was performed using the GeneSpring GX 11.5.1 software (Agilent Technologies, Madrid, Spain), which allows multifilter comparisons using data from different experiments to perform the normalization, generation of lists and the functional classification of the differentially expressed genes. After grouping the triplicates of each experimental condition, a preliminary list of differentially expressed genes could be generated by using an ANOVA analysis. The *P*-value cutoff was <0.01. Out of all these genes, separate volcano plot analyses were carried out for each experimental condition. Unpaired *t*-test was applied using asymptotic *P*-value computation. The expression of each gene was reported as the ratio of the value obtained for each condition relative to the control condition after normalization and statistical analysis of the data. The corrected *P*-value cutoff applied was <0.05; then the output of this statistical analysis was filtered by fold expression, selecting specifically those genes that had a differential expression of at least 1.2-fold. Extensive literature mining was performed on the list generated and differentially expressed genes in the mephedrone, mephedrone + ethanol, and ethanol groups with a potential role in neuronal plasticity and dependence were selected for further analyses and classified according to gene ontology biological processes. Principal component analysis (PCA) was also performed in order to determine the overall expression pattern for each treatment group.

Results

Effects on locomotor activity

Two different doses of mephedrone were tested, 10 and 25 mg·kg^{-1}, as well as their combinations with 0.5 or 1 g·kg^{-1} of ethanol. Locomotor activity was recorded for 120 min. ANOVA of cumulative breaks revealed an overall effect of treatment ($F_{8,43} = 9.50$, $P < 0.001$). As can be seen in Figure 1A, mephedrone induced significant increases in locomotor activity. Ethanol, at the doses used, had no significant effect on locomotion when administered alone. The effect of 10 mg·kg^{-1} mephedrone was similarly enhanced (around 40% increase) when combined with either of the ethanol doses, reaching statistical significance with respect to the mephedrone group at the dose of 1 g·kg^{-1}. The effect of the 25 mg·kg^{-1} of mephedrone was not modified by its association with ethanol, probably indicating a maximum effect of the cathinone. For this reason, we chose the 10 mg·kg^{-1} dose for further analysis.

Figure 1B shows the kinetics of locomotor activity for mephedrone 10 mg·kg^{-1}, both doses of ethanol and their association. Mephedrone-induced hyperlocomotion peaked shortly after administration and lasted for around 120 min. Two-way ANOVA denoted statistical significance of time ($F_{14,\ 480} = 131.6$, $P < 0.0001$), treatment ($F_{5,\ 480} = 64.30$, $P < 0.0001$) and their interaction ($F_{70,\ 480} = 3.77$, $P < 0.0001$). When mephedrone was administered concomitantly with ethanol, locomotor activity increased with respect to the mephedrone group; this became especially evident in the first time intervals and lasted approximately 1 h. After this period, all the mephedrone-treated groups showed analogous activity scores. The association of mephedrone with both doses of ethanol showed similar profiles, although only the association with 1 g·kg^{-1} reached statistical significance along all the first five points with respect to the mephedrone group (Tukey's multiple comparisons test), while that with 0.5 g·kg^{-1} was only statistically significant at the time point of 40 min.

As previously described (López-Arnau *et al.*, 2012), ketanserin and haloperidol affected the hyperlocomotion induced by mephedrone. Pretreatment with ketanserin (1 mg·kg^{-1}), a 5-HT$_{2A}$ receptor antagonist, reduced locomotion in both mephedrone and mephedrone + EtOH groups by 30.37% ($P < 0.01$ vs. mephedrone) and by 32.27% ($P < 0.001$ vs. mephedrone + EtOH) respectively. By contrast, haloperidol (0.25 mg·kg^{-1}), a dopamine receptor antagonist, reduced at a much higher degree the locomotor activity elicited by mephedrone + EtOH (52.09%; $P < 0.001$) than that of mephedrone alone (34.67%; $P < 0.05$), virtually bringing them to the same level as saline, as shown in Figure 1C. Both antagonists were administered at doses that did not significantly affect basal locomotor activity (cumulative breaks in 120 min: saline, 4515 ± 456; ketanserin, 5575 ± 422; haloperidol, 5413 ± 311; both drugs $P > 0.05$ vs. saline) but reported to have significant effects on hyperlocomotion (Kelly *et al.*, 1998; Williams *et al.*, 2012).

Effect of ethanol on the place conditioning induced by mephedrone

The CPP paradigm was used to study the conditioning properties of two different doses of mephedrone (10 and

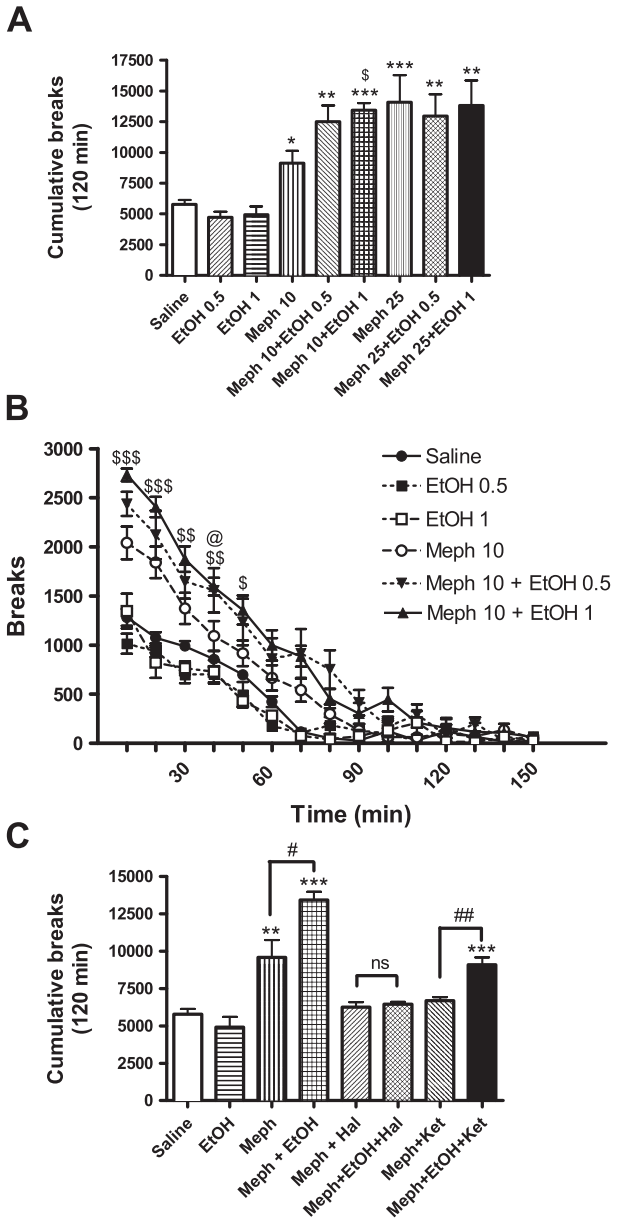

Figure 1

(A) Effect of a single injection of mephedrone (Meph; 10 or 25 mg·kg^{-1}), EtOH (0.5 or 1 g·kg^{-1}), mephedrone + EtOH combined or saline on locomotor activity of adolescent CD-1 mice. Activity was measured as interruption counts (breaks) in 10 min blocks and monitored for 120 min. Panel B depicts the time course evolution of locomotion from saline, EtOH (0.5 or 1 g·kg^{-1}), mephedrone (10 mg·kg^{-1}) and mephedrone + EtOH combinations. Panel C shows the cumulative breaks after 120 min for the effect of ketanserin (Ket, 1 mg·kg^{-1}), haloperidol (Hal, 0.25 mg·kg^{-1}) on mephedrone (10 mg·kg^{-1}) and mephedrone + EtOH (10 mg·kg^{-1} + 1 g·kg^{-1})-induced hyperlocomotion. Data are expressed as the mean ± SEM for all treatment groups ($n = 6$ to 8 animals per group). One-way (panels A and C) and two-way ANOVA (panel B) and *post hoc* Tukey–Kramer multiple comparisons test.*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$ significantly different from saline; \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.01$, comparisons between the mephedrone 10 + EtOH 1 versus mephedrone 10 group; @ $P < 0.05$, comparing mephedrone 10 + EtOH 0.5 with mephedrone 10 group; n.s., non-significant. #$P < 0.05$, ##$P < 0.01$ between the indicated groups.

25 mg·kg^{-1}) and how ethanol (0.75 mg·kg^{-1}, a dose that does not elicit CPP on its own) could enhance this effect (Figure 2).

Times (expressed as a percentage) spent in both compartments during the pre-conditioning phase were 49.5 ± 1.30 and 50.5 ± 1.30 ($P > 0.05$), respectively indicating a total lack of preference for either side.

We investigated the effect of mephedrone alone (10 and 25 mg·kg^{-1}) and in the presence of ethanol (0.75 g·kg^{-1}), administered s.c. in a single solution using the CPP paradigm (Figure 2). On the test day (day 10, post-conditioning), one-way ANOVA revealed a significant effect of the treatment ($F_{5,46} = 4.487$, $P < 0.01$). Ethanol by itself did not exert any effect on preference score. Both doses of mephedrone elicited a similar positive place preference (mephedrone 10: 152.3 ± 50.85 s; mephedrone 25: 158.9 ± 31.4 s), which showed statistical significance ($P < 0.05$ and $P < 0.01$ vs. saline respectively). The concomitant administration of ethanol increased mephedrone-induced CPP by 44% (mephedrone 10 mg·kg^{-1}; $P = 0.38$) and 70% (mephedrone 25 mg·kg^{-1}; $P < 0.05$ vs. its respective non-ethanol group) respectively.

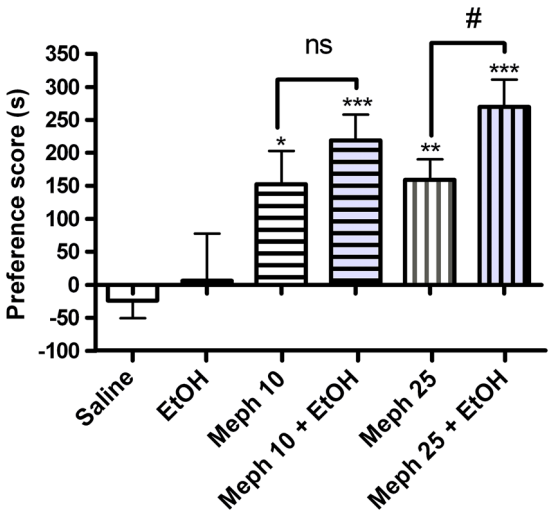

Figure 2

Effect of EtOH (0.75 g·kg^{-1}) on mephedrone (Meph; 10 and 25 mg·kg^{-1})-induced conditioned place preference. The *x*-axis represents the treatment group, and the *y*-axis represents the preference score (difference between the times, in s, spent in the drug-paired compartment on the test and pre-conditioning day). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significantly different from saline-treated group; n.s., non significant, $\#P < 0.05$ significantly different between the indicated groups ($n = 7$ to 15 mice per group).

Functional genomics and qPCR validation

Principal component analysis of microarrays was used in order to determine general transcriptional profiles for each treatment. PCA illustrates how animals are clustered in two clearly differentiated groups (Figure 3). Cluster 1 comprised saline and ethanol-treated groups, whereas cluster 2 included both mephedrone-treated groups. This reflects how mephedrone was responsible for the main transcriptional modifications, while ethanol did not cause important changes in expression patterns when compared with their respective control groups (Saline and mephedrone 25).

ANOVA performed on normalized microarray data identified 563 differentially regulated genes by the RMA method ($P < 0.01$). Hierarchical clustering of the obtained genes and samples was performed on the data and visually expressed as $\log_2$ of fold change (FC) (Figure 4). Unsupervised hierarchical clustering was achieved with unweighted pair-group method using arithmetic averages, using Pearson correlation distance as the similarity metric. As with PCA, hierarchical clustering of differentially expressed genes showed how gene expression profiles of all pooled animals fell into two major groups: those treated with mephedrone and those that were not.

Out of these genes, we generated a list for each treatment group of differentially expressed genes when compared with saline by means of volcano plot analysis. Unpaired *t*-test was applied using asymptotic *P*-value computation, at a

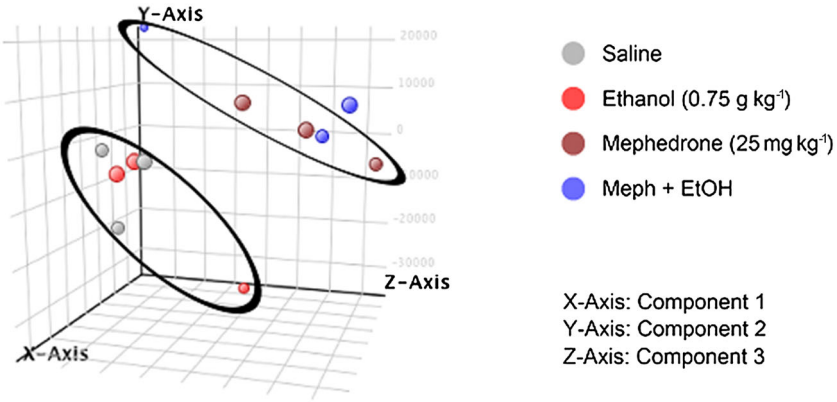

Figure 3

Principal component analysis of full genome array. The principal components represent the variability in gene expression levels observed within the dataset, with the top three principal components (X, Y and Z) used to generate the three-dimensional graph shown. Each dot represents two to three pooled animals from each respective treatment group. The analysis uses data from the PLIER microarray normalization method. Mephedrone-treated and non-mephedrone-treated animals are clustered into two clearly differentiated groups.

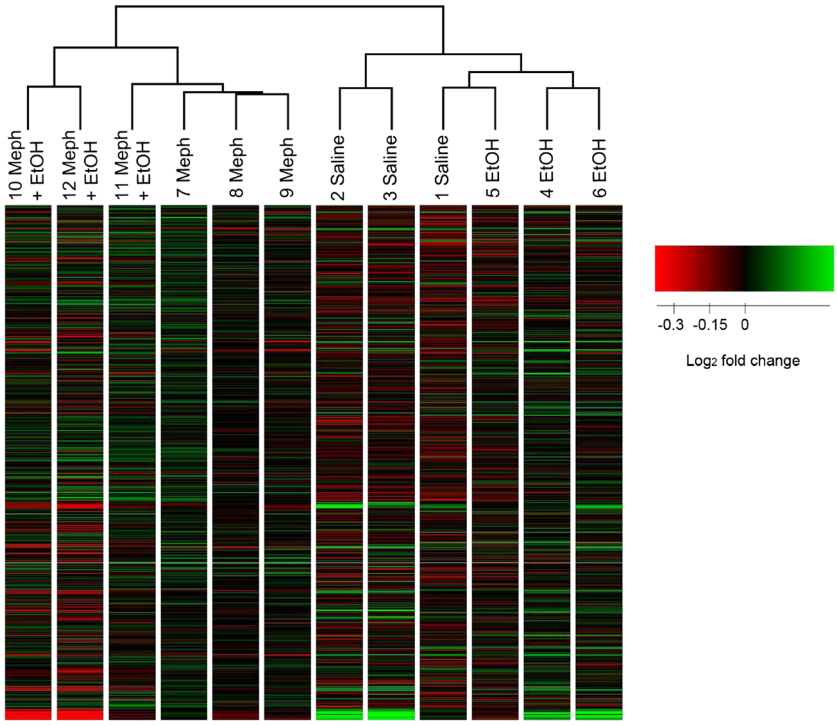

Figure 4

Heat map of microarray data using the RMA normalization method. Log2 signal values are standardized by row mean centring and scaled to row mean square. Clustering was performed by the unweighted pair group method with arithmetic mean algorithm using Pearson correlation distance as the similarity metric.

cutoff of $P < 0.05$ and FC > 1.2. A final list of 103 genes was generated (49 were up-regulated and 53 were down-regulated). Given its large size, extensive literature mining was performed, and 34 potentially interesting genes for the purpose of the study were selected. Table 1 classifies these entities in 12 gene ontology categories and shows log 2 of FC and statistical significance for each treatment group.

This list was subjected to literature mining to finally select the most potentially interesting genes according to their bibliographic interest (implicated in neuronal plasticity, which participate in addiction, as well as in neurotoxic or regulatory processes). Differential expression for *Drd3*, *Arpc5*, *Nfu1*, *Gpx6*, *Muted* and *Syt10* was validated by qPCR and shown in Figure 5. Similar increases in gene expression were found for *Drd3* in the three drug-treated groups, and for *Muted*, *Nfu1* and *Syt10* only in the mephedrone and mephedrone + EtOH groups.

Out of the validated genes, we only found differences in *Arpc5* and *Gpx6* between the mephedrone and mephedrone + EtOH groups, which confirms that gene expression in this treatment is not massively modified by the concomitant administration of ethanol, as mentioned previously. In *Arpc5*, we might find an explanation for the notable ethanol-induced increase in CPP score, as it is involved in neuronal actin remodelling (as discussed below).

Effect of SB-277011A on CPP and D_3 receptor gene expression

Given the reported role of dopamine D_3 receptors in addiction and the increase we found in its gene expression, we sought to determine whether SB 277011-A (25 mg·kg^{-1}, SB), a selective D_3 receptor antagonist, could prevent CPP elicited by mephedrone (25 mg·kg^{-1}) and its association with ethanol (0.75 mg·kg^{-1}) and how that pretreatment would affect the observed increase in *Drd3* mRNA.

Overall, one-way ANOVA revealed a significant effect of treatment ($F_{7,59} = 6.179$, $P < 0.0001$). The results showed a total blockade of mephedrone-induced and mephedrone + ethanol-induced CPP (Figure 6A).

Twenty-four hours after the test, the animals were killed following the same procedure as in the first CPP experiment; *Drd3* mRNA expression was then determined through q-PCR. One-way ANOVA revealed a significant effect of treatment ($F_{7,50} = 8.254$, $P < 0.001$). We found that treatment with SB-277011A totally abolished drug-induced *Drd3* overexpression in all groups (Figure 6B).

Effect of ANA-12 on mephedrone's effects on CPP and D_3 receptor gene expression: assessment of BDNF mRNA levels after drug injection

Due to the robust changes observed with the administration of the D_3 antagonist and the fact that D_3 receptors did not seem to mediate the potentiation by ethanol, we decided to further explore the D_3 receptor regulation pathway on the conditioning effects of mephedrone, omitting the ethanol-treated groups for simplicity. As BDNF has been reported to control dopamine D_3 receptor expression (Guillin *et al.*, 2001) and its expression to be increased by psychostimulants

Table 1

Differential gene expression after microarray analysis

Affected genes classified by gene ontology	Gene name	EtOH		mephedrone		mephedrone + EtOH	
Neuronal changes							
Actin-related protein 2/3 complex, subunit 5	Arpc5	0.19	Up	0.27	Up**	0.23	Up*
Calcium/calmodulin-dependent protein kinase kinase 1, α	Camkk1	0	Down	0.3	Up***	0.3	Up**
Muted	Muted	0.21	Up	0.28	Up***	0.23	Up
Mitogen-activated protein kinase kinase kinase 12	Map3k12	−0.06	Down	−0.27	Down***	−0.14	Down
Neurotransmitter transport and synaptic transmission							
Solute carrier family 6 (neurotransmitter transporter), member 15	Slc6a15	0.12	Up	0.31	Up***	0.27	Up**
Synaptotagmin X	Syt10	0.19	Up	0.54	Up***	0.44	Up*
Islet cell autoantigen 1	Ica1	0	—	0.27	Up***	0.05	Up
Dopamine receptor 3	Drd3	0.53	Up**	0.34	Up*	0.31	Up*
Metabolic processes							
Hedgehog interacting protein-like 1	Hhipl1	0.19	Up	0.35	Up***	0.37	Up**
Coenzyme Q3 homologue, methyltransferase (yeast)	Coq3	0.12	Up	0.29	Up***	0.33	Up***
Glutamate oxaloacetate transaminase 2, mitochondrial	Got2	0	—	−0.43	Down**	0.06	Up
StAR-related lipid transfer (START) domain containing 5	Stard5	0.2	Up	0.34	Up***	0.24	Up
Apoptosis							
B-cell receptor-associated protein 29	Bcap29	0.1	Up	0.31	Up***	0.29	Up**
Proteasome (prosome, macropain) subunit, α type 2	Psma2	0.28	Up*	0.3	Up***	0.35	Up***
Survival motor neuron domain containing 1	Smndc1	0.09	Up	0.27	Up**	0.19	Up
Glucocorticoid induced transcript 1	Glcci1	0	—	−0.38	Down**	−0.2	Down
D site albumin promoter binding protein | sphingosine kinase 2	Sphk2	−0.04	Down	0.26	Up***	0.31	Up***
Brain-expressed X-linked 2	Bex2	0.14	Up	0.29	Up**	0.33	Up**
Gliosis							
Cyclin-dependent kinase-like 1 (CDC2-related kinase)	Cdkl1	0.03	Up	0.31	Up***	0.23	Up
Gene expression							
Mediator of RNA polymerase II transcription, subunit 6 homologue	Med6	0.13	Up	0.27	Up**	0.3	Up***
RNA (guanine-7-) methyltransferase	Rnmt	0.17	Up	0.27	Up**	0.35	Up***
Eukaryotic translation initiation factor 2C, 4	Eif2c4	−0.01	Down	−0.27	Down**	−0.42	Down**
tRNA splicing endonuclease 15 homologue (S. cerevisiae)	Tsen15	0.27	Up***	0.32	Up**	0.26	Up
Oxidative stress							
Glutathione peroxidase 6	Gpx6	−0.22	down	0.69	Up***	0.04	Up
Mitosis							
HAUS augmin-like complex, subunit 1	Haus1	0.02	Up	0.28	Up**	0.27	Up**
Histone aminotransferase 1	Hat1	0.12	Up	0.27	Up**	0.25	Up
Signal transduction							
GNAS complex locus	Gnas	0.17	Up	−0.61	Down***	0.1	Up
Inositol 1,4,5-triphosphate receptor 1	Itpr1	0.04	Up	−0.31	Down***	−0.08	Down
Circadian rhythms							
Basic helix-loop-helix family, member e41	Bhlhe41	0.03	Up	0.33	Up*	0.42	Up**
Period homologue 3 (Drosophila)	Per3	−0.03	Down	0.34	Up***	0.26	Up
Aryl hydrocarbon receptor nuclear translocator-like	Arntl	0.11	Up	−0.35	Down***	−0.46	Down***

(Continues)

Table 1 (Continued)

Affected genes classified by gene ontology	Gene name	EtOH		mephedrone		mephedrone + EtOH	
Regulation of cell shape							
Family with sequence similarity 40, member B	Fam40b	0	—	0.35	Up***	0.23	Up
Mitocondrial function							
NFU1 iron-sulfur cluster scaffold homologue (S. cerevisiae)	Nfu1	0.38	Up***	0.79	Up***	0.68	Up***
Coiled-coil-helix-coiled-coil-helix domain containing 4	Chchd4	0.14	Up	0.34	Up***	0.2	Up

Differentially expressed genes in adolescent mice after CPP, where animals were treated with saline, mephedrone, ethanol and mephedrone + ethanol. Mice were treated and subjected to the CPP protocol as described in Methods. Pooled mRNAs from ventral striata were hybridized to GeneChip Mouse Gene 1.0 ST Affymetrix microarrays. After an initial overall ANOVA with a cutoff *P*-value of <0.01, genes, which were significantly differentially expressed ($P < 0.05$ vs. saline) by at least 1.2-fold, were selected and summarized in this table.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. saline-treated mice.

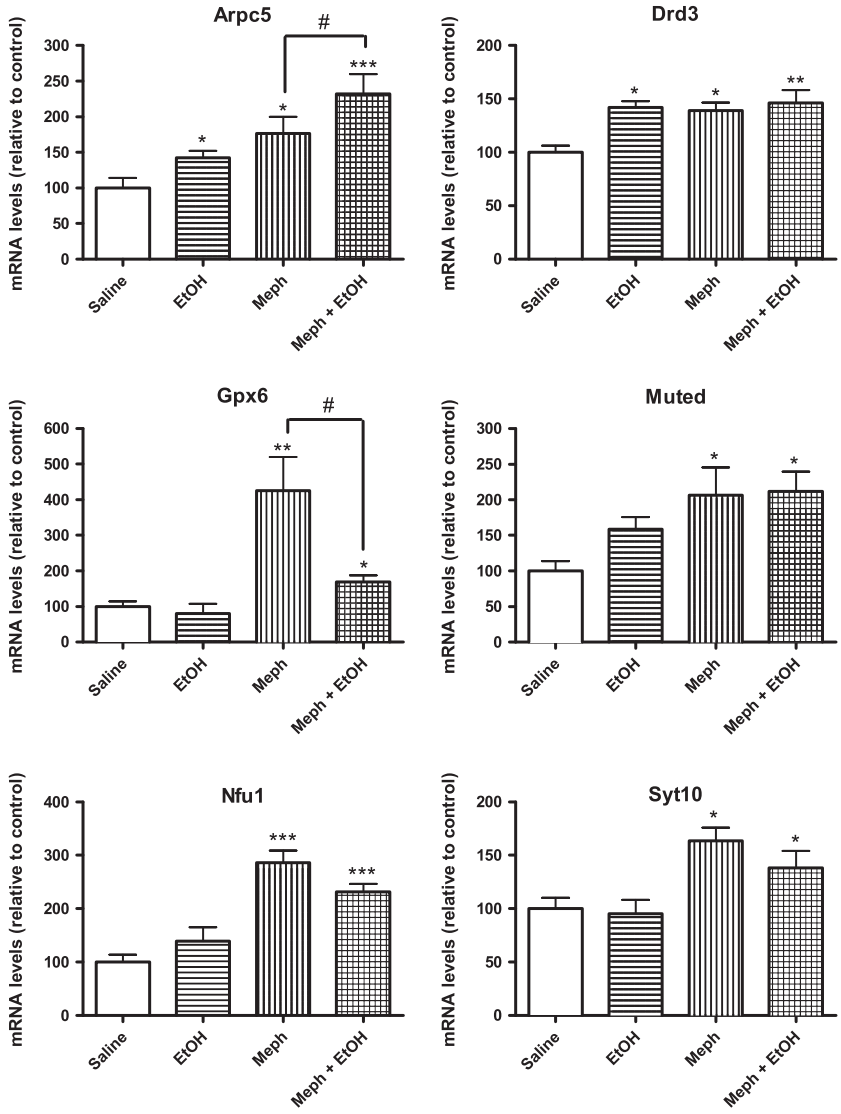

Figure 5

Quantitative real-time PCR confirmed changes in the genes selected from previous microarray analysis. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, significantly different from saline-treated group; #$P < 0.05$, significantly different from the corresponding mephedrone (Meph) group.

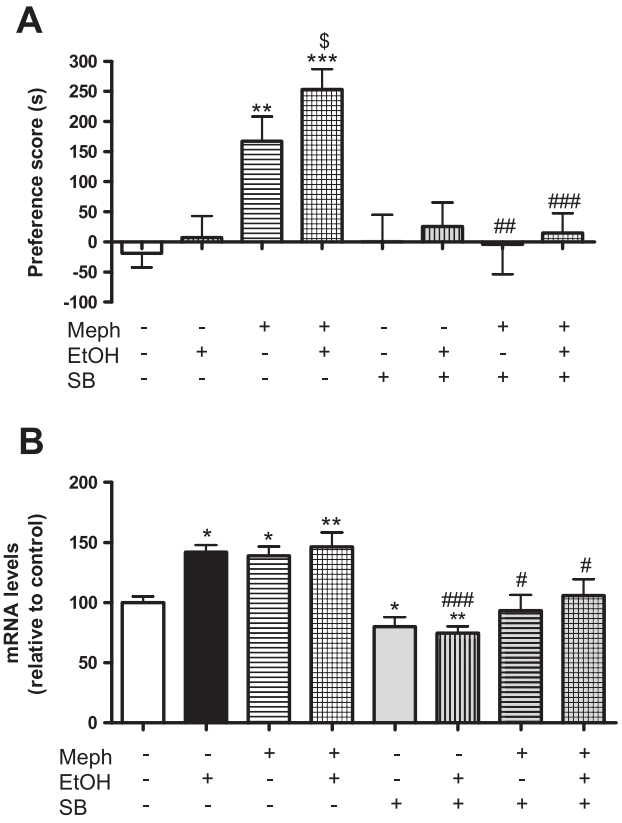

Figure 6

Effect of SB-277011A (SB, 25 mg·kg^{-1}) on mephedrone (Meph; 25 mg·kg^{-1})- and mephedrone + EtOH (25 mg·kg^{-1} + 0.75 g·kg^{-1})-induced conditioned place preference (panel A) and *Drd3* mRNA expression (panel B). $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from saline-treated group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ significantly different from the corresponding value of the non-SB-277011A-treated group ($n = 8$ to 12 mice per group).

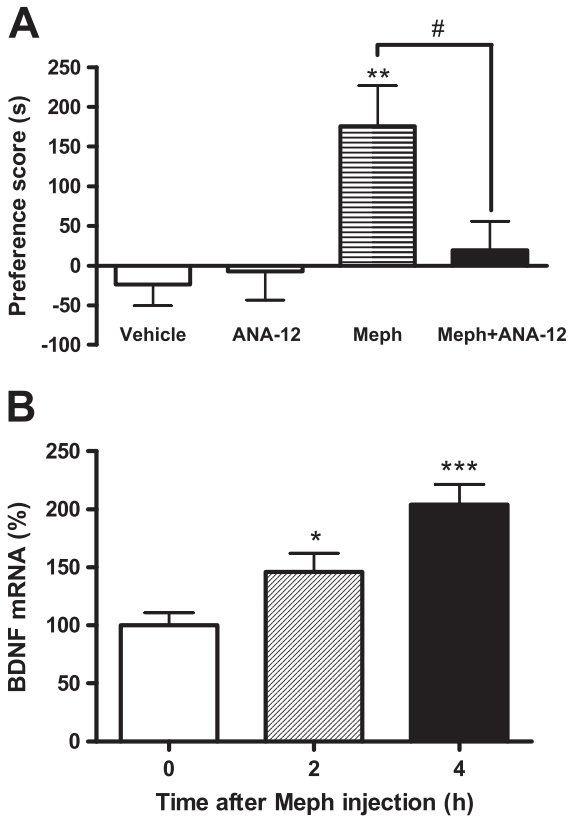

Figure 7

(A) Effect of ANA-12, a selective trkB receptor antagonist on mephedrone (Meph; 25 mg·kg^{-1})-induced conditioned place preference. The *x*-axis represents the treatment group and the *y*-axis represents the preference score (s). $^{**}P < 0.01$ significantly different from saline-treated group; $^{\#}P < 0.05$ between the indicated groups ($n = 6$ to 10 mice per group). (B) Effect of a single injection of mephedrone (25 mg·kg^{-1}) on *BDNF* mRNA levels in the medial prefrontal cortex, measured at 0, 2 and 4 h after administration. $^{*}P < 0.05$ and $^{***}P < 0.001$ are significantly different from animals killed immediately after administration ($n = 6$ mice per group).

(Graham *et al.*, 2007), we hypothesized that the indirect blockade of *Drd3* up-regulation induced by interferring with the BDNF pathway using ANA-12, a trkB antagonist, could also result in a decrease in the rewarding properties of mephedrone.

Overall, one-way ANOVA revealed a significant effect of treatment ($F_{3,24} = 4.36$, $P < 0.05$). The results showed a total blockade of mephedrone-induced CPP by ANA-12. ANA-12 did not induce any conditioning effect by itself (Figure 7A) .

Similarly as described above, *Drd3* mRNA expression was then determined through q-PCR and compared with that of non-ANA-12-treated animals. One-way ANOVA revealed a significant effect of treatment ($F_{3,24} = 3.12$, $P < 0.05$). ANA-12 totally blocked mephedrone-induced *Drd3* overexpression (mRNA levels, relative to saline group: mephedrone, 142.00 ± 11**; mephedrone + ANA-12, 96.11 ± 12, $P < 0.05$ between groups; $^{**}P < 0.01$ vs. saline). ANA-12, administered alone, had no effect on *Drd3* levels (mRNA levels: saline: 100.00 ± 6; ANA-12, 103.00 ± 8.89, n.s.).

Finally, to confirm a role for BDNF, we studied, in a new set of mice, the effects of a single injection of mephedrone (25 mg·kg^{-1}; s.c.) on BDNF mRNA through q-PCR in samples of the medial prefrontal cortex from animals killed 0, 2 and 4 h after drug administration. As described by other authors (Le Foll *et al.*, 2005), a time-dependent increase in mRNA was detected in this area (Figure 7B).

Discussion

Amphetamines exert their psychostimulant effect through activation of the mesolimbic dopamine system, leading to dose-dependent increases in locomotor activity in rodents (Izawa *et al.*, 2006). This hyperlocomotor activity of amphetamines is directly correlated with blockade of dopamine uptake and with a non-exocytotic transporter-mediated, dopamine release. Also, the release of dopamine induced after 5-HT$_2$ receptor activation is involved in the hyperlocomotion induced by derivatives such as MDMA and mephedrone, which similarly inhibit 5-HT uptake and induce its release;

furthermore, they are also moderate 5-HT_2 agonists (Nash *et al.*, 1994; López-Arnau *et al.*, 2012).

In this sense, the rapid increase in locomotor activity we observed with mephedrone is in agreement with previous findings (Kehr *et al.*, 2011; Lisek *et al.*, 2012; López-Arnau *et al.*, 2012). When administered concominantly with ethanol, locomotor activity was significantly increased. In this sense, ethanol exhibits an effect on mephedrone comparable with that exerted on MDMA at a dose of 1.5 $g \cdot kg^{-1}$ which, by itself, was devoid of significant effects on locomotion (Cassel *et al.*, 2004; Ben Hamida *et al.*, 2007, 2009). Moreover, the dose of ethanol that we used (1 $g \cdot kg^{-1}$) was even lower than reported in the experiments with MDMA. Interestingly, the increase in locomotor activity after the concomitant administration of EtOH 0.5 $g \cdot kg^{-1}$ was similar to that obtained for the dose of 1 $g \cdot kg^{-1}$. This points towards the possibility that potentiation by EtOH at the assessed dose range might not be dose-dependent or, more probably, that the sensitivity of the method employed is not enough to discern between the potentiation elicited by such similar doses of ethanol.

The effects of ethanol in the brain are numerous due to its ability to cross biological membranes and to interact on several molecular targets (i.e. ligand-gated ion channels). One of the main mechanisms by which it is capable of increasing hyperlocomotion is the inhibition of GABAergic interneurons in the substantia nigra reticulata, which leads to disinhibition and increased burst firing of dopamine neurons in the nucleus accumbens, but it also directly increases dopamine release in other areas of mesocortical pathways (see Siggins *et al.*, 2005 for a review). Also, activation of the opioid reward pathway has been reported (Mitchell *et al.*, 2012). These mechanisms are different from those of mephedrone but, in turn, would converge in increased dopamine release and/or disinhibition in certain brain areas, which could explain the observed increased effect.

To assess the participation of 5-hydroxytryptaminergic and dopaminergic pathways on the increase in mephedrone-induced hyperlocomotion by EtOH, we tested the effects of ketanserin and haloperidol. The fact that both antagonists reduced mephedrone-induced hyperlocomotion, as previously reported by us (López-Arnau *et al.*, 2012), but that only haloperidol was able to completely block the increase elicited by EtOH, suggests that the increase in locomotor activity caused by EtOH might be mediated by an enhancement of dopaminergic neurotransmission. In fact, Riegert *et al.* (2008) demonstrated, *in vitro*, that MDMA-induced dopamine outflow is facilitated by EtOH, whereas that of 5-HT is barely modified, implying that the dopamine/5-HT release ratio is increased when MDMA is administered concomitantly with EtOH. Thus, a similar phenomenon could occur with mephedrone, although more experiments with a wider range of ketanserin/haloperidol doses or locomotor activity assessment coupled to microdialysis should be performed to confirm this assertion.

Furthermore, Ben Hamida *et al.* (2009) reported that ethanol is capable of increasing the concentration of MDMA in areas with high dopamine transmission (striatum and frontal cortex) in a much higher proportion than in the hippocampus, which is richer in 5-HT terminals. Although no mechanistic description was found for this effect, we cannot rule out a similar pharmacokinetic interaction between ethanol and mephedrone as an additional underlying cause for the dopamine-mediated increase in locomotor activity.

A second objective of this work was to investigate whether EtOH could enhance the conditioning properties of mephedrone, by means of the CPP paradigm. The acquisition of conditioning after repeated administration of a drug suggests that it induces rewarding effects that, in turn, can be indicative of abuse liability. Mephedrone, given alone, induced similar place preference scores at 10 and 25 $mg \cdot kg^{-1}$, which is in accordance with results recently described by Karlsson *et al.* (2014).

At both mephedrone doses tested, ethanol increased their preference score, although statistical significance was only reached at 25 $mg \cdot kg^{-1}$ of mephedrone. As mentioned previously, alcoholic drinks are very often combined with psychostimulants; therefore, this result is of importance because a similar effect in humans could result in increased abuse potential.

Because CPP depends on the mesolimbic pathway, addictive drugs are expected to evoke synaptic plasticity in the areas that it comprises including the NAc, the ventral tegmental area, the hippocampus and the medial prefrontal cortex (Everitt and Wolf, 2002). For this reason, one of our aims was to characterize these changes by determining major transcriptional modifications in the ventral striatum (comprising the NAc) after completing the whole conditioning process.

A number of studies using the microarray approach with psychostimulants (mainly cocaine, methamphetamine and amphetamine) in rodents have been published (reviewed by Yuferov *et al.*, 2005). More recently, similar studies have been carried out with alcohol (Mulligan *et al.*, 2011) or heroin and methamphetamine (Piechota *et al.*, 2012). From these studies, it is concluded that differential gene expression for a given drug depends on many factors such as dose, schedule, mode of administration (non-contingent or self-administration), studied tissue, animal strain and time of withdrawal or at which time point the expression is measured. In this study, we focused on the remaining expression changes in the ventral striatum 48 h after the end of a conditioning treatment, an approach that had not been yet taken for any drug of abuse.

After full genome microarray screening, we validated a list of six genes that could play a potentially important role in the acquisition of addiction as well as in the regulatory processes induced by mephedrone, ethanol and their combination. One of the most notable differentially expressed gene was the D_3 dopamine receptor gene (*Drd3*). It was similarly increased in all drug-treated animals. D_3 dopamine receptors (see Levant, 1997 for a review) are a subtype of D_2-like receptors with both presynaptic and postsynaptic locations, negatively coupled to adenylyl cyclase and acting as autoreceptors modulating dopamine release and/or synthesis. D_3 receptors are known to be implicated in reinforcement and reward induced by many drugs, including ethanol (Leggio *et al.*, 2014), cocaine (Vorel *et al.*, 2002; Song *et al.*, 2012), morphine (Liang *et al.*, 2011) and methamphetamine (Higley *et al.*, 2011), and they have been portrayed as a target for treating addiction (Vorel *et al.*, 2002; Newman *et al.*, 2012; Song *et al.*, 2012; Leggio *et al.*, 2014). D_3 receptors are mainly localized in limbic brain regions, especially the NAc (Diaz *et al.*, 1994). Ethanol, morphine and cocaine are all capable of up-regulating *Drd3* mRNA in rodents (Spangler *et al.*,

2003; Le Foll *et al.*, 2005; Vengeliene *et al.*, 2006) and in human addicts (Mash and Staley, 1999).

Based on these antecedents, we tested whether blocking D_3 receptors affected CPP and *Drd3* up-regulation induced by mephedrone and its combination with ethanol. The D_3 antagonist SB-277011A was able to completely block mephedrone-induced CPP and *Drd3* mRNA up-regulation. The fact that *Drd3* was also increased in the EtOH group, which did not show CPP at the dose used, suggests that it is not the sole player in establishing conditioning (discussed in the succeeding discussions). However, due to the robust blockade obtained with the D_3 antagonist, we sought to further explore the mechanisms involved in mephedrone-induced CPP and *Drd3* up-regulation.

BDNF has been reported to control dopamine D_3 receptor expression (Guillin *et al.*, 2001) and its expression to be increased by psychostimulants (Graham *et al.*, 2007). An increase in dopamine in the NAc (i.e. by psychostimulants) stimulates D_1/D_5 receptors of cortico-striatal neurons which, in turn, activate the cAMP pathway, thereby increasing the phosphorylation of CREB, which is required for BDNF production in certain cortical neurons. BDNF is then anterogradely transported and released in projecting areas, leading to induction of D_3 receptors in the striatum (Guillin *et al.*, 2001).

In fact, BDNF and D_3 receptors share common pathways in their respective signalling cascades, such as the kinases MEK-ERK and PI3K-Akt-mTOR, both involved in neuronal plasticity (reviewed by Collo *et al.*, 2014). Furthermore, Le Foll *et al.* (2005) demonstrated that *Drd3* mRNA and D_3 receptor binding are significantly increased after a single dose of cocaine and preceded by a transient increase in BDNF mRNA. Thus, increased BDNF expression has been suggested to alter the response to drug-associated cues by affecting the D_3 receptors in the nucleus accumbens (Le Foll *et al.*, 2005). In our experiments ANA-12, a selective trkB (BDNF receptor) antagonist, blocked both CPP and *Drd3* up-regulation induced by mephedrone. Moreover, mephedrone administration acutely increased BDNF mRNA in medial prefrontal cortex. Both results confirm that D_3 receptor differential expression can be mediated by BDNF and point to the fact that blocking their signalling can reduce the rewarding properties of mephedrone.

Interestingly, in our first treatment, we found *Drd3* mRNA in all three drug-treated groups to be equally increased, including ethanol-treated animals, which did not show CPP. This suggests that although D_3 receptors clearly play a role in the rewarding effects of mephedrone, there are also other changes needed to establish conditioning. D_3 receptor activity modulation by dopamine activation-dependent phosphorylation may also play a role (Liu *et al.*, 2009) but also other synaptic plasticity-related changes must occur for CPP.

To establish other possible candidates with a key role in the establisment of CPP and in the potentiation of mephedrone effects by ethanol, we screened the microarray results in search of other differentially expressed genes, which could be implicated in CPP-related synaptic plasticity. Of these, we consider it worth mentioning *Syt10* and *Muted*, which were only significantly increased in the groups receiving mephedrone and therefore could be the other partners needed for CPP and *Arpc5*, whose expression was increased in all drug-treated groups and potentiated in the mephedrone + EtOH group.

Syt10 encodes synaptotagmin 10, a calcium sensor involved in the regulation of neuron size and arborization through the exocytosis of the insulin-like growth factor 1 (IGF-1) which, in turn, mediates membrane expansion and axonal and dendritic growth (Scolnick *et al.*, 2008). Furthermore, the Muted gene codifies for a subunit of the BLOC-1 complex, which is involved in the activation of ARP2/3 (Ryder *et al.*, 2013). BLOC-1 also plays a key role in endosomal trafficking and as such has been found to regulate cell-surface abundance of the D_2 dopamine receptor, the biogenesis and fusion of synaptic vesicles, and neurite outgrowth. Therefore, it is possible that changes in synaptic membrane trafficking in the context of synaptic plasticity may contribute to the acquisition of CPP, together with the regulation of actin polymerization, *Syt10*-dependent IGF-1 secretion and D_3 receptor expression.

As mentioned previously, *Arpc5* mRNA was the only gene related with synaptic plasticity whose expression was directly correlated with CPP preference score and enhanced when associating mephedrone and ethanol. Its product, *Arpc5*, plays an important role in maintaining the ARP2/3 complex nucleating capability, which is essential for actin remodelling and synaptic plasticity at a presynaptic and postsynaptic level (Stradal and Scita, 2006; Cingolani and Goda, 2008). The ARP2/3 complex is associated with F-actin in the spinoskeleton core and acts to nucleate new actin filament branches from existing actin filaments. It is therefore essential in the activity-dependent enlargement of dendritic spines. Similarly, *Camkk1*, whose codified protein plays an important role in actin dynamics, was significantly up-regulated.

However, additional and very extensive work should be performed to investigate and demonstrate the hypothetical role of these candidates on CPP acquisition and potentiation. Moreover, other transient factors that returned to basal levels in less than 24 h (i.e. BDNF), and therefore were left out from microarray screening, may also play a role.

In the present study, we also found five notably up-regulated apoptosis-related genes as well as a robust increase in the expression of *Nfu1* in mephedrone-treated groups. Amphetamines cause oxidative stress and mitochondrial dysfunctions in rat brain, which can can induce from cellular malfunction to apoptosis (Beauvais *et al.*, 2011). NFU1 protein activity is essential in the mitochondrial respiratory chain and the citric acid cycle (Mühlenhoff *et al.*, 2002) so the increase in *Nfu1* expression could be explained by higher energetic demand due to metabolic stress. These results are in accordance with the high increases in glutathione peroxidase in the same groups, as this enzyme family is one of the most highly implicated in the detoxification of ROS. Interestingly, this enzyme was expressed significantly less in animals treated with the drug combination, compared with mephedrone alone. This unexpected phenomenon is being further explored in work focussed on the neurotoxic effects of this combination (unpublished results).

To sum up, the co-administration of ethanol with mephedrone in adolescent mice increases its psychostimulant and rewarding properties, which suggests an increased risk of drug abuse if translated to humans. Thus, an experimental-based warning about the risks of combined consumption of these drugs should be given to the youth

population. 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 is neede to determine whether adults could be susceptible to changes of the same nature and degree. The establisment of conditioning by mephedrone requires changes in the expression of genes related to neurotransmitter (dopamine) receptors, among which D_3 receptors and BDNF appear to play a key role, although other factors that require investigation may participate as well.

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Author contributions

D. P., E. E. and J. C. were responsible for the study concept and design. A. C. and D. P. performed experiments with animals and qPCR. C. J. C. and A. C. performed the microarray analysis. J. C. and E. E. performed statistics and interpretation of behavioural experiments. D. P. and A. C. wrote the manuscript draft. All authors critically reviewed content and approved the final version.

Conflict of interest

None.

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Article 4 (accepted but unpublished): The combination of ethanol with mephedrone increases the sings of neurotoxicity and impairs neurogenesis and learning in adolescent CD-1 mice. *Toxicology and applied pharmacology*

Neurotoxicity of amphetamine derivatives is a matter of concern and has been subject of a great amount of studies. Recently, a new family of amphetamine derivatives under the name of cathinones, mephedrone being the most widely consumed, broke into the illegal market. In light of the fact that around 95% of cathinone consumers have been reported to combine them with ethanol, we sought to study the consequences of the concomitant consumption of ethanol on mephedrone-induced neurotoxicity.

Adolescent (5 weeks) male Swiss-CD1 mice were treated four times in one day with a dose of mephedrone of 25 mg/kg and changing doses of ethanol (2; 1.5; 1.5; 1 g/kg; obtaining a steady plasma concentration of around 1.5 g/l) each separated by 2 hours in a room with set temperature at 27ºC, emulating common ambient conditions found in dance clubs. Following, several neurochemical, histological and behavioral parameters were measured.

7 days post-treatment (PT), the concomitant administration of ethanol enhanced mephedrone-induced decreases in tryptophan hydroxylase and serotonin transporter density in the hippocampus, as well as in tyrosine hydroxylase and dopamine transporter density in the frontal cortex by approximately 2-fold. Furthermore, these decreases correlated with a 2-fold increase in lipid peroxidation in both areas, measured as concentration of malondialdehyde (MDA) 24 hours PT. This effect was accompanied by increases in the oxidative stress-related enzymes glutathione peroxidase, catalase and superoxide dismutase in both mephedrone-treated groups.

In a separate experiment, animals were injected with Bromo-deoxy-Uridine (BrdU) following the same treatment schedule described above, and sacrificed 28 days PT, with the objective of measuring neurogenesis in the dentate gyrus. During this time period, animals underwent a general Morris water maze (MWM) protocol, starting on day 7 PT.

MWM showed an effect of Meph treatment on multiple learning and memory parameters, which correlated with a BrdU count 25% lower than that of control animals. These changes were enhanced in the mephedrone+ethanol group, which showed a decrease higher than 2-fold in BrdU labeling. The drop in hippocampal

neurogenesis 28 days PT in mephedrone-treated animals and its potentiation by ethanol could be caused by a reduction in cell proliferation, increase in cell death, or a combination of both factors.

The fact that this effect was accompanied long-term decreases in serotonergic and dopaminergic markers of a similar magnitude, points to a clear enhancement of mephedrone-induced neurotoxicity by ethanol, which could be directly related with the increase shown in oxidative stress. 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. In this sense, the effects of the concomitant use of mephedrone and ethanol on the hippocampus are especially noteworthy, since this area is greatly involved in learning and memory processes, and could affect normal brain development in young adults with long-term behavioral consequences, as suggested by results obtained in the MWM paradigm.

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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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 ºC. 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 hours 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.

Keywords: adolescence, alcohol, cathinones, mephedrone, neurogenesis, neurotoxicity.

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 et al., 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).

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.

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).

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; López-Arnau et al., 2012; Martínez-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 (Sánchez 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 (Martínez-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 (Martínez-Clemente et al., 2014). Moreover, similar results have recently been reported in rats (López-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. Hernández-Rabaza el 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 (Martínez-Clemente et al., 2014; López-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 (Martínez-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 to 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, Martínez-Clemente et al., 2013). Furthermore, during the whole duration of the treatment, room temperature was set at 26±2ºC, at which this drug has been reported to induce signs of neurotoxicity (Martínez-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; Martínez-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 hour 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, difering 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 ºC until use. When required, tissue samples were thawed and homogenized through sonication at 4 ºC. The homogenates were centrifuged at 1,000 x g for 15 min at 4 ºC. Aliquots of the resulting supernatants were stored at -80 ºC until use for Western blot assays. The rest of the samples were resuspended and centrifuged at 15,000 x g for 30 min at 4ºC. The pellets were resuspended in buffer and incubated at 37 ºC for 5 min. The final pellets were resuspended in the appropriate buffer and stored at -80 ºC 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 x 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 4ºC. 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 25ºC 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. 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 2h at room temperature with a primary mouse monoclonal antibody against TH (Transduction Laboratories, Lexington, KY, USA) dil. 1:5000; rabbit polyclonal anti-TPH2 (Millipore, Billerica, MA, USA) dil.1:1000; sheep

polyclonal anti-SOD Cu/Zn (EMD Chemicals, La Jolla, CA, USA) dil.1:2000; mouse monoclonal 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 ± 1ºC) to a depth of 25 cm and rendered opaque by the addition of a non-toxic latex solution. The pool was in an isolated room and black curtains were closed around it to suppress room cues. Four positions around the edge of the tank were designated as north (N), south (S), east (E), and west (W) and also defined the division of the tank into four quadrants: NE, SE, SW, and NW, providing alternative start positions. 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 Bromo-deoxyuridine (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 -20ºC until used for immunohistochemical analysis.

Immunohistochemistry and its quantification

For BrdU detection, sections of interest were selected and washed with PBS, incubated in 2N HCl at 37°C for 30 minutes, washed in PBS, incubated in 0.1M boric acid at 37ºC, washed in PBS and blocked for 1 hour in a blocking solution (PBS containing 0.2M 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 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 ($F_{3,20} = 17.04$, $P < 0.001$).

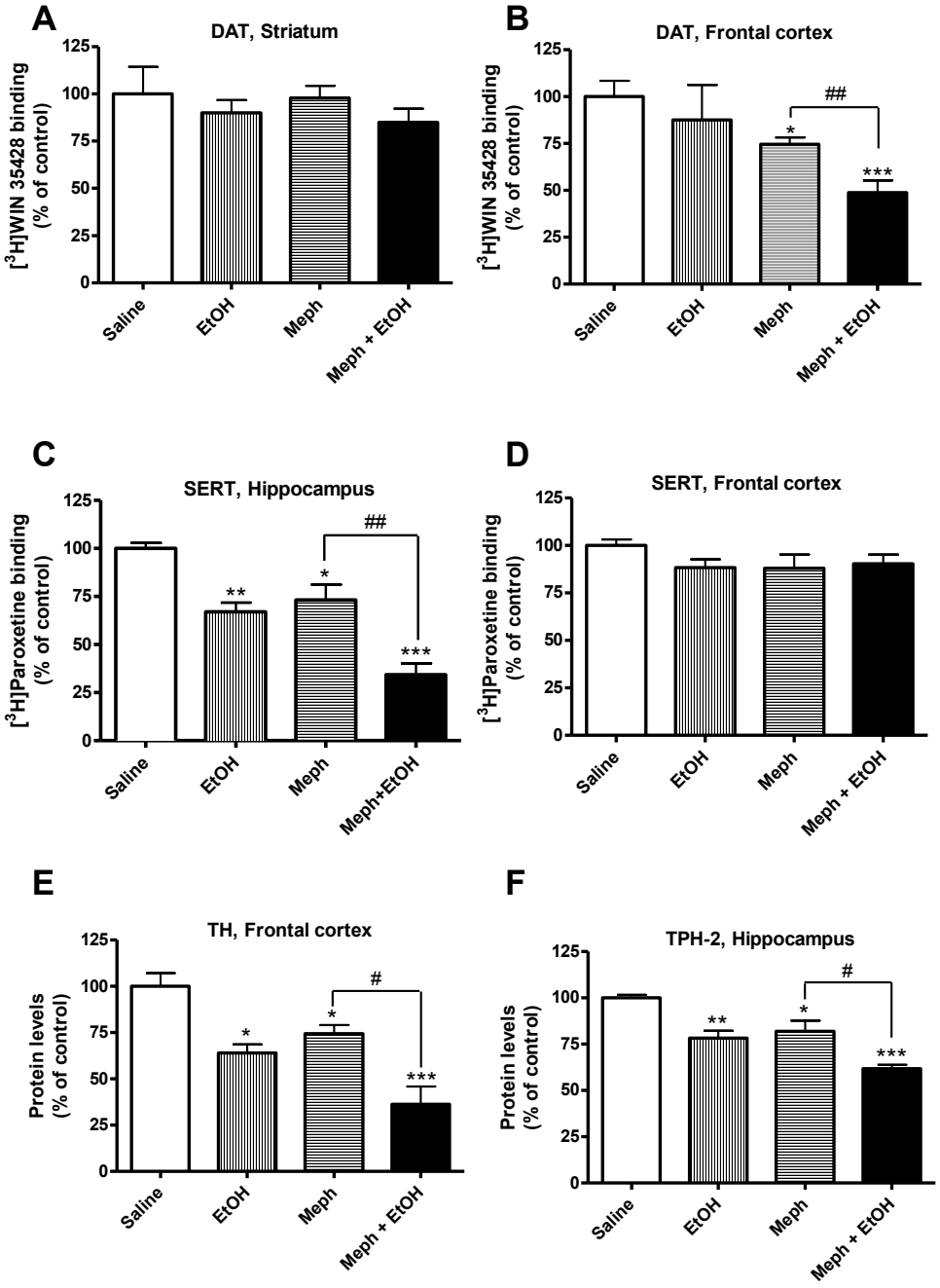

Figure 1.- Levels of dopamine (DAT, panels A and B) and serotonin (SERT, panels C and D) transporters in specific brain areas of adolescent CD-1 mice 7 days after being treated with either saline, ethanol, mephedrone or their combination, following the schedule described in the Materials and methods 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.

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 hours 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 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.

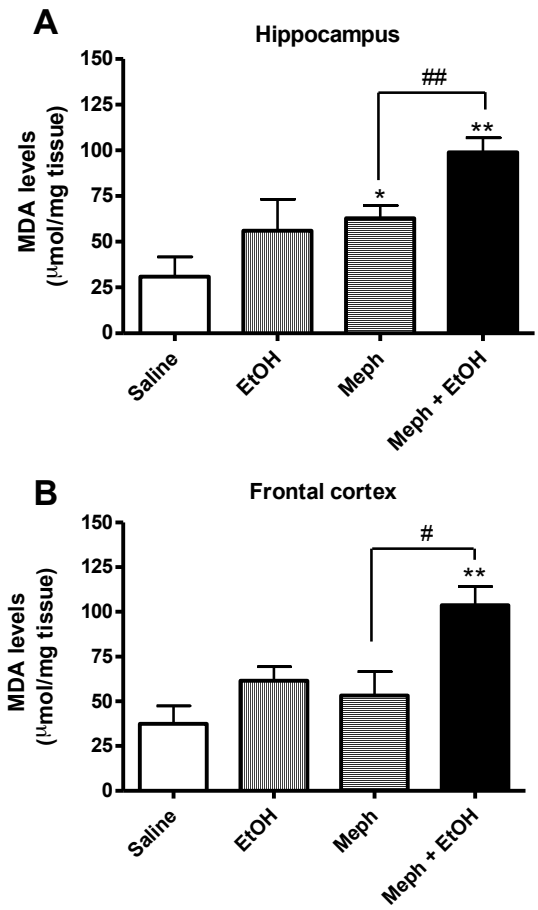

Figure 2.- Assessment of lipid peroxidation measured as levels of malondialdehyde (MDA) in hippocampus (A) and frontal cortex (B) from adolescent CD-1 mice, 24 h after being treated with either saline, ethanol, mephedrone or their combination, following the schedule described in the Materials and methods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

Gpx was significantly overexpressed in both mephedrone-treated groups (Fig. 3A), although no significant difference could be found between them ($F_{3,17}$ = 4.19, P < 0.05) and similarly occurred with CAT levels ($F_{3,17}$ = 16.89, P < 0.001) (Fig. 3B).

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).

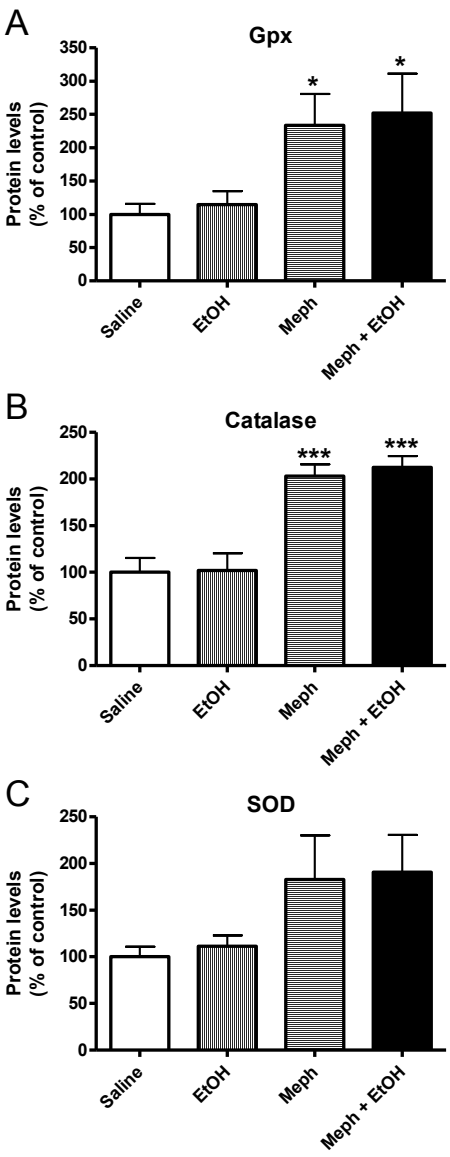

Figure 3.- Levels of enzymes related with antioxidant response in frontal cortex from adolescent CD-1 mice, 24 h after being treated with either saline, ethanol, mephedrone or their combination, following the schedule described in the Materials and methods section. Gpx1 (panel A), catalase (panel B) 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 6-8 animals per group. *P < 0.05, ***P < 0.001 vs. saline.

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 x 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 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.

24 h 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 seconds

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).

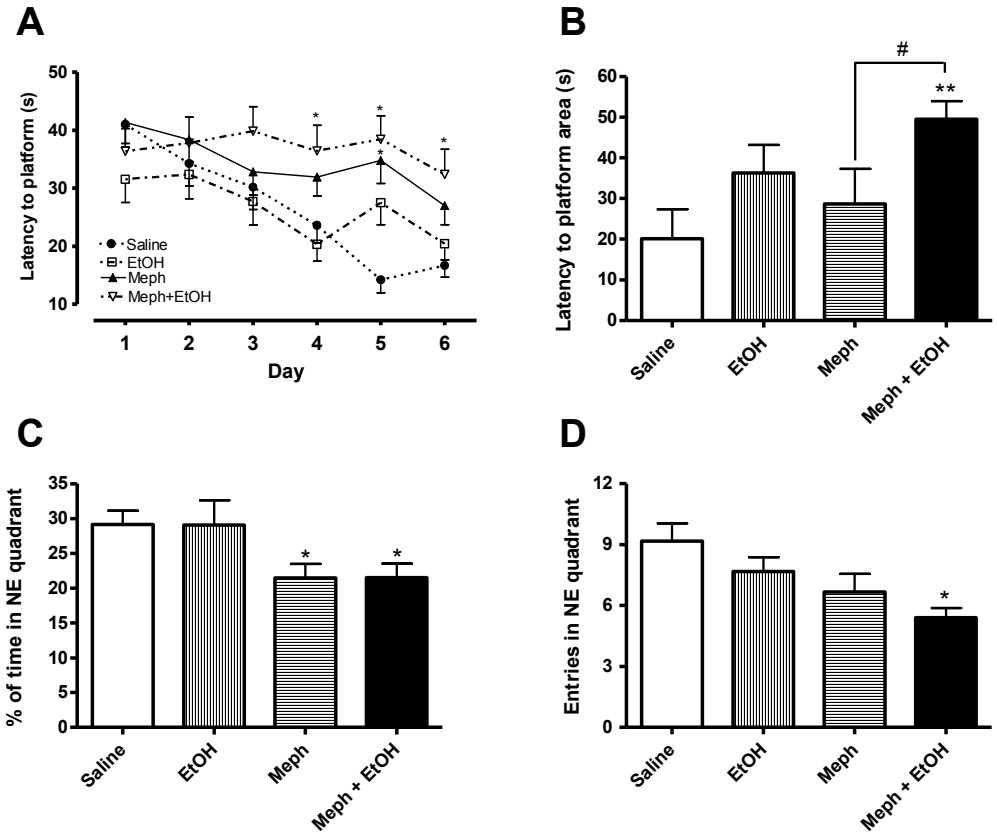

Figure 4.- Effects of treatment with either saline, ethanol, mephedrone or their combination on spatial learning and memory. Adolescent mice were treated as described in Materials and Methods and seven days after they were submitted to the Morris water maze paradigm, consisting of 6 days of training 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 < 0.01vs. saline; #P < 0.05 between the indicated groups.

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. 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.

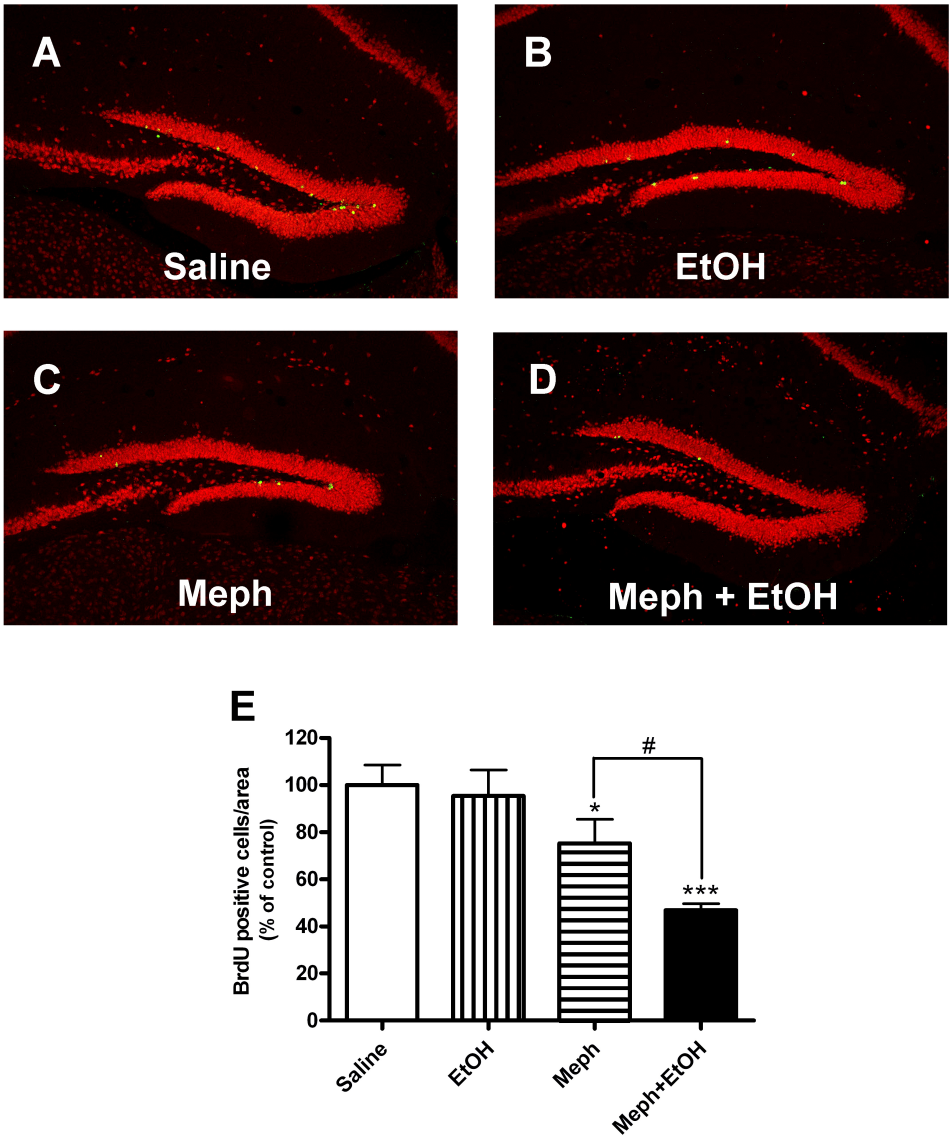

Figure 5.- Neurogenesis assessment in the dentate gyrus of the hippocampus of mice, 28 days after treatment with either saline, ethanol, mephedrone or their combination. Adolescent mice were treated as described in Materials and Methods, and received two injections of BrdU, 2 and 12 h after the last 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 of the slices and size of the signal, 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.

DISCUSSION

Alcoholic drinks are frequently combined with the new psychostimulant substances (Elliott and Evans, 2014). It has been reported 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., 2009). 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 (Martínez-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 hours, at a room temperature of 26ºC), 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. These transporters are integral membrane neuronal proteins that function to terminate neurotransmission by the rapid reuptake of synaptic neurotransmitters into presynaptic neurons. 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 (Martínez-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 of the effect on a given brain area also depend on the dosing schedule. In the case of mephedrone, an effect on serotonergic terminals in the frontal cortex only appeared after 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) (Martínez-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 et al., 1997; Wu et al., 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 et al., 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 1g/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.

ACKNOWLEDGEMENTS

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Article 5 (unsent): Ethanol decreases the psychostimulant but not the conditioning properties of MDPV: postulation of a pharmacokinetic interaction.

Methylenedioxypyrovalerone (MDPV) is a newly abused psychostimulant of the cathinone family. It has strong cocaine-like effects, as it is a fairly selective dopamine transporter (DAT) blocker, exhibiting notable psychostimulant and conditioning properties. Currently, most drug use occurs in leisure environments, such as dance clubs and parties, leading to a preference for the use of psychostimulants (i.e. cocaine and amphetamine derivatives such as MDPV) and alcohol (ethanol), which is omnipresent due to its legal drug status. Thus, the combination of psychostimulants and ethanol is frequent. Ethanol has been found to potentiate the psychostimulant and rewarding properties of many drugs of abuse, such as MDMA, cocaine or mephedrone. Thus, it proved important to explore the potential pharmacological interaction between MDPV and ethanol.

Locomotor activity assays evidenced that 1 g/kg ethanol (a dose that is inactive on its own) elicited a reduction, rather than a potentiation, in the stimulant properties of low doses of MDPV (0.1-0.3 mg/kg). Conversely, high doses of MDPV (1-3 mg/kg) were unaffected. The rewarding properties (measured as conditioned place preference, CPP) of a low and a high dose of MDPV (0.3 and 3 mg/kg) were unaltered by ethanol.

MDPV brain and blood levels were assessed at different time points after an acute administration of MDPV (0.3 or 3 mg/kg) and their combination with ethanol (1 g/kg). In agreement with behavioral experiments, no effect of ethanol was evidenced when combined with 3 mg/kg MDPV. Conversely, when administering 0.3 mg/kg MDPV, the combination group showed significantly decreased MDPV levels in the brain and blood (by around 50%) at the first assessed time point (20 min). MDPV concentration leveled off in both groups thereafter. Given that the decrease in MDPV occurred in parallel in both tissues, an effect of ethanol on normal blood-brain crossing rate was discarded. In light of the fact that ethanol and MDPV share several cytochromes (CYP) in their respective metabolic transformations, we postulate that a pharmacokinetic interaction between these two compounds may be taking place, where CYP inhibition by ethanol shifts normal MDPV metabolism into an alternative route involving enzymatic reactions with a high Vmax.

Ethanol decreases the psychostimulant but not the conditioning properties of MDPV: postulation of a pharmacokinetic interaction.

ABSTRACT

Methylenedioxypyrovalerone (MDPV) is a newly abused psychostimulant of the cathinone family with strong cocaine-like effects, as it is a fairly selective dopamine transporter (DAT) blocker. Ethanol has been found to potentiate the psychostimulant and rewarding properties of many drugs of abuse, such as MDMA, cocaine or mephedrone. Thus, it proved important to explore the potential pharmacological interaction between MDPV and ethanol.

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MDPV brain and blood levels were assessed at different time points after an acute administration of MDPV (0.3 or 3 mg/kg) and their combination with ethanol (1 g/kg). In agreement with behavioral experiments, when administering 0.3 mg/kg MDPV, the combination group showed significantly decreased MDPV levels in the brain and blood (by around 50%) at the first assessed time point (20 min). MDPV concentration leveled off in both groups thereafter. We postulate that a pharmacokinetic interaction between these two compounds may be taking place, where CYP inhibition by ethanol shifts normal MDPV metabolism into an alternative route involving enzymatic reactions with a high Vmax.

1. INTRODUCTION

The popularity of cathinones as recreational drugs has increased since they first appeared in the illicit drug market, to the extent where their use has become prevalent (Karila et al., 2015) Methylenedioxypyrovalerone (MDPV) is a synthetic cathinone that shares pharmacodynamic and structural similarities with cocaine and MDMA, but differs from most cathinones by acting preferentially as a monoamine reuptake inhibitor. It selectively inhibits dopamine (DA) and norepinephrine transporters, while serotonin uptake is less affected (Cameron et al., 2013; Marusich et al., 2014). Some studies have demonstrated that MDPV is even more potent than cocaine in blocking DA transporter, as well as in producing locomotor activation (Baumann et al., 2013; Cameron et al., 2013). Moreover, it is known that

MDPV shows rewarding and reinforcing properties similar to those of cocaine (Baumann et al., 2013; King et al., 2015).

In humans, recreational polydrug use is quite common (Pedersen and Skrondal, 1999). New psychoactive substances, such as MDPV, are also commonly combined with many other drugs, especially alcohol (ethanol, EtOH), which, in turn, is the most consumed legal drug in western countries (Elliot and Evans, 2014). The reasons for polydrug use are diverse, and range from the desire to enhance the effect or counteract the unwanted effects of some drugs, to the substitution of another substance, easy availability or social pressure, among others. In this sense, cocaine is known to aid in counteracting the subjective feeling of inebriation after heavy ethanol binges, while increasing the "high"; furthermore, ethanol counters anxiety precipitated by cocaine withdrawal albeit it can also facilitate cocaine craving (Lacoste et al., 2010). Many studies conducted in rodents indicate that EtOH can alter the pharmacological and metabolic profile of cocaine, and vice versa (Busse et al., 2004, 2005; Masur, Souza-Formigoni and Pires, 1989; Sobel and Riley, 1997). Even a dose-dependent attenuation of cocaine-induced increase in locomotor activity when ethanol is administered prior to cocaine has been described (Dewey et al., 1997). Moreover, the simultaneous use of cocaine and ethanol yields an anomalous byproduct of cocaine, cocaethylene, which is endowed with markedly different pharmacodynamic properties (Pérez et al., 1994), as well as increased cardiotoxic effects (Wilson et al., 2001).

In rats, EtOH-MDMA co-administration potentiates MDMA-induced hyperlocomotion and rewarding effects, while attenuating MDMA-induced hyperthermia (Ben Hamida et al., 2007; Jones et al., 2010). In fact, EtOH increases the concentrations of MDMA and its main metabolite, MDA, in blood and brain (Ben Hamida et al., 2007; Cassel et al., 2007). Additionally, Riegert et al (2008) described a local synergistic interaction of EtOH and MDMA on the spontaneous outflow of striatal DA and 5-HT, which could be relevant to the EtOH-induced potentiation of MDMA effects.

Recently, our group published the first study focusing on the effects of ethanol on the psychostimulant and rewarding properties of a cathinone derivative (mephedrone). We found that, at low (non stimulant) doses, ethanol significantly enhances the psychostimulant effects of mephedrone. This effect is mediated by an increase in synaptic dopamine, as haloperidol, but not ketanserin, is able of blocking the potentiation by alcohol. Similarly, the conditioning properties of mephedrone are enhanced by a low non-conditioning dose of alcohol (Ciudad-Roberts et al., 2015).

The effects of ethanol on the brain are numerous and complex. In the ventral tegmental area (VTA), its effect is mediated by an inhibition of NMDA receptors that normally serve to increase GABA release in response to signals mediated by glutamate (Steffensen et al., 1998). Accordingly, an ethanol-induced decrease in GABAergic neurotransmission leads to increased mesolimbic dopamine release, a classic mechanism involved in ethanol dependence. Ethanol is metabolized in the liver, through three different enzymatic pathways: alcohol dehydrogenase, catalase and a microsomal ethanol oxidizing system involving the CYP450 system, and it can alter the expression and/or activity of its isoenzymes (Lieber, 1990).

Thus, when investigating possible drug-drug interactions such as MDPV-EtOH, both pharmacokinetics and pharmacodynamics must be taken into consideration. Very few studies have been published regarding MDPV metabolism in vitro or in vivo in rodents and humans (Meyer et al., 2010; Negreira et al., 2015; Anizan et al., 2014). Using recombinant human cytochrome P450 (CYP) enzymes, the isoforms that have been found to be involved in MDPV biotransformation are CYP1A2, CYP2D6, CYP2C19, CYP2B6 and CYP2C9, the resulting primary metabolites being 3,4-dihydroxypyrovalerone (3,4-catechol-PV) and 4-hydroxy-3-methoxypyrovalerone (4-OH-3-MeO-PV) (Anizan et al., 2015; Novellas et al., 2015; Negreira et al., 2015). In previous findings from our lab we have demonstrated a good correlation between MDPV brain concentrations and the increase in locomotor activity and stereotypies induced by this cathinone (Novellas et al., 2015).

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 is capable of modifying the psychostimulant (locomotor activity) and conditioning effects of MDPV when administered concomitantly. The surprising results we obtained warranted a short pharmacokinetic study aimed at determining the effects of this combination on MDPV levels in blood and rat striatum.

Furthermore, is known that the nucleus accumbens (NAcc) and the rest of the striatum (Str) play a key role in the neural circuitry underlying psychostimulant action and the constructs of reward and reinforcement. These phenomena are mediated by changes in DA levels in these areas (Krasnova et al., 2013), in such a way that a good correlation has been reported between cocaine induced increases of DA concentrations in the NAc and the enhanced locomotor responses to this psychostimulant (Kalivas et al., 1993). We therefore investigated the effects of MDPV and their combination with ethanol on the concentration of accumbal extracellular DA and its main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA), in order to correlate them with the behavioral effects (i.e. place conditioning and locomotor activity).

2. MATERIALS AND METHODS

2.1 Subjects and Drugs

Male Sprague-Dawley rats (Charles River, Spain), weighing 250-300 g were used. Animals were housed at 22 ± 1 ^{o}C with food and water ad libitum. The Animal Ethics Committee of the University of Barcelona, following the 86/609/EEC guidelines, approved the experimental protocols for the use of animals in this study.

MDPV and methylone was synthesized in racemic form as HCl salt in our laboratory as described previously (Novellas et al., 2015; López-Arnau et al., 2012). Dopamine.HCl, DOPAC and HVA, as well as chemicals for mobile phase and perfusion medium preparations were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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.

2.2 Locomotor activity

Prior to the experiments, animals were randomly assigned to 4 groups (N= 3–5/group) and received two habituation sessions (48 and 24 h). During these sessions, each rat received saline and was placed in a Plexiglas cage without registering locomotor activity. This cage constituted the activity box that was later placed inside a frame system of 16 infrared photocells (LE8811, Panlab, Barcelona, Spain). Occlusions of the photo beams (breaks) were recorded over a 10-min block and sent to a computerized system (SedaCom32, Panlab, Barcelona). On the testing day, the animals received saline, MDPV (0.1, 0.3, 1 or 3 mg/kg, s.c.), EtOH (1g/kg, i.p.) or both. Since ethanol, at certain doses, can impair or enhance locomotion, it was administered at doses reported to not affect basal activity (Cassel et al., 2004). After drug administration, animals were immediately placed in the activity box and horizontal locomotor activity was monitored during 60, 120 or 360 min. Results are expressed as the area under the curve (AUC), which was measured as the total changes from baseline at each recording interval.

2.3 Conditioned place preference (CPP) test

The place conditioning protocol used was non-biased (Robledo et al., 2004). 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 pre-conditioning phase (day 0,1), 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 by computerized monitoring software (Smart, Panlab, Barcelona, Spain).

The CPP experiment was designed to assess the conditioning properties of two doses of MDPV (0.3 or 3.0 mg/kg s.c) and their association with ethanol (1 g/kg). We intended to use a dose of ethanol that did not produce CPP on its own (Tzschentke et al., 2007).

During the conditioning phase (days 2, 4, 6 and 8), rats (N= 8-12 /group) were treated with saline, MDPV (0.3 or 3.0 mg/kg s.c.), EtOH (1g/kg i.p.) or their combination, and immediatly confined into one of the two conditioning compartments for 30 min. On days 3, 5, 7 and 9 of the conditioning phase, animals received saline and were confined to the opposite compartment. The animals were exposed to only one pairing per day and treatments were counterbalanced to assure that drugs were equally administered in both compartments.

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 pre-conditioning phase.

2.4 MDPV levels in rat striatum

As mentioned above, a correlation between brain concentrations and enhancement of the locomotor activity and stereotypies induced by MDPV has been demonstrated previously (Novellas et al., 2015). Moreover, it is know that MDPV increases the synaptic concentrations of DA in the nucleus accumbens (Baumann et al., 2013). Accordingly, we quantified MDPV levels after its administration alone (0.3 or 3 mg/kg s.c.) or with EtOH (1 g/kg i.p.) in rat striatum.

This experiment was carried out as described by Novellas et al., 2015, with minor modifications. Briefly, after drug administration, rats were killed by decapitation under isoflurane anaesthesia 20, 40, 60 and 80 min after drug administration, and the striatum was quickly dissected out and stored at -80C until use. The striatum was homogenized in ice-cold sodium carbonate-sodium bicarbonate (pH: 11.5) using an ultrasonicator. The homogenate was centrifuged at 1000 × g for 20 min at 4°C. The sample (400 µL supernatant + 250 µL distilled H2O + 100 µL methylone (Martínez-Clemente et al., 2013) as internal standard (IS)) 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. Each point on the curve is the mean of five animals.

A PE Sciex API3000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was used to quantify MDPV in brain and blood samples. This system was coupled to a refrigerated autosampler, a photodiode array detector and a column oven set to 40°C. 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 formic acid in both solvents. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows, (0, 5), (20, 95), (22, 95), (22.5, 5) and (27.5, 5), at a constant flow rate (150 µL/min). The biological samples were refrigerated at 4°C and 5 µL were injected into the LC-MS/MS system. For MDPV, two transitions were followed (m/z 276.1 → 126.2 and 276.1 → 175.3, collision energy of 35 and 30 V respectively). Two transitions were followed for methylone (m/z 208.1→190.1 and 208.1→160.0, collision energies of 17 and 22 V respectively), and both were used for the quantification.

2.5 Surgery and microdialysis experiments

The microdialysis experiments were carried out on awake rats (N=3-5/group) according to the protocol described by Yoshitake et al. (2006), with some modifications. Rats were anesthetized with ketamine (90mg/kg i.p) plus xylazine (10mg/kg, i.p.). The animal was placed in a stereotaxic frame and a middle scalp incision was made. After exposure of the skull, a hole for a probe 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 nucleus accumbens, according the coordinates: 1.6 mm lateral, 1.8 mm anterior to bregma and 6.0 mm ventral to the dura surface according to the stereotaxic atlas of Paxinos et al. (2009) 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 15000 Da cut-off) was inserted into the guide cannula and perfused overnight with artificial cerebrospinal fluid (aCSF) solution (148mM Nacl, 2.2mM $CaCl_2$, 0.8 mM $MgCl_2$, 1.2 mM Na_2HPO_4 and 0.3mM NaH_2PO_4) at a flow rate of 0.6µL/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 composed by 0.1 M acetic acid plus 0.27 mM Na_2EDTA and 0.5 mM ascorbic acid in order to prevent monoamine degradation (Thorré et al., 1997). The first three samples were used for estimation of basal levels of DA, 5-HT, DOPAC, HVA and 5-HIAA. Thereafter, saline, MDPV (0.3 or 3.0 mg/kg s.c.), EtOH (1g/kg i.p.), or both were injected to separate groups of rats and the fractions were collected for 240 min and stored at -80 ^{o}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 those rats with correct placements were included.

2.6 LC-MS/MS determination of DA and metabolites in dialysate samples

A 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 x 4 mm, 3 µm, Sigma-Aldrich, St. Louis, MO, USA) pentafluorophenyl column thermostated at 37 ^{o}C. The mobile phase was water (A) and methanol (B) with 0.1% of formic acid in both solvents. An increasing linear gradient (v/v) of B was used (t(min), %B), as follows, (X, X) FLOW RATE. 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 microdialysates samples were refrigerated at 4 ^{o}C and 20 µL were injected, without sample pretreatment, into the LC-MS/MS system. The LC-MS/MS conditions were optimized by direct infusion of monoamine and metabolites standards (1 µg/ml) dissolved in 50,50 (v/v) water (0.1 % formic acid)/methanol (0.1 % formic acid) into the MS at a constant flow rate (X µL/min). Mass spectrometric quantification in positive ion mode was carried out using the following transitions: DA (*m/z 154 → 137; 154 → 91)* and DOPAC (*m/z 123 → 77*). A negative ion mode was used in the analysis of HVA (*m/z 181 → 122*).

Six standars (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 less than 15%.

2.7 MDPV levels in blood

Blood samples (150–200 µL) were collected from awake rats through the tail vein 20, 40 and 80 min after MDPV injection (0.3 or 3 mg/kg, s.c.) alone or in combination with EtOH (1g/kg, i.p.) and transferred to 300 uL Microvette CB 300 EDTA (Sarstedt, Nümbrecht, Germany) tubes on ice. Samples were centrifuged (2000 × g for 10 min) and 90 µL of plasma was mixed with 10 µL of IS solution (methylone, 200 ng/ml). The mixture was extracted by adding 250 µL of methanol up to a final concentration of 70%. The denatured proteins were precipitated by centrifugation at 10,000 × g for 4 min. Of the clear supernatant, 250 µl was acidified with formic acid (50/50, v/v) to a pH of 2.5–3.0 to obtain stable extracts since cathinones degraded relatively fast in non-acidified live blood extracts (Sørensen 2011). The mixture was ultrafiltered by centrifugation (35,000×g for 30 min)

through a 30-kDa regenerated cellulose membrane (Microcon 10®, Millipore, Bedford, MA, USA) to remove high-molecular-weight components. Finally, 100 µl of the filtrate was transferred to an autosampler vial to quantify MDPV levels by LC-MS/MS as above.

2.8 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) and significant differences ($P < 0.05$) were analyzed using the Tukey's post hoc test for multiple comparison measures (InVivoStat software package). Analyst v1.4.2 software was used to calculate the areas of the peaks of chromatograms.

3. RESULTS

Four different doses of MDPV were tested, 0.1, 0.3, 1 and 3 mg/kg, as well as their combination with 1 g/kg of ethanol. Locomotor activity was recorded for 60 min (MDPV 0.1), 120 min (MDPV 0.3 and 1 mg/kg) or 360 min (MDPV 3 mg/kg), due to the difference between the psychostimulant effects at each dose. ANOVA of area under the curve (AUC) revealed an overall effect of treatment for all three designs (60 min: $F_{3,12} = 9.73$, $P < 0.01$; 120 min: $F_{5,16} = 30.11$, $P < 0.001$; 360 min: $F_{3,12} = 8.33$, $P < 0.01$; n=4 per group). As can be seen in table 1, MDPV induced significant increases in locomotor activity. Ethanol, at the doses used, had no significant effect on locomotion when administered alone.

With regards to the combination treatments, ethanol significantly reduced locomotor activity elicited by MDPV at 0.1 ($P<0.01$) and 0.3 mg/kg ($P<0.05$) by 70 and 65%, respectively, yielding, in both cases, activity levels that are not significantly different from those for the saline group. Conversely, no significant effect ($P>0.05$) was observed for ethanol on the higher doses of MDPV (1 and 3 mg/kg). Furthermore, the reduction effect is also dependent on ethanol concentration, as locomotor activity elicited by MDPV 0.3 mg/kg was further reduced by ethanol 2 g/kg (data not shown). The time course for locomotor activity induced by MDPV 0.3 and 3 mg/kg and their combination with ethanol 1 g/kg is displayed in Figure 1.

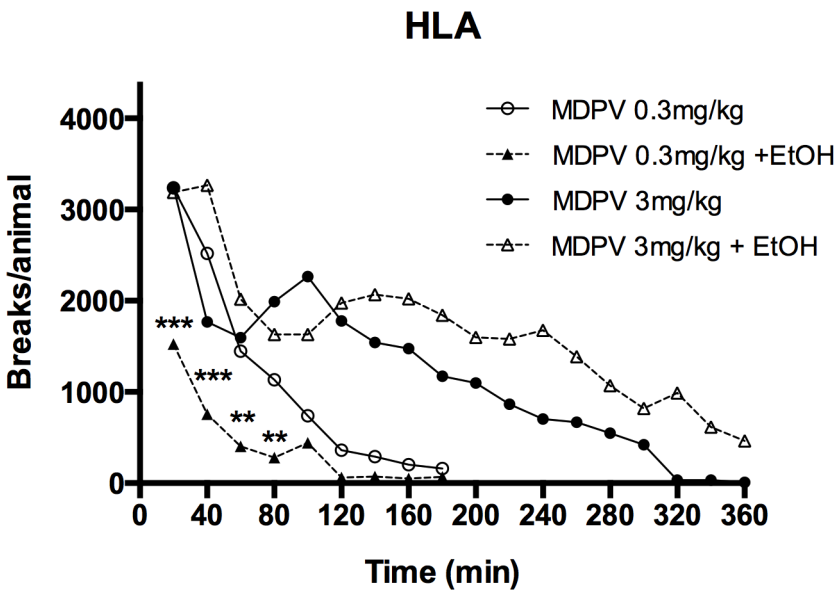

Figure 5: Time course of locomotor activity expressed as breaks/10 min intervals. ** (P<0.01) * (P<0.001) comparison with respective non-ethanol treated group.**

The CPP paradigm was used to study the conditioning properties of two different doses of MDPV (0.3 and 3 mg/kg) and how ethanol (1 g/kg, a dose that does not elicit CPP on its own) could modify this effect (Figure 2). Times (expressed as a percentage) spent in both compartments during the pre-conditioning phase were 50.1 ± 2.42 and 49.9 ± 2.42 (P>0.05), respectively indicating a total lack of preference for either side. On the test day (day 10, post-conditioning), one way ANOVA revealed a significant effect of the treatment ($F_{5,36}$ =1.647, P<0.001, n= 6-7/group). Ethanol by itself did not exert any effect on preference score (Saline: -50.75 ±35.13, ethanol: -40.52 ±39.1). Both doses of MDPV elicited place preference, albeit to a different degree (MDPV 0.3: 134.8 ±77.87 s; MDPV 3: 241.6±23.40 s), which showed statistical significance (P<0.05 and P<0.01 vs. saline respectively). The concomitant administration of ethanol did not reduce MDPV-induced CPP by MDPV at the dose of 0.3 mg/kg. Furthermore, it caused a significant CPP over control animals (MDPV+ethanol 0.3: 146.0 ±92.56. Similarly, at the dose of 3 mg/kg, both MDPV-treated groups showed virtually the same preference score (MDPV 3: 241.6 ±23.40 vs MDPV 3+ethanol 1: 235 ± 57.31s, n.s.).

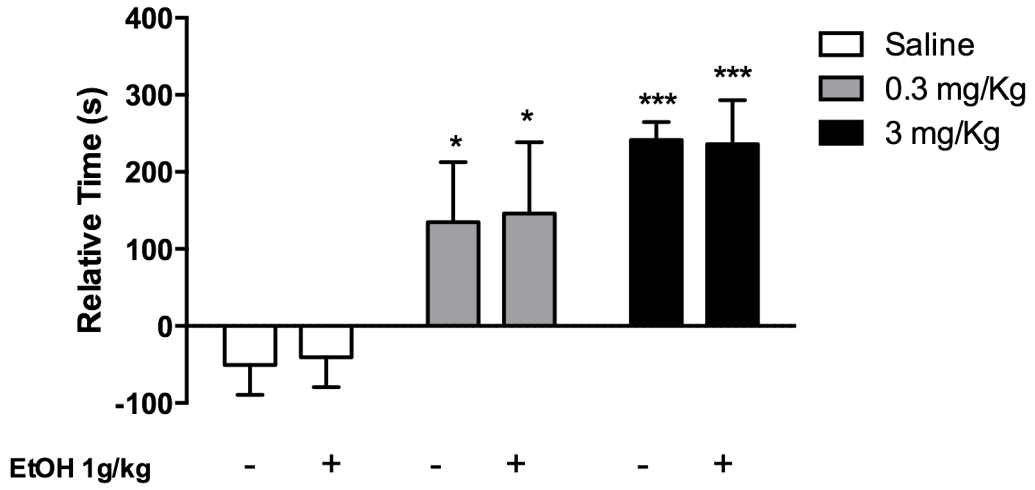

Figure 6: CPP score represented as relative time in drug-paired compartment on the test day minus relative time in drug-paired compartment on the pre-conditioning day. * (P<0.5) **(P<0.001) compared with the control group.

Results on locomotor activity suggested a differential effect of ethanol on high and low doses of MDPV. Thus, It proved important to measure MDPV brain levels caused by the tested drug-treatments. In this sense, a full kinetic study was not performed, due to the large number of animals needed (4-5 animals are required for each point); furthermore, reports on MDPV kinetics have already been published by others (Anizan et al., 2014; Novellas et al., 2015). Rather, a simpler approach was used, focusing on early time points, as this is when the reduction in locomotor activity by ethanol is shown. Furthermore, as in CPP experiments, both a low and a high dose of MDPV were assessed (0.3 and 3 mg/kg).

When administering 0.3 mg/kg MDPV in combination with 1 g/kg ethanol, brain levels were significantly decreased, when compared to animals treated with MDPV alone. This decrease, of around 42%, was only evident at the earliest assessed time point (20 min), and yielded statistical significance (P<0.05). Conversely, at later time points (40, and 80 min), MDPV levels were similar in both treatment groups; this contrasts with results from locomotor activity, which remained significantly lower throughout the first 80 min. When administering 3 mg/kg, MDPV levels were unchanged by the combination with ethanol at all measured time points, and were in good agreement with the locomotor activity profile. The above results are depicted in Figure 3, which shows MDPV brain levels plotted against locomotor activity values.

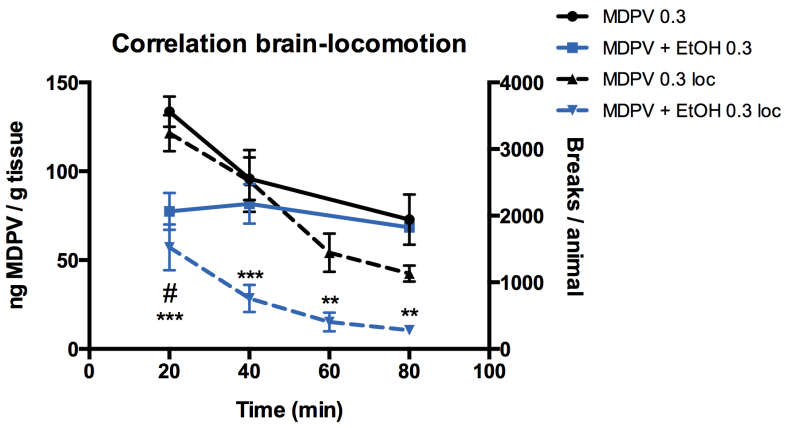

Figure 7: Brain MDVP concentration in the striatum at different time points. # (P<0.05) compared to non-ethanol treated group. Locomotor activity at different time points. ** (P<0.01) *(P<0.001) compared to non-ethanol treated group.**

We initially hypothesized that a plausible explanation for the changes in MDPV brain levels was an affectation of the normal crossing rate of MDPV through the blood-brain barrier (BBB), as ethanol has been described to alter its normal functionality (Haorah et al., 2007). To test this hypothesis, blood MDPV levels were measured and compared to those found in the striatum at three different time points (20, 40 and 80 min) for the 0.3 mg/kg dose. Only one time point (20 min) was assessed for the 3 mg/kg dose, as no significant differences between MDPV and MDPV+ethanol groups had been detected in any of the previous experiments.

As expected, 3 mg/kg MDPV and its combination with ethanol did not yield significantly different blood levels at the assessed time point (20 min, MDPV: 446 ±39.5 vs MDPV+ethanol: 385.8 ±25.44 ng/ml, P > 0.05). When analyzing MDPV blood levels after the administration of 0.3 mg/kg MDPV, a significant reduction was found in the combination group 20 min after injection; by contrast, in the following assessed time points (40 and 80 min) MDPV levels were similar in both groups. This is in agreement with findings for brain MDPV levels (Figure 4).

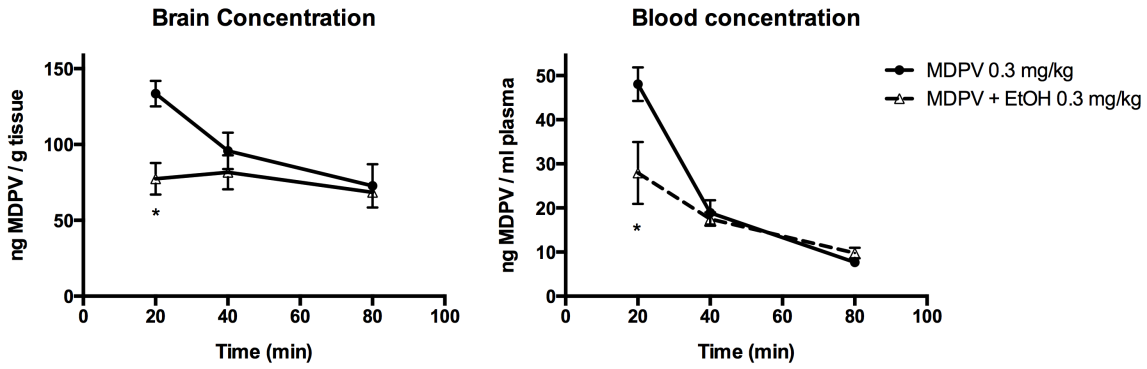

Figure 8: MDPV concentration in the striatum and blood, expressed as ng MDPV/g tissue and ng MDPV/ml plasma, respectively. * (P<0.05) compared with non-ethanol treated group.

Finally, the combination with ethanol was found to have divergent effects on MDPV-induced locomotor activity and conditioning, as the former was significantly reduced, while the latter was unaffected by ethanol. This phenomenon led us to assess the changes in dopamine (DA) levels in the nucleus accumbens (NAc) elicited by and acute dose of MDPV or MDPV+ethanol, as this region is directly implicated in the conditioning effects of drugs of abuse.

For MDPV at the dose of 0.3 mg/kg, the DA levels between groups were significantly different for the treatment [$F_{(3, 11)}$ = 8,318]. The administration of MDPV caused a rapid increase in extracellular DA, reaching an AUC increase of 150 ± 35% (P<0.01) over control values. Ethanol 1g/kg caused a visible increase of 21 ± 1.8%, although this did not attain statistical significance, due to the higher variability in the MDPV-treated groups. Furthermore, the combination treatment yielded a 118 ± 31.5% increase (P<0.05), which was not statistically different with respect to the group treated with MDPV alone (Figure 5).

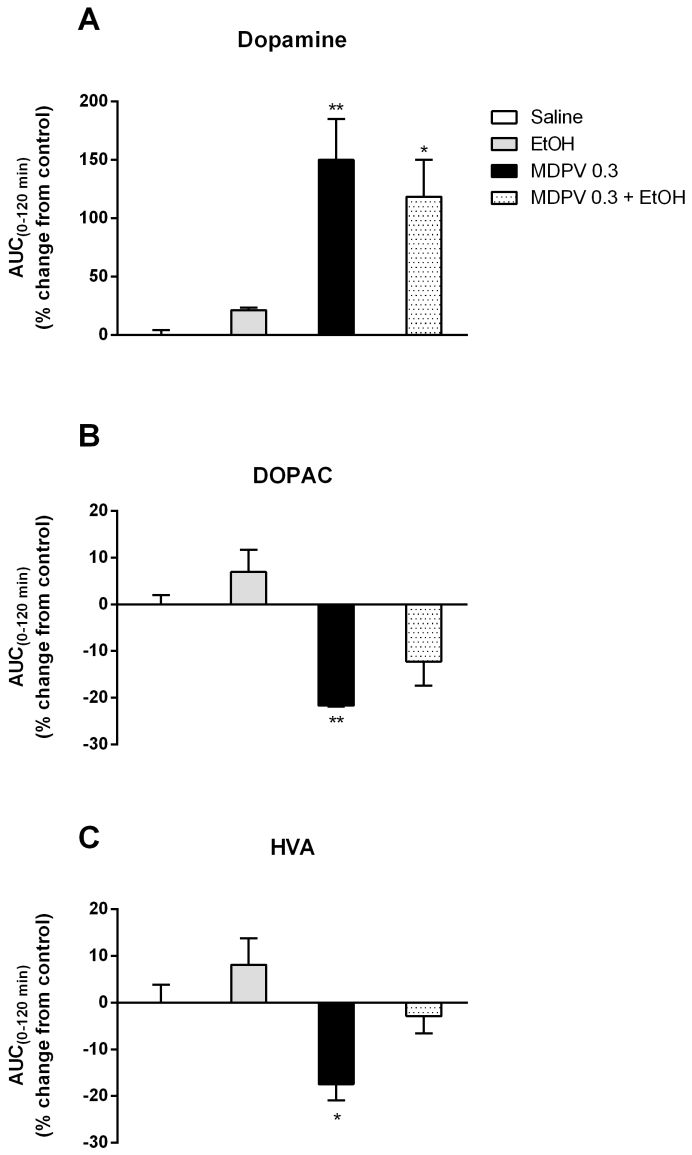

Figure 9: Dopamine (A), DOPAC (B) and HVA (C) levels in the core of the nucleus accumbens after the administration of 0.3 mg/kg MDPV and 1 g/kg ethanol, expressed as % change over control of AUC during 120 minutes. * (P<0.05), ** (P<0.01) compared with the control group.

For MDPV at the dose of 3 mg/kg, a similar, yet notably stronger effect was evidenced. DA levels between groups were significantly different for the treatment [$F_{(3, 11)}$ = 8,096]. The administration of MDPV caused an increase in extracellular DA, reaching an AUC increase of 891 ± 218.7% (P<0.01) over control values. Ethanol 1g/kg caused an increase of 23 ± 1.7%. Finally, the combination treatment elicited a 680 ± 126.4% increase (P<0.01) (Figure 6). The time course for both treatments is depicted in Figure 7.

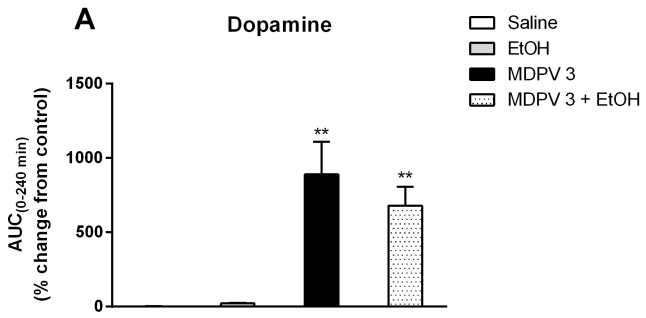

Figure 10: Dopamine levels in the core of the nucleus accumbens after the administration of 3 mg/kg MDPV and 1 g/kg ethanol, expressed as % change over control of AUC during 120 minutes. ** (P<0.01) compared with the control group.

The same samples analyzed for DA concentration were also analyzed for the content of metabolites DOPAC and HVA. For the treatment with 0.3 mg/kg MDPV, there was an overall treatment effect on DOPAC [$F_{(3, 12)}$ = 12,20; P<0.01] and HVA [$F_{(3, 12)}$ = 7,365; P<0.01] levels. Individual AUC values for each treatment are depicted in Figure 5. Conversely, for 3 mg/kg MDPV, no significant treatment effect was evidenced on DOPAC [$F_{(3, 14)}$ = 2,640] and HVA [$F_{(3, 12)}$ = 1,207].

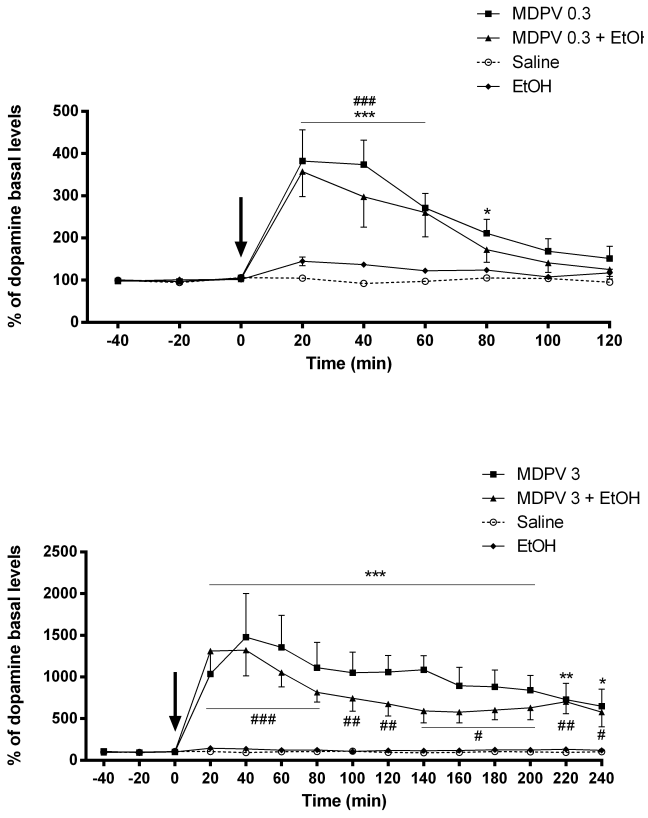

Figure 11: Time course of dopamine levels expressed as % over basal levels at the 0 time points, for

animals treated with 0.3 mg/kg MDPV and 1 g/kg ethanol, as well as 3 mg/kg and 1 g/kg ethanol.

4. DISCUSSION

The initial approach of this work was to assess the effect of ethanol on MDPV-induced changes in behavioral parameters for psychostimulant and conditioning properties (i.e. locomotor activity and CPP). Our initial finding was of great interest, as ethanol significantly reduced locomotor activity counts induced by low doses of MDPV. Four different doses of MDPV were assessed; we evidenced a clear dose-dependent susceptibility for MDPV to the effects of ethanol on locomotor activity: as MDPV doses were lower, the magnitude of the reduction effect of ethanol on activity counts increased. In this sense, ethanol caused the highest reduction in locomotor activity when administered concomitantly with 0.1 mg/kg MDPV (70%); this reduction was slightly more modest at 0.3 mg/kg MDPV (60%) and negligible at 1 and 3 mg/kg MDPV. Furthermore, as the dose of ethanol is increased, this reduction in locomotor activity seems to be higher, as ethanol 2 g/kg further decreased activity counts elicited by 0.3 mg/kg MDPV.

The attenuation of the psychostimulant effects of MDPV by ethanol was both unexpected and of great interest, as they contrasted with the potentiation previously found by us for the other main cathinone mephedrone (Ciudad-Roberts et al., 2015). In an attempt to shed a light on the potential underlying mechanisms for this phenomenon, MDPV concentration was assessed in the striatum in the MDPV and MDPV+ethanol groups, with the aim of determining whether this phenomenon is due to a pharmacokinetic interaction, manifested as a decrease in MDPV levels in the brain. In agreement, we revealed a significant reduction (around 50%) in MDPV levels, when the cathinone was combined with ethanol. Nonetheless, this reduction was only evidenced in the first assessed time point (20 min), and brain MDPV concentration for both groups leveled off in the subsequent time points (40 and 80 min).

It is known that MDPV crosses the blood-brain barrier (BBB) through passive diffusion and active transport. This last feature is a differential trait of this compound with respect to the rest of the studied synthetic cathinones studied in the literature, such as mephedrone (Simmler et al., 2012). Thus, we initially hypothesized that ethanol could be disrupting active transport through the BBB, hindering the entrance of MDPV into the central nervous system. This hypothesis was challenged by the data obtained in the subsequent experiment, as MDPV levels were also found to be decreased in blood when co-administering MDPV and ethanol.

In summary, ethanol, at low doses, seems to trigger a strong decrease in overall

levels of MDPV, which, in turn, translates into lower psychostimulant effects. This phenomenon could be explained by a pharmacokinetic interaction, where the metabolism of MDPV would be accelerated by a change in the normal metabolic route for MDPV. In this sense, 3,4-cathecol-PV and 4-OH-3MeO-PV are believed to be the main metabolites of MDPV (Anizan et al., 2014; Novellas et al., 2015); the main enzymes responsible for the transformation from MDPV into these metabolites are CYP2D6, CYP2C19, CYP2B6, CYP2C9, CYP1A2 and CYP3A4. (Meyer et al., 2010, Negreira et al., 2015). The former three enzymes are inhibited by ethanol (Busby et al., 1999; Hellum and Nilsen, 2007); furthermore, they also mediate other significant transformations for MDPV, such as the formation of M4 (view Figure 8 for all proposed metabolite structures) (through a reduction of MDPV followed by a hydroxylation), M5 (resulting from the hydroxylation of M4), M3 (corresponding to a dihydroxylation of MDPV on the pyrrolidine ring and the propyl side chain) or M2 (resulting from the transformation of the pyrrolidine ring of MDPV to a primary amine). Conversely, CYP3A4 and CYP2C9 (which mediate the transformation of MDPV into its principal metabolites 3,4-cathecol-PV and 4-OH-3MeO-PV) are not affected by ethanol (Busby et al., 1999; Negreira et al., 2015).

It is feasible that, by inhibiting CYP2D6, CYP2C19 and CYP2B6, other enzymes, such as CYP3A4, CYP1A2 and CYP2C9, take over and become dominant in the transformation of MDPV. In this sense, the enzymatic reactions that are not susceptible to ethanol inhibition could show a higher Vmax, as well as a higher Km. The lower affinity for these enzymes (higher Km) would explain why other CYPs (i.e. CYP2D6, CYP2C19 and CYP2B6) would preferentially metabolize MDPV in basal conditions,. Thus, when the enzymatic inhibition by ethanol takes place, MDPV metabolism would be shifted into faster enzymatic reactions, and would explain the lower levels of MDPV in the drug combination group. This proposed mechanism is depicted in Figure 8.

40 minutes after drug injection, MDPV levels, which are initially decreased by about 50% in the combination group, level off in the brain and blood in both treatment groups. This is probably explained by a saturation of the enzymatic function due to a decrease in the number of functional cytochromes. Interestingly, despite the equivalent MDPV levels 40, 60 and 80 minutes after drug treatment, locomotor activity is markedly lower in the combination group. Here, we postulate two mechanisms through which this phenomenon may be taking place.

Firstly, it is known that MDPV has very high affinity for DAT. As stated by Baumann et al. (2013), due to its high potency at DAT, MDPV may display a slow dissociation from the site (i.e. persistent binding), thereby augmenting and extending its pharmacological effects at time points when MDPV brain levels have already become low or below threshold. This hypothesis is supported by a previous study by

us (Novellas et al., 2015), which shows a counter clockwise hysteresis when plotting MDPV brain concentration vs locomotor activity. This is evidenced by equivalent locomotor activity 40 and 60 min after administration, despite brain levels being reduced by one half during this time period. In this sense, we hypothesize that, in the present study, MDPV concentration is reduced to sub-threshold levels, where DA reuptake in the synapse occurs at a higher rate, due to a low overall occupancy of this transporter type by MDPV, thus causing lower locomotor activity.

Furthermore, it has been suggested that 3,4-cathecol-PV and 4-OH-3MeO-PV have little or no psychostimulant effect (Anizan et al., 2014; Novellas et al., 2015). The shift of MDPV transformation into the formation of these "inactive" metabolites (see Figure 5) could also explain the significantly lower locomotor activity in the MDPV+ethanol group, when compared to animals treated with MDPV alone, at time points when MDPV levels are equivalent in both groups. Nonetheless, it is unclear whether the metabolites resulting from other metabolic routes (i.e. M2, M3, M4 and M5) are active, which would back this hypothesis. Interestingly, M2, M3, M4, M5 keep the methylenedioxy group on the phenyl ring, which is lost for a cathecol group in 3,4-cathecol-PV (and further methylated to 4-OH-3MeO-PV) and M1. This transformation could be responsible for the hypothetically lower psychostimulant properties of these metabolites. Further assays should be performed in this regard, although it is beyond the scope of this work.

As has been mentioned above, as MDPV doses are increased, the reduction effect of ethanol becomes less significant. At the dose of 3 mg/kg MDPV, ethanol has no perceivable effect on locomotor activity and MDPV brain and blood levels. This phenomenon fits into our working hypothesis, and points to the fact that, as MDPV concentration becomes higher, it overcomes the inhibitory effect of ethanol on the various CYPs.

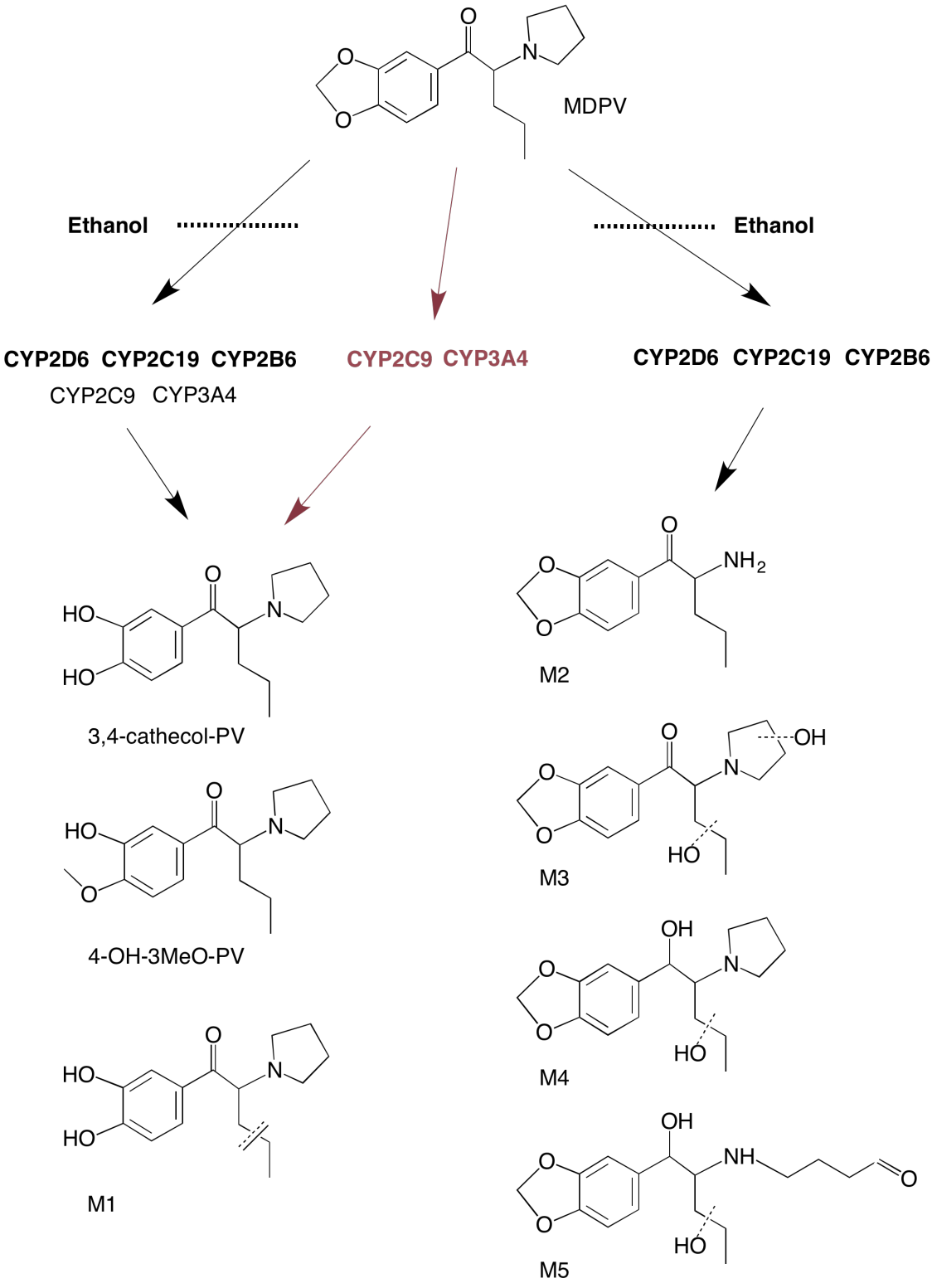

Figure 8: Scheme of the postulated effect of ethanol on MDPV metabolism

It is also feasible that the reduction in MDPV levels is due to changes in normal absorption. This is improbable though, as a delay in the increase in MDPV levels, as

well as locomotor activity would then be expected. As shown by the pharmacokinetic profile described herein, this does not occur: MDPV concentrations of both treatment groups level off 40 minutes post-administration, and decrease in parallel thereafter. This is further backed by the time course for locomotor activity; in this sense, not only is a delay not evidenced, but AUC values are also notably lower in the combination treatment (i.e. total activity throughout the duration of the effect). Furthermore, Pan et al., (199) demonstrated that ethanol could accelerate, rather than delay the absorption of cocaine, due to high vasodilation.

Given that locomotor activity, as well as MDPV levels, were markedly reduced by the concomitant administration of ethanol, it proved interesting to explore a potential explanation for its conditioning properties not being similarly affected. For this purpose, microdialysis experiments were performed, and changes in DA, DOPAC and HVA were assessed. Interestingly, DA levels were not notably modified by ethanol. Nonetheless, although non-statistically significant, a mild decrease in DA is evidenced at early time points in the combination group with respect to animals treated with MDPV alone. Similarly, ethanol-treated animals show increased DA levels with respect to those treated with saline. This observation is further supported by the comparison of AUCs for each group.

It is feasible that the decrease in DA (as a consequence of lower amounts of striatal MDPV) is compensated by the increase in DA elicited by ethanol, yielding similarly conditioning effects. This hypothesis is backed by the fact that DOPAC and HVA levels were increased in the drug combination group with respect to animals treated with MDPV alone. This evidences that, although synaptic DA levels are similar, the amount of DA transported back into the terminal (and thus metabolized into DOPAC and HVA) is higher, pointing to a milder blockade of DAT, due to lower concentrations of synaptic MDPV. Accordingly, DOPAC and HVA levels in the ethanol group are visibly higher than control animals, as the mechanism through which ethanol increases DA in the NAc is different to that of MDPV, and does not involve DAT blockade, thus allowing the entry (and subsequent metabolism) of DA in the presynaptic terminal.

Finally, it is evident that DA levels do not change in parallel with locomotor activity counts at each time point. In this regard, it is known that the involvement of each area of the ventral striatum in psychostimulant-induced locomotor activity varies for each individual psychostimulant (Ikemoto, 2002). In the present work, the microdialysis probe was located in the NAc core; it is probable that other regions (e.g. olfactory tubercle, NAc shell) are significantly implicated in the psychostimulant properties of MDPV, thus accounting for this divergence.

In sum, in this study we demonstrate that ethanol decreases MDPV levels in the

brain and blood, which is reflected by a decrease in its psychostimulant but not its conditioning properties; this effect is more significant for lower doses of MDPV. These findings are important, as the simultaneous use of MDPV and ethanol could result in a reduction in the stimulant effects of MDPV without affecting its rewarding properties, which could increase users' tendency to re-dose and, in turn, the risk of overdosing. Therefore, a warning should be issued to the general population regarding the potential dangers associated with the combination of these two substances.

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Article 6 (in press): Changes in CREB and ΔFosB are associated with the behavioral sensitization induced by MDPV. *Journal of psychopharmacology*

Methylenedioxypyrovalerone (MDPV) is one of the most popular synthetic cathinones together with mephedrone. It is exceptionally potent, and is used predominantly by young adults as an alternative to classic psychostimulants, such as cocaine or MDMA, due to its easy availability and a widespread perception of innocuousness. In contrast to most cathinone derivatives, it is a dopamine transporter blocker; for this reason, the subjective effects it elicits are similar to those of cocaine, causing euphoria, potent stimulation and self-confidence.

In the present study, we sought to investigate the sensitizing potential of a recreational dose of MDPV. Simultaneously, we measured the levels of synaptic plasticity-related proteins that might play an important role in the development of behavioral sensitization, focusing on all key time points of the sensitizing procedure. Furthermore, given the mechanistic similarities between MDPV and cocaine, cross-sensitization between these two psychostimulants was also assessed.

The experimental protocol consisted of 3 phases: Phase I (sensitization): MDPV (0.3 mg/kg) was administered once a day for 5 consecutive days. Phase II (withdrawal): animals remained MDPV-free for 8 days (days 6-14). Phase III (challenge): animals were administered saline on day 15 and MDPV (0.3 mg/kg) or cocaine (10 mg/kg) on day 16; locomotor activity was recorded immediately after both administrations. cAMP-response element-binding protein (CREB) and phosphor-CREB (p-CREB) were measured 2h after Phase III; ΔFosB was measured 24h after Phase I, II and III.

Results revealed that animals repeatedly exposed to MDPV for 5 consecutive days showed increases in locomotor activity that reached levels ranging 160 to 200% those of control groups after a challenge dose of MDPV or cocaine. Furthermore, exposure to this sensitizing MDPV regimen increased total CREB and p-CREB in the striatum and the nucleus accumbens after a MDPV challenge. Both increases were of the same magnitude, pointing to the possibility that the increase in p-CREB is due to an increase in total CREB, rather that a transient effect caused by the acute MDPV challenge. Previous time-course studies on the progression of p-CREB levels after an acute dose of cocaine back this hypothesis. ΔFosB was increased by the sensitizing regimen, an effect which persisted throughout the three measured timepoints.

The present study shows that repeated use of MDPV can enhance the psychostimulant effect of future doses of MDPV and cocaine. Results are of great significance, as they suggest that early-life experimentation with MDPV could sensitize users to the effects of future exposures to cocaine; this, in turn, could result in a significant increase in its reinforcing properties and thus a rise in the susceptibility to cocaine addiction. We hypothesize that, as has been described previously for cocaine, both CREB and ΔFosB are transcription factors which play a role in the induction of behavioral sensitization to MDPV; furthermore, the up-regulation of the proteins described herein provides a neurobiological basis for the potential relationship between MDPV and cocaine abuse.

Changes in CREB and deltaFosB are associated with the behavioral sensitization induced by MDPV

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ABSTRACT

Objective: To investigate the locomotor sensitization induced by methylenedioxypyrovalerone (MDPV) in adolescent mice.

Methods: Behavioral testing consisted of 3 phases: Phase I: MDPV (0.3 mg/kg s.c. x day for five days) or saline. Phase II: resting (days 6-14). Phase III: Both groups were administered with MDPV (0.3 mg/Kg) or cocaine (10 mg/kg) challenge on day 16. We quantified CREB and phospho-CREB 2h after Phase III as well as deltaFosB 24h after the Phase I, II and III.

Results: Mice repeatedly exposed to MDPV increased locomotor activity by 160-200% following acute MDPV or cocaine administration after an eleven-day resting period, evidencing a MDPV-induced sensitization to cocaine. An explanation for this phenomenon is the common mechanism of action between these two psychostimulants. Furthermore, the MDPV challenge resulted in higher levels of phosphorylated CREB in MDPV-conditioned mice than in MDPV-naïve mice, probably as a consequence of an up-regulation of the cAMP pathway.Likewise, MDPV exposure induced a persistent increase in the striatal expression of deltaFosB; the priming dose of MDPV also produced a significant increase in the accumbal expression of this transcription factor.

Discussion: This study constitutes the first evidence that an exposure to a low/threshold dose of MDPV during adolescence induces behavioral sensitization and provides a neurobiological basis for a relationship between MDPV use and future cocaine abuse. We hypothesize that, as cocaine, both CREB and deltaFosB play a role in the induction of this behavioral sensitization.

Keywords: MDPV; Sensitization; CREB; phospho-CREB; deltaFosB; Cocaine

1. INTRODUCTION

Methylenedioxypyrovalerone (MDPV) is a synthetic cathinone, which has recently emerged as a designer drug of abuse. Synthetic cathinones seem to produce their effect primarily via monoamine transporters (López-Arnau et al., 2012), which resemble those of cocaine or ecstasy (Glennon, 2014). MDPV differs from other cathinones by acting preferentially as a monoamine reuptake inhibitor, selectively blocking dopamine and norepinephrine transporters (Cameron et al., 2013). It is more selective and potent than cocaine in blocking dopamine transporter, as well as in producing locomotor activation (Baumann et al., 2013, Novellas et al., 2015). Furthermore, it shows rewarding and reinforcing properties (King et al., 2015), pointing to an abuse liability similar to that of cocaine (Baumann et al., 2013). However, to the best of our knowledge, behavioral sensitization has not yet been reported after repeated MDPV exposure. This feature has been reported for mephedrone (Gregg et al., 2013), however due to the different mechanism of action between the two cathinones, the results are not extrapolable.

In rodents, behavioral sensitization is induced by psychostimulants and is present when motor activity induced by repeated drug exposure significantly increases over the hyperlocomotion produced by the initial exposure. Because of the similarities in the mechanism of action between MDPV and cocaine, it proved important to determine whether the use of MDPV could increase the susceptibility towards cocaine consumption in future stages in life. In this sense, assessing the sensitizing potential of MDPV over cocaine was found to be a good initial screening strategy to shed a light on this matter.

Behavioral sensitization results from neuroadaptive changes associated mainly with the development of increased sensitivity to re-exposure (Robinson and Berridge, 1993).The nucleus accumbens (NAcc) and the striatum (Str) play a key function in the neural circuitry underlying psychostimulant action and the constructs of reward. In particular, neuroplastic changes in these areas participate in the transition from casual to compulsive drug use and might play a critical role in the development of addiction (Krasnova et al., 2013).

Of particular interest is the transcription factor deltaFosB (ΔFosB), whose expression in NAcc is induced by chronic exposure to virtually all drugs of abuse, and regulates their psychomotor and rewarding effects (Colby et al., 2003).

Evidence points towards changes in the expression of cAMP-response element-binding protein (CREB) being involved in the development of behavioral sensitization to psychostimulants (Madsen et al., 2012). It also has been shown that

the repeated administration of cocaine enhanced the amount of phosphorylated CREB (P-CREB) in the NAcc (Mattson et al., 2005).

Therefore, given these antecedents, we investigated whether changes in ΔFosB and CREB/P-CREB expression in the NAcc and the Str could be involved in MDPV-induced behavioral changes. This would allow us to suggest an underlying mechanism for MDPV-induced sensitization, which could be of importance in finding therapeutic strategies for the treatment of MDPV addiction.

2. MATERIALS AND METHODS

2.1. Subjects and drugs

Adolescent Swiss CD1 male mice (Charles River, Spain), aged 4-5 weeks were used. Animals were housed at 22 ± 1 °C with food and water ad libitum. The Animal Ethics Committee of the University of Barcelona, following the 86/609/EEC guidelines, approved the experimental protocols for the use of animals in this study. Pure racemic MDPV HCl was synthesized and characterized by us as described (Novellas et al., 2015). Cocaine was provided by the Spanish National Institute of Toxicology. The other drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) and all buffer reagents were of analytical grade. A low dose of MDPV eliciting hyperlocomotion was chosen for this study (0.3 mg/kg). This is equivalent to a dose of 2 mg in humans, in which threshold dosages are around 1-5 mg and strong effects are shown with 10-25 mg (EMCDDA, 2014).

2.2. Experimental design and locomotor activity tracking

Before testing, mice were handled for 10 min during 2 days. Behavioral testing for locomotor sensitization to MDPV consisted of 3 phases (see Fig 1): Phase I: The conditioning regimen (days 1-5). One group of animals received daily subcutaneous injections of MDPV (0.3 mg/kg) for five days (MDPV pretreated group), while the other group received daily saline injections (1 ml/kg) (saline pretreated). Immediately following each injection, horizontal locomotor activity was monitored (Smart 3.0, Panlab) in 10-min blocks for 90 min, in the open field. Phase II: After the fifth day, injections ceased, and animals remained in their home cages for 10 days of resting period (days 6-15). Phase III: On day 16 (locomotor conditioning) both groups were challenged subcutaneously with MDPV (0.3 mg/Kg) or cocaine (10 mg/kg). Locomotor activity was registered during this phase in the same open field arena.

For Western blot analysis, animals (n = 4 - 8 in each group, randomly selected) were sacrificed at different times: CREB and P-CREB 2h after Phase III; ΔFosB 24h after

the Phase I, II and III (days 6, 15 and 17). This time schedule is in accordance with their maximum expression pattern.

2.3. Tissue sample preparation

Mice were killed by cervical dislocation at time points described before. Str and NAcc were quickly dissected out and stored at -80ºC until use. Tissues were homogenized in lysis buffer and processed as described by Pubill et al. (2013). When required, proteins were separated by standard techniques and stored at -80°C until use.

2.4. Western blotting and immunodetection.

Western blot analysis was performed using conventional techniques with anti-CREB(48H2), anti-P-CREB(Ser133) antibodies (both from Cell Signal), and anti-fosB(83B1138) antibody (Abcam). As a control for load, beta-actin (Healthcare) or beta-tubulin (Sigma-Aldrich) antibodies were used.

In order to identify significant differences between groups, results were compared by Student-t test or two-way ANOVA. When significant, ANOVA was followed by the Tukey's post-hoc tests, and the criterion for significance was set at $p<0.05$.

RESULTS AND DISCUSSION

3.1. Locomotor sensitization

Adolescent rodents show higher sensitization to the locomotor-activating effect of cocaine or amphetamine (Niculescu et al., 2005); becoming an excellent model for the study of these drugs Therefore, in order to evaluate behavioral sensitization to MDPV we assessed the hyperlocomotion induced by MDPV in adolescent mice. In the Phase I of the present study, a two-way ANOVA revealed that the distance travelled was significantly affected by the day ($F_{4,72} = 5.80$, $p<0.001$) and treatment ($F_{1,18} = 56.76$, $p<0.001$) variables. MDPV-induced hyperlocomotion ranged from 172.3 ± 12.2 % versus saline the first day to 493.0 ± 37.3 % the 5th day. In phase III, MDPV or saline pretreated mice were tested after acute MDPV (0.3 mg/kg). Two-way ANOVA evidenced significant differences for variables time, pretreatment and the interaction between time and pretreatment (time variable $F_{8,120} = 18.88$, $p<0.001$; pretreatment variable $F_{1,15} = 12.39$, $p<0.01$; interaction time X pretreatment $F_{8,120} = 2.73$, $p<0.01$). Post-hoc analysis revealed that distance travelled was significantly greater during the first 30 min in mice receiving MDPV pretreatment (Fig 2A). The assessment of cumulative data (0–90 min) also evidenced a significant difference between both pretreated groups ($p<0.01$; Fig 2A inset). Interestingly, we have demonstrated, for the first time that the new

psychostimulant drug MDPV induces locomotor sensitization because response to acute MDPV challenge was stronger in mice that were previously exposed to repeated MDPV injections ($p<0.001$).

In a second set of experiments (Fig. 2B) animals were challenged in Phase III with cocaine (10 mg/kg, s.c.). As above, two-way ANOVA revealed differences among groups (time variable $F_{8,120} = 81.49$, $p<0.001$; pretreatment variable $F_{1,15} = 41.95$, $p<0.001$; interaction time X pretreatment $F_{8,120} = 14.78$, $p<0.001$), which has also been assessed in cumulative data ($p<0.001$; Fig 2B inset). Mice which have been conditioned with MDPV during adolescence, showed a dramatic increase (by 300%, $p<0.001$) in locomotor activity following acute cocaine administration eleven days after the cessation of the cathinone treatment, evidencing the MDPV-induced sensitization to cocaine. The sensitization effect of MDPV is greater for cocaine than for MDPV itself.

Gregg et al., 2013 tested the hypothesis that prior mephedrone exposure could enhance the stimulant effects of cocaine in rats. In their study, authors demonstrate that cocaine-induced locomotor activation is enhanced by prior mephedrone exposure. However, the mechanism of action of both cathinones, mephedrone and MDPV are different; consequently, the interactions of mephedrone cannot be directly extrapolated to MDPV. To our knowledge, this is the first study to investigate the effects of repeated MDPV administrations on cocaine-induced locomotion in adolescent mice.

3.2. Changes in the expression of proteins associated with sensitization

To address whether MDPV treatment was associated with alterations in some transcription factors involved in locomotor sensitization of MDPV, we investigated the expression of CREB, P-CREB and ΔFosB proteins in the Str and the NAcc. For total CREB (Fig. 3A, 3D), MDPV pretreatment induced a significant increase in protein expression in both brain areas 2 hours after MDPV challenge, pointing to changes in the CREB gene expression, and thus, to an up-regulation of the cAMP pathway. This up-regulation is a common feature in the neurobiology of opiate and cocaine addiction (Nestler, 2005), which seems to be reproduced for MDPV.

Regarding CREB activity (evidenced by an increase in P-CREB), it is a typical phenomenon linked to sensitization. The phosphorylated form (Fig. 3B, 3E), is notably higher in animals that have been pretreated repeatedly with MDPV (i.e., MDPV pretreatment hypersensitises the response to an acute MDPV challenge). Increases in the phosphorylated form were in accordance with the changes in total CREB.

A prolonged induction of ΔFosB promotes reward and motivation, and serves as a key mechanism of drug sensitization (Nestler, 2013). Because of its stability, the ΔFosB protein persists in neurons for at least several weeks after cessation of drug exposure (McClung and Nestler, 2003). In the present study, ΔFosB expression was analyzed in the Str and NAcc 24 h after Phase I, II and III of the sensitization paradigm. Fig. 3C and 3F show the Western blot analyses of ΔFosB expression in the two brain regions. As can be seen, in the Str, two-way ANOVA (pretreatment variable $F_{1,19}$ = 84.66, p<0.001; day variable $F_{2,19}$ = 0.44, n.s.;) followed by Tukey's post-hoc tests revealed that repeated treatment with MDPV induced a significant increase in ΔFosB expression not only after dosing (day 6) but also after the resting period (day 15), pointing to an enduring expression of this transcription factor, which does not increase further in spite of the additional MDPV dose/challenge. However, in the NAcc, MDPV treatment induced a rise in ΔFosB levels in the first two time points. Nonetheless, only after an additional dose (MDPV challenge) did ΔFosB levels increase further, reaching statistical significance (pretreatment variable $F_{1,20}$ = 29.10, p<0.001; day variable $F_{2,20}$ = 0.2.34, n.s).

Because CREB and ΔFosB are involved in the control of the action of psychostimulants in both the Str and NAcc, we suggest that changes in these transcription factors are a relevant step towards the induction of neuroplastic changes that lead to behavioral sensitization of MDPV.

Overall, the present findings extend the knowledge on the behavioral effects of a low dose of MDPV in an animal model of locomotor sensitization. These results constitute the first evidence that an exposure to a threshold dose of this cathinone during adolescence induces behavioral sensitization, which is common to other psychostimulants. A likely explanation for the MDPV-induced sensitization to cocaine is their shared mechanism of action. This is accompanied by a parallel increase in key transcription factors, providing a neurobiological basis for a relationship between MDPV use and cocaine abuse. All these results are relevant because adolescents increasingly consume MDPV, which is perceived as "safe" drug, devoid of the adverse effects associated to classical psychostimulants.

The consumption of MDPV, and cathinones in general, responds to rapidly changing drug use trends. However, the long-term consequences of their consumption are not well known. From our results, it can be suggested that MDPV consumption during adolescence could increase susceptibility towards future cocaine abuse. Thus, an experimental-based warning concerning the risks associated to the use of MDPV should be issued, focusing especially on the young population.

We believe that the conclusions set forth herein are important and must be transmitted to the scientific community and the public at large in an urgent manner.

Nonetheless, clearly more studies are needed focusing on the exact mechanism whereby this sensitization occurs, as well as its impact on the effects of other drugs of abuse.

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LEGENDS AND FIGURES

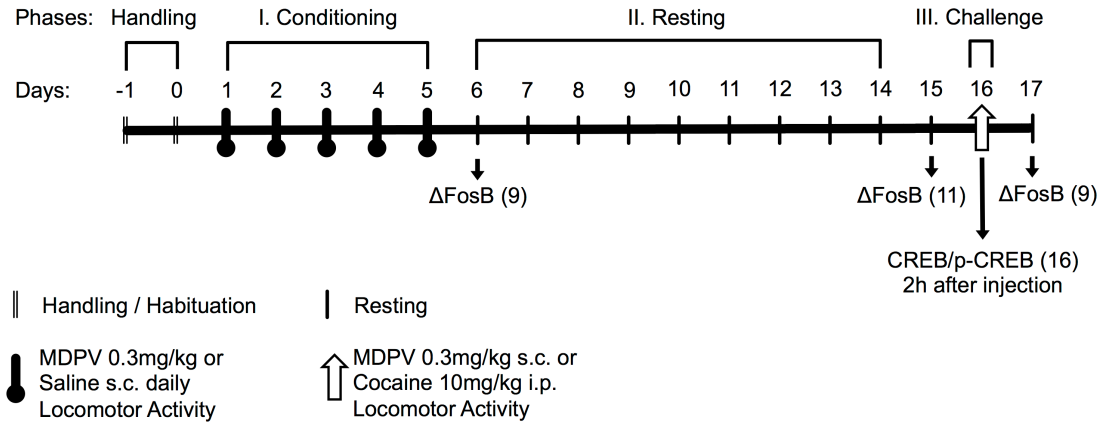

Figure 1.-

Schematic representation of the paradigm for acquisition and expression of MDPV-induced sensitization (number of mice indicated in parenthesis).

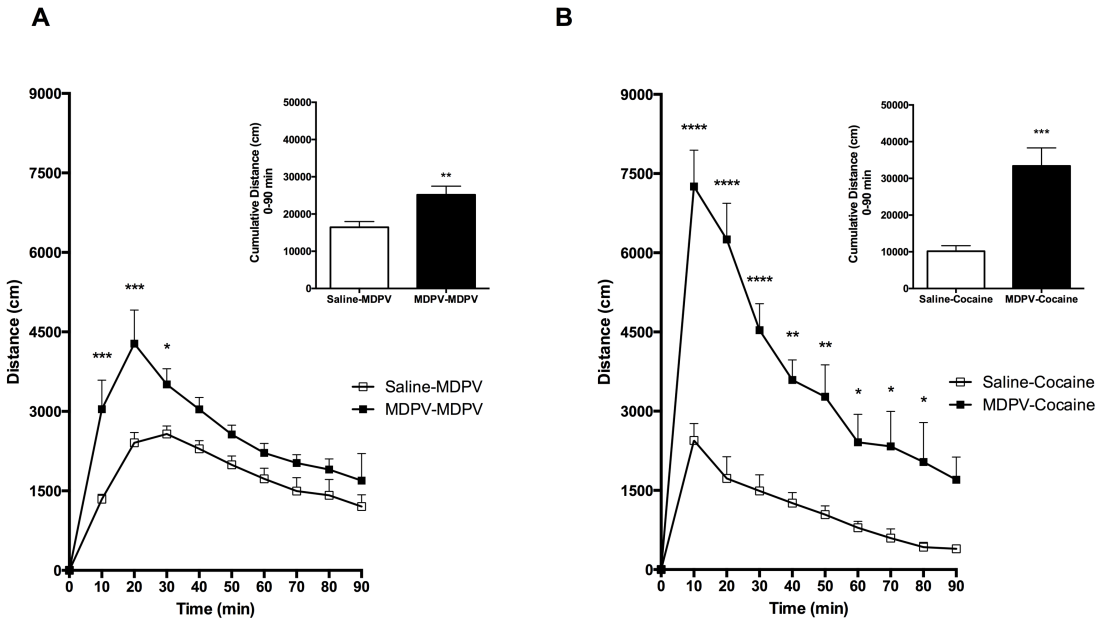

Figure 2.-

Expression of behavioral sensitization to MDPV. Effect of a challenge of MDPV (0.3 mg/kg; s.c.; Panel A) or cocaine (10 mg/kg; s.c.; Panel B) administered following a resting period from MDPV (0.3 mg/Kg; s.c.) or Saline (5 ml/Kg) pretreatment. Time-course data are expressed as distance covered mean ± s.e.m. of n = 16-18 animals

per group (two-way ANOVA and Tukey's post-hoc analysis) ** P<0.01, *** P<0.001 versus MDPV or cocaine challenge in saline pretreated group. Cumulative data (0–90 min) are expressed as total distance covered following MDPV (inset A) or cocaine (inset B) injection. ** P<0.01, , *** P<0.001 versus saline pretreated group (Student's t-test).

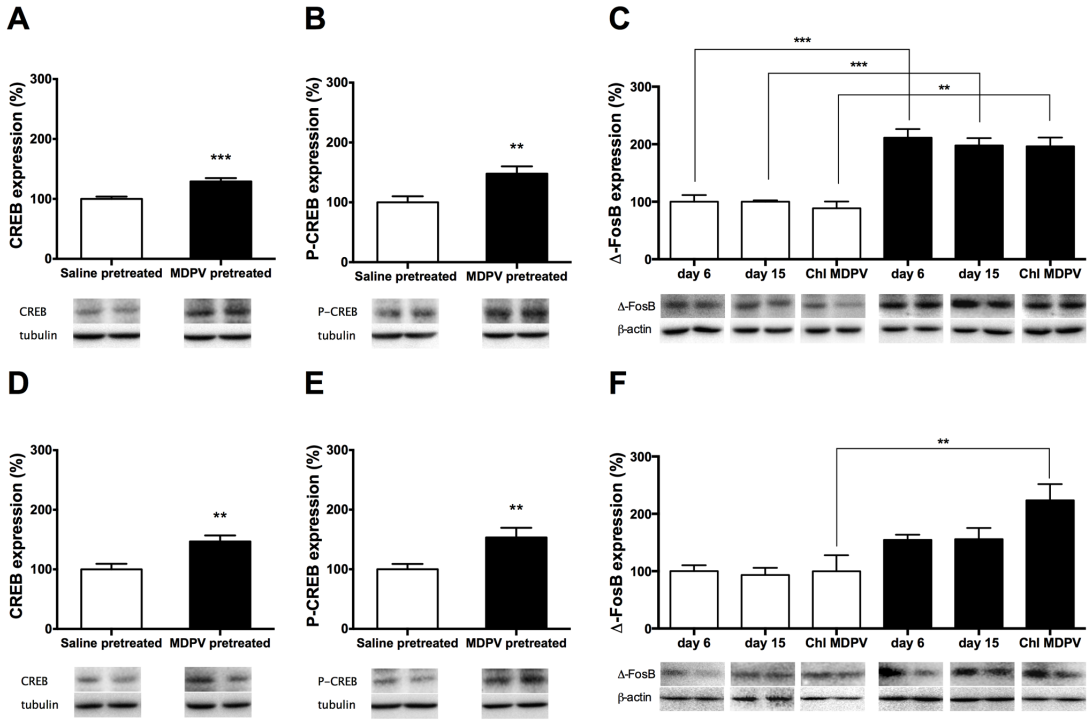

Figure 3.-

Effect of MDPV-conditioning (0.3 mg/kg, daily for five days) (full bars) or saline pretreatment (5 ml/kg) (empty bars) on factor expression in the Str (panels A to C) and NAcc (panels D to F). CREB (panels A and D) and phospho-CREB (panels B and E) values correspond to animals killed 2h after MDPV challenge (0.3 mg/kg). ΔFosB (panels C and F) values correspond to animals killed 24h after Phase I (day 6); after Phase II (day 15) and after MDPV challenge (Chl MDPV). Below each bar graph, the corresponding representative Western blots are shown. Results are expressed as mean ± s.e.m. from 4-6 animals per group for ΔFosB (two-way ANOVA and Tuckey's post hoc analysis), and 7-8 animals per group for CREB and phospho-CREB (Student-t test). ** p<0.01; ***p<0.001 versus saline-pretreated mice of the matched time group

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DISCUSSION

MDMA and its interaction with nicotinic receptors

In the first study, we sought to explore the role of α4β2 nAChRs in the acute psychostimulant effects and development of behavioral sensitization to MDMA. In order to do so, we administered MDMA together with α4β2 antagonist and partial agonist DHβE and varenicline, respectively, throughout a sensitizing protocol.

Previous studies have described how DHβE is capable of attenuating behavioral sensitization elicited by amphetamine, cocaine, ephedrine and methylphenidate (Karler et al., 1996; Miller and Segert, 2005; Schoffelmeer et al., 2002; Wooters and Bardo, 2009). Taking this into account, and given that evidence points to α4β2 as a potential target for drug dependence to a number of drugs (Crunelle et al., 2009), we also sought to explore the effect of varenicline (in addition to DHβE) on sensitization to MDMA, as it is already commercialized as a drug for smoking cessation.

A clear role of α4β2 nAChRs in the acute psychostimulant effects of MDMA was revealed, as both DHβE and varenicline significantly attenuated hyperlocomotion elicited by MDMA. We hypothesize that nicotinic receptors could be modulating the release of dopamine and serotonin acutely elicited by MDMA, and that their blockade may be attenuating its psychostimulant effects.

Furthermore, we showed that the administration of these α4β2 ligands also blocks delayed sensitization (although not early sensitization) to MDMA. Accordingly, animals treated with MDMA + varenicline/DHβE were increasingly sensitized during the 10-day treatment, yet they did not experience any increase in locomotor activity after the withdrawal period the way animals treated solely with MDMA did.

In an attempt to explain this effect of nAChR ligands on delayed sensitization, and given that our group previously described that MDMA can differentially regulate α4β2 nAChR levels, we hypothesized that a sensitizing treatment regimen could exert a similar effect, which could play an important role in the development of behavioral sensitization. Thus, a main objective of this work was to elucidate whether behavioral sensitization occurred in parallel to α4β2 up-regulation; if so, it proved important to determine whether the blockade of long-term sensitization by both DHβE and varenicline was accompanied by changes in α4β2 nAChR levels.

In this sense, we found a good correlation between sensitization and α4β2 nAChR expression in the frontal cortex. In this brain region, α4β2 were up-regulated in all drug-treated groups after the 10-day sensitizing treatment, which matched early sensitization for all groups. Similarly, after the withdrawal period, only animals

treated solely with MDMA showed up-regulated α4β2 nAChRs in the cortex, which is in agreement with the delayed sensitization exhibited by this group. Furthermore, the role of α4β2 nAChR up-regulation in the increase of the psychostimulant properties of certain drugs is also backed by previous results from our group, which showed that a protracted nicotine treatment potentiates methamphetamine- (Camarasa et al., 2009) and MDMA-induced (unpublished) hyperlocomotion.

Evidence presented herein suggests an important role of the cortex in the development of behavioral sensitization. It is known that the prefrontal cortex and the hippocampus exhibit converging projections to the NAc and have functional reciprocal connections via indirect pathways (Day et al., 1991; Goto and Grace, 2008). Medial prefrontal neurons, including those projecting to the NAc, are also excited by conditioned stimuli (Laviolette, 2007), demonstrating that long-lasting locomotor sensitization to MDMA is accompanied by reorganization of synaptic connectivity, not only in NAc, but also in the medial prefrontal cortex (mPFCx).

Results are of special importance since the expression of behavioral sensitization in animals is related and is believed to contribute to craving and drug relapse in human addicts (Robinson and Berridge, 2003).

Given the role of α4β2 nAChRs in the acute psychostimulant effects of MDMA, as well as in behavioral sensitization to MDMA, we hypothesized that this receptor type could also play an important function in the mediation of its rewarding effects.

Conditioned place preference (CPP) is elicited by most rewarding substances, and it is a ubiquitously accepted parameter that measures the motivational value of a particular compound, which, in turn, can be indicative of its abuse liability. Indeed, both DHβE and varenicline effectively blocked CPP for MDMA (10 mg/kg). This result on its own clearly demonstrates that α4β2 nAChRs are pivotal to MDMA reward; nonetheless, it became important to also assess whether the differential expression of these receptors could play a role in the mediation of these effects.

In this sense, by means of a protracted nicotine treatment, we were capable of effectively up-regulating α4β2 nAChRs; when doing so, a non-conditioning dose of MDMA (3 mg/kg) showed clear CPP. This is of capital importance, since many compounds, such as MDMA itself or nicotine are capable of up-regulating α4β2, as has been discussed extensively herein. Thus, the concomitant use of α4β2 nAChR up-regulating agents (such as, but not restricted to, nicotine) could potentiate the rewarding properties of MDMA.

The up-regulated nAChRs could mediate enhanced synaptic transmission when stimulated by local and brief releases of acetylcholine at synapses. Stimulation of

dopamine neurons in the VTA via the α4β2 nAChR leads to an increase of dopamine in the NAc that plays a crucial role in drug reward as measured by CPP (Di Chiara and Imperato, 1998). Consequently, the modulation of dopamine release by means of α4β2 nAChR up-regulation, and subsequent activation, could result in a modification of the CPP induced by MDMA.

A further implication of this finding is the potential use of α4β2 ligands for the attenuation of the rewarding effects of amphetamine derivatives such as MDMA. In this sense, it is not clear whether the commercialized drug varenicline, tested herein, attenuates the conditioning properties of MDMA by a direct action of the blockade of α4β2 nAChRs on neurotransmission, by preventing receptor up-regulation though this blockade, or by a combination of both factors. Regardless, although this matter requires further research, varenicline reveals itself as a potential candidate drug for substance use disorders. Furthermore, present findings evidence how nicotine can increase the rewarding properties of MDMA; thus, a public health warning should be issued regarding the polysubstance use of nicotine and MDMA, emphasizing the implications their concomitant consumption could have on MDMA abuse.

As has been extensively discussed in the introduction section, nicotine is believed to up-regulate nAChRs through post-translational mechanisms, rather than by changes in gene expression. Nonetheless, there is still much controversy in the literature as to the exact mechanism behind this phenomenon, and two main hypotheses have been postulated to explain this phenomenon: "chaperone-like maturation enhancing effect" and the "stabilization of the high affinity state of the receptors". Moreover, a combination of both mechanisms could be taking place. In light of this, we sought to investigate whether the nAChR up-regulation induced by MDMA found *in vitro* also occurred *in vivo* and if MDMA-induced nAChR up-regulation would also be mediated by a post-translational mechanism. To confirm this hypothesis, we submitted Sprague-Dawley rats to a protracted (10-day) MDMA treatment schedule; following, animals were sacrificed and gross brain areas were dissected and membrane α4β2 nAChRs were measured through radioligand binding assays with [^{3}H]epibatidine. Significant increases (ranging 25%) were found in the frontal cortex and the coronal slice delimited by the thickness of superior colliculi, after removal of the cortex and hippocampus, containing superior colliculi, lateral geniculate nuclei, substantia nigra and ventral tegmental area). In order to determine whether these changes were due to an increase in protein expression or post-translational modifications, total $\alpha 4$ content was measured by though immunoblotting. In agreement with what occurs for nicotine, $\alpha 4$ subunit concentration was not increased in those areas where binding to α4β2 nAChRs had been up-regulated, pointing to a post-translational effect.

Given the clear role of α4β2 nAChRs and their up-regulation in the mediation of MDMA-induced psychostimulant and rewarding effects, it proved important to map the differential expression pattern of this receptor type elicited by MDMA. For this purpose, we repeated the protracted MDMA administration schedule described above and mapped [^{125}I]epibatidine binding through autoradiography assays on coronal brain slices from the entire brain.

This exhaustive study yielded several interesting results. α4β2 nAChRs were up-regulated in several key areas closely involved in reward and addiction, such as the VTA by 17%, NAc by 17% and the olfactory tubercle by 20%. Furthermore, other cortical areas implicated in sensory and motor functions also underwent significant up-regulation.

As has been discussed above, an increase in α4β2 nAChRs in the VTA and NAc could results in an increase in synaptic dopamine in the NAc as a response to basal acetylcholine; this phenomenon would, in turn, enhance the rewarding properties of MDMA.

The olfactory tubercle, like the NAc, is a component of the ventral striatum, which is connected to multiple affective-, reward- and motivation-related areas in the brain (Wesson and Wilson, 2011), and has been especially implicated in addictive behavior (Ikemoto, 2003). Furthermore, in the olfactory tubercle, the stimulation of α4β2 nAChRs causes dopamine release (Grady et al., 2001); thus, evidence points to receptor up-regulation in this area being pivotal in the mediation of MDMA-induced reward.

α4β2 nAChRs in the nigrostriatal pathway are also affected by MDMA, as demonstrated by the 33% and 16% increases in the substantia nigra (SN) and the caudate putamen (CPu), respectively. In this sense, this increase could correspond to dopaminergic neurons projecting from the SN into the CPu (located in the striatum); accordingly, Jones et al. (2001) described that α4β2 nAChRs had been found both in the axonal terminals (located in the CPu) and the soma and dendrites (found in the SN) of these neurons. The stimulation of these receptors possesses the ability of eliciting dopamine release, not only from the terminal in the striatum, but also from the soma and dendrites within the SN (Cheramy et al., 1981). Given that this is a crucial structure of the nigrostriatal pathway, in can be hypothesized that the up-regulation of α4β2 nAChRs in these neurons has important effects on locomotion.

Given that nicotine and MDMA are capable of similarly up-regulating α4β2 nAChRs, it would be expected that the pattern in which they do so within the brain would be similar. Accordingly, when comparing our study with that by Nguyen et al. (2003), a

clear homology could be detected. For example, in both studies, the substantia nigra and the amygdala underwent the most drastic up-regulations, while other areas, such as the interpeduncular nucleus or the medial habenula, exhibited no significant changes. Similarly, within the striatum, the NAc undergoes the most notable up-regulation, while in the CPu it is more modest, although still significant.

Interestingly, this homology did not hold true throughout all areas of the brain. In this sense, the hippocampus was unaffected in our study, whereas it is robustly affected by nicotine. As discussed above, nAChR up-regulation is mechanistically complex, and requires the interaction of the ligand (i.e. nicotine or MDMA) with intracellular immature forms of the receptor. We hypothesize differences between ligands could be explained by distinct mechanisms through which they are capable of entering the cell. Accordingly, nicotine penetrates through the cell membrane (Whiteaker et al., 1998), while MDMA enters through reverse transport across SERT.

In agreement with this hypothesis, we found a good homology between α4β2 nAChR up-regulation in our study and results published by Battaglia et al. (1991), where they examined the differential expression of SERT, after a similar MDMA treatment, using rats of the same strain and age as us. In this sense, the areas that underwent SERT up-regulation (amygdala and VTA) or no changes (substantia nigra and nucleus accumbens) exhibited up-regulated α4β2 nAChRs, whereas the areas that underwent SERT down-regulation (hippocampus and certain cortical areas) tended to show no changes in nAChR density. SERT down-regulation would therefore be hindering MDMA internalization and, thus, effects on α4β2 nAChR expression.

β-Ketoamphetamines and their interaction with ethanol and other psychostimulants

Mephedrone

Amphetamine-induced psychostimulant effects are believed to be mediated by an activation of the mesolimbic dopamine system (Izawa et al., 2006). Dopamine and serotonin are released into the synaptic cleft, and their reuptake by DAT and SERT is blocked, yielding a high increase in extracellular levels of both neurotransmitters. Through the alluded dopaminergic activation, mephedrone elicits a rapid and notable increase in locomotor activity, an effect that was significantly potentiated by the concomitant administration of ethanol. For this purpose, a low (10 mg/kg) and a high (25 mg/kg) dose of mephedrone were assessed. We only detected a

clear potentiation at the low dose; this can be explained by the fact that the higher dose probably elicited peak locomotion levels, thus masking any enhancement effect by ethanol. Interestingly, the two doses of ethanol that were tested caused a similar potentiation.

By the administration of haloperidol and ketanserin, we were capable of discerning, that this potentiation is most probably due to an increase in synaptic dopamine, as haloperidol, but not ketanserin, fully blocked the potentiating effect of ethanol on mephedrone-induced locomotor activity. This phenomenon could be due to a pharmacokinetic or pharmacodynamic interaction or a combination of both.

As has been discussed in the introduction, the effects of ethanol on the central nervous system are multiple and complex. Ethanol increases GABA function through an activation of $GABA_A$ receptors; in this sense, the activation of $GABA_A$ in the substantia nigra pars reticulata is known to cause locomotor activation. Ethanol is also capable of indirectly decreasing GABA function in the VTA . This effect, which is exclusively localized to this region of the brain, is due to a blockade of glutamate receptors on GABA neurons. This phenomenon causes a reduction in the inhibitory signal of these GABA neurons on the dopaminergic neurons to which they project. Consequently, the disinhibition of these dopaminergic neurons, which project into the NAc, causes an increase of dopamine in this brain area, potentially contributing to the psychostimulant and reinforcing effects of ethanol. Furthermore, ethanol also induces endogenous opioid release in the NAc, elicits dopamine increases in other mesocortical pathways and acts as a glutamate neurotransmission inhibitor in several brain areas, including the cortex, NAc and VTA.

These mechanisms are clearly different to that of mephedrone; thus, we hypothesize that their interaction yields the synergistic psychostimulant effect described herein. In this sense, ethanol could increase accumbal dopamine through the disinhibition of dopamine neurons projecting from the VTA. This dopamine, which would get transported back into the terminal in basal conditions, would accumulate in the synaptic cleft due to the blockade of DAT by Meph. Synaptic dopamine would further increase due to the amphetamine-mediated dopamine release from the vesicles. This example, although conjectural, is illustrative of the potential interactions that might occur between the mechanisms of these two drugs of abuse. Furthermore, Riegert et al. (2008) showed how ethanol, in vitro, was capable of increasing the dopamine/serotonin release ratio for MDMA in striatal slices. A similar phenomenon could be expected for mephedrone, given its homology with MDMA.

As to potential pharmacokinetic interactions between mephedrone and ethanol, Ben Hamida et al., (2009) showed that ethanol could cause an increase in MDMA

brain concentration. Interestingly, this increase was not homogenous throughout the brain; instead, MDMA increased more notably in areas with higher dopaminergic transmission, such as the striatum or the frontal cortex, whereas other areas were less affected, such as the hippocampus, which is richer in serotonin terminals. This, a similar effect could also be expected for mephedrone, and would also contribute to the dopamine-mediated potentiation of locomotor activity by ethanol.

Given the clear effects of ethanol on mephedrone-induced psychostimulant effects, we sought to investigate how this would translate in the mediation of its rewarding effects. For this purpose, we used the CPP approach. ethanol, at a dose that was not conditioning by itself, was capable of significantly increasing the preference score of mephedrone at 25 mg/kg; furthermore, it also notably increased the preference score of mephedrone at 10 mg/kg, although this result did not yield statistical significance.

CPP is highly dependent on the activation of the mesolimbic pathway, and addictive substances are believed to cause synaptic plasticity in the areas that it comprises, mainly the NAc and the VTA. It is believed that rewarding stimuli modulate behavior through an increase in dopamine in the nucleus accumbens (NAc), which is endowed with numerous dopaminergic terminals that project from cell somas located in the ventral tegmental area (VTA). Therefore, all addictive drugs evoke dopamine increases in the NAc, whether acting through direct or indirect mechanisms (discussed in “introduction” section). For this reason, through full genome microarray screening, we sought to assess what long-term changes in the expression of synaptic plasticity-related genes were elicited by the conditioning mephedrone treatment, as well as by the combination of mephedrone and ethanol. This experiment had two purposes: A) identifying important changes that could underlie the advent of positive conditioning caused by mephedrone and B) determining differentially expressed genes in the combination group, with the objective of finding candidates that could be responsible for the robust enhancement of CPP score elicited by ethanol.

Upon microarray screening, 6 potentially interesting genes were short-listed. One of the most interesting genes was the D3 dopamine receptor gene (Drd3). It was consistently up-regulated in all drug-treated groups. D3 dopamine receptors are a subtype of D2-like receptor which is mainly located in the limbic regions of the brain and is known to be strongly implicated in the reinforcing effects of a wide range of drugs of abuse (Diaz et al., 2014; Leggio et al., 2014; Levant, 1997; Vorel et al., 2002). Furthermore, Drd3 mRNA had been found to be up-regulated after exposure to ethanol, cocaine and morphine. Taking these antecedents into account, we sought to further explore the specific role of D3Rs in the acquisition of CPP. For this

purpose, a pharmacological approach was taken, in which we assessed the effect of SB-277011A (a selective D3R antagonist) on the conditioning treatment initially conducted. SB-277011A completely blocked CPP in both mephedrone and mephedrone+ethanol groups. Furthermore, the up-regulation of Drd3 mRNA was also prevented.

The mechanism through which D3Rs are up-regulated in the NAc has been studied in the literature. It has been described that BDNF is increased upon exposure to cocaine and subsequently controls D3R expression (Graham et al., 2007; Guillin et al., 2001). It has been postulated that an increase in dopamine in the NAc activates D1-like (Gs GPCR) receptors, thus increasing CREB phosphorylation and consequently increasing BDNF production in the mPFCx; it would subsequently be anterogradely transported into the terminals and release in the striatum. Interestingly, in the present work, we found Drd3 up-regulation to be inhibited by the blockade of D3Rs, which are a subtype of D2-like receptors with both presynaptic and postsynaptic locations, negatively coupled to adenylyl cyclase and acting as autoreceptors modulating dopamine release and/or synthesis (Levant, 1997). In this sense, their activation does not cause the same downstream reactions as those evoked by the activation of D1-like receptors, which, as discussed above, are believed to be responsible for D3R up-regulation.

These data add further complexity to the matter of D3R up-regulation. We were therefore interested in elucidating whether the up-regulation in Drd3 reported herein was mediated by a pathway involving BDNF (through D1R activation), as suggested by the literature. For this purpose, another pharmacological approach was undertaken to modulate this phenomenon. Firstly, we assessed whether an acute dose of mephedrone was capable of increasing BDNF expression in the mPFCx, where it is synthesized. Certainly, we reported a time-dependent increase in BDNF mRNA, which reached highest levels 4h after mephedrone administration. Following, CPP treatment was repeated again (only with Saline and mephedrone groups, for increased simplicity) after pretreating animals with ANA-12, a trkB (BDNF receptor) antagonist. This experiment yielded a total blockade of CPP; furthermore, animals given ANA-12 showed baseline levels of Drd3 mRNA expression in the ventral striatum, as opposed to animals treated with mephedrone alone. These results point to the fact that Drd3 up-regulation is mediated by an acute increase in BDNF in neurons from the mPFCx projecting into the NAc. This increase in BDNF could be mediated by the activation of D1Rs, as occurs for cocaine; in this sense, further research must be performed to explain why the D3R selective antagonist SB-277011A was capable of preventing Drd3 up-regulation.

In our initial treatment, we found Drd3 mRNA in all three drug-treated groups to be equally increased, including ethanol-treated animals, which did not show CPP. This

suggests that, although D3 receptors clearly play a role in the rewarding effects of mephedrone, there are also other changes needed to establish conditioning.

Long-term potentiation (LTP) and long-term depression (LTD) are the most widely studied mechanisms mediating neuroplasticity (Malenka and Bear, 2004). These changes in synaptic transmission are triggered in response to stimuli, and are pivotal in the development of memories. In this sense, LTP and LTD are highly dependent on changes in gene expression, and play an essential role in the establishment of addiction in response to a rewarding stimulus (i.e. exposure to a drug of abuse) (Hyman et al., 2006; Stanton and Sarvey, 1984). Actin polymerization permits neuron arborization, which in turn underlies LTP. Out of all synaptic plasticity-related genes that were modified by the conditioning treatment, only Arpc5 was differentially regulated in the mephedrone+ethanol group. This gene encodes for Arpc5, a protein that plays an important role in maintaining the ARP2/3 complex nucleating capability, which is essential for actin remodeling and synaptic plasticity at a presynaptic and postsynaptic level (Stradal and Scita, 2006; Cingolani and Goda, 2008). The ARP2/3 complex is associated with F-actin in the spinoskeleton core and acts to nucleate new actin filament branches from existing actin filaments. It is therefore essential in the activity-dependent enlargement of dendritic spines. Although speculative, Arpc5 could play an important part in establishing reward-related memories. This could account for the matching profiles between Arpc5 expression and CPP score elicited by the different treatments. It must be taken into consideration that changes in gene expression found herein can be considered long-term, and with high probability related to the conditioning reward-chamber association, as the last drug administration is given 72 hours prior to animal sacrifice. All this, taken together, evidences that further research on the role of actin remodeling in drug-induced LTP is warranted, with special focus on Arpc5.

Both Drd3 and Arpc5 are regulated upstream by a common kinase of the Ca2+/calmodulin family: calcium/calmodulin-dependent protein kinase kinase 1 (CAMKK1). It is encoded by the Camkk1 gene; interestingly, its expression was significantly up-regulated in both mephedrone-treated groups by around 30%. The role of this protein in LTP, general synaptic plasticity and memory consolidation is being increasingly explored (Tronson and Taylor, 2007); the potential relationship shown herein between drug-induced conditioning and Camkk1 expression (accompanied by downstream increases in Drd3 and Arpc5) could be a valuable addition to existing evidence on this matter.

Among the list of significant genes that were differentially regulated upon the conditioning treatment, we found five notably up-regulated apoptosis-related genes, as well as a very significant increase in Nfu1 (an essential protein for

mitochondrial function) in mephedrone-treated groups. This was of special interest, since amphetamine derivatives are known to cause oxidative stress and mitochondrial dysfunction, which, in turn, can cause cellular malfunction and apoptosis (Beauvais et al., 2011). The current results pointed towards a similar phenomenon for mephedrone and its association with ethanol. This was further backed by the increase in glutathione peroxidase expression, as the protein it codes for is pivotal in the physiological process of detoxification of reactive oxygen species. Surprisingly, this enzyme was significantly more expressed in animals treated solely with mephedrone, when compared to the mephedrone+ethanol group.

This interesting phenomenon pointed to potential differential toxic effects between a mephedrone treatment and its combination with ethanol. This warranted further study on the matter, which drove us to outline the following study, focusing on the neurotoxic effects of an acute treatment with mephedrone or its association with ethanol.

For this work, the first challenge was to find an acute binge-like mephedrone and ethanol treatment schedule that would be equivalent to common use patterns. In humans, the typical amount of mephedrone consumed over an evening/night is about 0.5 to 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, Martínez-Clemente et al., 2013). Furthermore, during the whole duration of the treatment, room temperature was set at 26±2ºC, at which this drug has been reported to induce signs of neurotoxicity (Martínez-Clemente et al., 2014), in order to reproduce the common hot conditions found in crowded dance clubs.

The same rationale was followed in choosing the administration pattern for ethanol. 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; Martínez-Clemente et al., 2013), we administered changing doses of ethanol throughout the treatment schedule. After testing several combinations, we chose decreasing doses of ethanol every two hours as follows: 2, 1.5, 1.5, 1 g/kg which yielded uniform blood concentrations ranging between 1 and 1.5 g/l during the whole duration of treatment.

As an initial screening, animals were tested for long-term signs of neurotoxicity; for this purpose, they were sacrificed 7 days post-treatment (PT), in order to give the

organism enough time to recover from any homeostatic dysregulation resulting from drug-treatment. At that time point, DAT and SERT density were significantly decreased in the frontal cortex and hippocampus, respectively. This effect was further potentiated by the concomitant administration of ethanol. The potential deleterious effect on the dopaminergic and serotonergic systems were further confirmed by measuring the dopamine and serotonin synthesis-limiting enzymes tyrosine hydroxylase and tryptophan hydroxylase, respectively, in the affected areas. Results were in good agreement with those found for neurotransmitter transporters: mephedrone caused light depletion, which was notably and significantly enhanced by the co-administration with ethanol.

The region-specific affectation found in these experiments is in agreement with reports for other amphetamine derivatives, such as MDMA (Green et al., 2003; Yamamoto and Bankson, 2005). Furthermore, this region-specificity of signs of neurotoxicity seems to be dose-dependent, as in a previous study conducted in our laboratory, a more aggressive mephedrone treatment (4 doses of 50 mg/kg in one day) also elicited dopaminergic depletion in the striatum; similarly, a less aggressive treatment (3 doses of 25mg/kg per day during two days) only caused significant effects in the frontal cortex up to two days after treatment (Martínez-Clemente et al., 2014).

The significant potentiation of neurotoxic effects caused by the combination of mephedrone and ethanol warranted further experiments in search for a mechanistic explanation for this phenomenon. In this sense, oxidative stress is believed to be a main mechanism in the mediation of neurotoxic effects of many drugs of abuse (Yamamoto and Bankson, 2005). We hypothesized that a similar phenomenon could be occurring for the present treatment. This possibility was backed by the different expression levels for the Gpx6 gene between animals treated with mephedrone and its combination with ethanol reported for animals that had undergone the conditioning treatment discussed above. This difference seemed especially noteworthy, taking into consideration that animals were sacrificed 72 hours after the last mephedrone administration, in addition to the fact that a conditioning treatment is relatively mild when compared to acute neurotoxic treatments.

Certainly, the binge-like mephedrone treatment caused significant increases in lipid peroxidation in the frontal cortex and hippocampus, a good marker for oxidative stress-related damage (Wu and Cederbaum, 2003). This effect was very notably potentiated by the combination with ethanol, while the increase caused by ethanol did not attain statistical significance. Furthermore, when measuring oxidative stress-related enzymes levels (i.e. glutathione peroxidase, catalase and superoxide dismutase), an interesting phenomenon could be observed. In contrast to the large

difference found in lipid peroxidation assays, the increase in enzyme levels was equivalent in both mephedrone-treated groups; enzyme levels were unchanged in response to exposure to ethanol alone.

Ethanol has been shown to increase the presence of ROS through several mechanisms, such as the decrease in functional glutathione, the induction of the enzyme CYPE1 or the formation of ethanol-breakdown products (Montoliu et al., 1995; Lieber et al., 1997; Wu et al., 2003). Taking this into consideration, we hypothesize that the effects of mephedrone exceed the antioxidant response (i.e. saturation of enzyme levels), leading to an increased deleterious effect of ethanol-induced ROS, which would otherwise be neutralized. This phenomenon is well reflected by the synergistic increase in lipid peroxidation in the mephedrone+ethanol group, which seems higher than that resulting from the mere addition of the effects elicited by mephedrone and ethanol alone.

Due to the strong effect on serotonergic markers in the hippocampus, it became of interest to assess whether this could have any repercussion on behavioral parameters, namely learning and memory, as this brain area is directly related with these functions (Squire, 1992). Thus, after the binge-like treatment, animals underwent the Morris water maze (MWM) protocol.

During the learning phase of the MWM protocol, animals treated with the drug combination presented significantly worse performance than the other three groups, 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 fell between those of the vehicle and the combination groups, indicating a milder affectation of 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.

Given the robust deleterious effects described above, measuring neurogenesis in animals that had undergone the MWM test was of great interest. 28 days after the binge treatment, neurogenesis was decreased in both mephedrone-treated animals, an effect that was significantly enhanced in the combination group.

It has been widely suggested that the generation of new neurons is implicated in correct learning and memory processes, including MWM performance in rodents (Garthe et al., 2013). Furthermore, neurotoxic processes are closely related to a decrease in cell proliferation and an increase in cell death. Serotonin input to the hippocampus positively regulates adult neurogenesis (Brezun and Daszuta, 1999). In this sense, serotonin 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 serotonin depletion can, in turn, cause decreased cell survival in the dentate gyrus (Brezun and Daszuta, 2000). Our results are in good agreement with this, and evidence a good correlation between neurogenesis in the dentate gyrus, hippocampal neurotoxicity markers and MWM performance.

Finally, it is noteworthy to mention that ethanol alone had a significant effect on some of the assessed biochemical parameters, namely SERT, tryptophan hydroxylase and tyrosine hydroxylase. Furthermore, lipid peroxidation was visibly increased in this group, although these changes did not attain statistical significance. Blood ethanol concentration ranged around 1.5 g/L during a period of 8 hours. Although this concentration can be considered high, it is equivalent to a "common" weekend binge-like pattern; thus, such a marked affectation (consistent throughout the different measured markers) was initially surprising. Supporting this finding was challenging, as this is an unusual administration schedule for ethanol on rodents. Though literature mining, we found that Landau et al. (2007) administered a similar treatment (6 doses at 1g/kg), and detected a dramatic drop in tyrosine hydroxylase, dopamine and its main metabolites. The fact that the decrease in tyrosine hydroxylase was not accompanied by significant changes in DAT suggests that this phenomenon responds to a non-neurotoxic homeostatic regulation as a response to treatment.

As for the hippocampus is concerned, it seems as though, although ethanol alone was capable of altering markers for neurotoxicity and generating ROS, this phenomenon did not cause any significant protective response (oxidative stress-related enzymes) or negative effect on cell proliferation of survival (neurogenesis). As discussed above, serotonergic depletion is known to significantly affect neurogenesis (Nixon, 2006). Furthermore, behavioral markers for learning and memory were unaffected by ethanol alone; all the above suggests that ethanol was non-neurotoxic on its own, and that changes in biochemical markers could be a homeostatic response to increased ROS and a dysregulation in neurotransmission. This is only conjectural and, although of great interest, is beyond the scope of the present doctoral thesis; nonetheless, further studies looking into this phenomenon elicited by ethanol alone are warranted.

To sum up the results from this second chapter, these two studies demonstrate that ethanol is capable of potentiating the psychostimulant and conditioning effects of mephedrone. Furthermore, neurotoxicity is also increased by this combination. This phenomenon is mediated, at least in part, by a synergistic effect between

mephedrone- and ethanol-induced ROS, and has significant consequences on neurogenesis, as well as on learning and memory.

MDPV

Research chemical use is highly and rapidly changing, due to variations in legislation and the ability of the underground market to come up with new molecules. In this sense, following the rise in the use of mephedrone and methylone (the two first widely abused cathinone derivatives), came the entry of MDPV into the black market. This compound has attracted a lot of attention, due to the number of overdoses and hospitalizations related to its use, as a result of its very high potency. Furthermore, shocking episodes, such as reports of cannibalism after MDPV use, have also contributed to the general awareness of the existence of this new compound, which led the EMCDDA to publish, in 2014, a risk assessment report warning of the potential dangers associated to its use. Similarly to what occurred for mephedrone, it became of interest to explore the interrelation between MDPV and ethanol consumption, focusing on the effects of the concomitant use of ethanol on the psychostimulant and rewarding properties of MDPV (i.e. locomotor activity and CPP)..

Our initial finding was of great interest, as ethanol significantly reduced locomotor activity counts induced by low doses of MDPV. Four different doses of MDPV were assessed; we evidenced a clear dose-dependent susceptibility for MDPV to the effects of ethanol on locomotor activity: as MDPV doses were lower, the magnitude of the reduction effect of ethanol on activity counts increased. In this sense, ethanol caused the highest reduction in locomotor activity when administered concomitantly with 0.1 mg/kg MDPV (70%); this reduction was slightly more modest at 0.3 mg/kg MDPV (60%) and negligible at 1 and 3 mg/kg MDPV. Furthermore, as the dose of ethanol is increased, this reduction in locomotor activity seems to be higher, as ethanol 2 g/kg further decreased activity counts elicited by 0.3 mg/kg MDPV.

When analyzing the conditioning effects of MDPV and their combination with ethanol, a similar phenomenon takes place. When combining 0.3 mg/kg MDPV and 1 g/kg ethanol, animals show no conditioning, which contrasts with the significant conditioning shown by animals treated with MDPV alone. Conversely, ethanol has no effect on the conditioning properties of 3 mg/kg MDPV.

The attenuation of the psychostimulant and conditioning effects of MDPV by ethanol was both unexpected and of great interest, as they contrasted with the potentiation previously found by us for the other main cathinone mephedrone (Ciudad-Roberts et al., 2015). In an attempt to shed a light on the potential underlying mechanisms for this phenomenon, MDPV concentration was assessed in

the striatum in the MDPV and MDPV+ethanol groups, with the aim of determining whether this phenomenon is due to a pharmacokinetic interaction, manifested as a decrease in MDPV levels in the brain. In agreement, we revealed a significant reduction (around 50%) in MDPV levels, when the cathinone was combined with ethanol. Nonetheless, this reduction was only evidenced in the first assessed time point (20 min), and brain MDPV concentration for both groups leveled off in the subsequent time points (40 and 80 min).

It is known that MDPV crosses the blood-brain barrier (BBB) through passive diffusion and active transport. This last feature is a differential trait of this compound with respect to the rest of the studied synthetic cathinones studied in the literature, such as mephedrone (Simmler et al., 2012). Thus, we initially hypothesized that ethanol could be disrupting active transport through the BBB, hindering the entrance of MDPV into the central nervous system. This hypothesis was challenged by the data obtained in the subsequent experiment, as MDPV levels were also found to be decreased in blood when co-administering MDPV and ethanol.

In summary, ethanol, at low doses, seems to trigger a strong decrease in overall levels of MDPV, which, in turn, translates into lower psychostimulant and conditioning effects. This phenomenon could be explained by a pharmacokinetic interaction, where the metabolism of MDPV would be accelerated by a change in the normal metabolic route for MDPV. In this sense, 3,4-cathecol-PV and 4-OH-3MeO-PV are believed to be the main metabolites of MDPV (Anizan et al., 2014; Novellas et al., 2015); the main enzymes responsible for the transformation from MDPV into these metabolites are CYP2D6, CYP2C19, CYP2B6, CYP2C9 and CYP3A4. (Meyer et al., 2010, Negreira et al., 2015). The former three enzymes are inhibited by ethanol (Busby et al., 1999; Hellum and Nilsen, 2007); furthermore, they also mediate other significant transformations for MDPV, such as the formation of M8 (through a reduction of MDPV followed by a hydroxylation), M4 (resulting from the hydroxylation of M8), M9 (corresponding to a dihydroxylation of MDPV on the pyrrolidine ring and the propyl side chain) or M5 (resulting from the transformation of the pyrrolidine ring of MDPV to a primary amine). Conversely, CYP3A4 and CYP2C9 (which mediate the transformation of MDPV into its principal metabolites 3,4-cathecol-PV and 4-OH-3MeO-PV) are not affected by ethanol (Busby et al., 1999; Negreira et al., 2015).

It is feasible that, by inhibiting CYP2D6, CYP2C19 and CYP2B6, other enzymes, such as CYP3A4 and CYP2C9, take over and become dominant in the transformation of MDPV. In this sense, the enzymatic reactions that are not susceptible to ethanol inhibition could show a higher Vmax, as well as a higher Km. The lower affinity for these enzymes (higher Km) would explain why other CYPs (i.e. CYP2D6, CYP2C19

and CYP2B6) would preferentially metabolize MDPV in basal conditions, albeit at a slower rate, due to their hypothetically lower Vmax. Thus, when the enzymatic inhibition by ethanol takes place, MDPV metabolism would be shifted into faster enzymatic reactions, and would explain the lower levels of MDPV in the drug combination group. This proposed mechanism is depicted in Figure 5 of the article manuscript.

40 minutes after drug injection, MDPV levels, which are initially decreased by about 50% in the combination group, level off in the brain and blood in both treatment groups. This is probably explained by a saturation of the enzymatic function due to a decrease in the number of functional cytochromes. Interestingly, despite the equivalent MDPV levels 40, 60 and 80 minutes after drug treatment, locomotor activity is markedly lower in the combination group. Here, we postulate two mechanisms through which this phenomenon may be taking place.

Firstly, it is known that MDPV has very high affinity for DAT. As stated by Baumann et al. (2013), due to its high potency at DAT, MDPV may display a slow dissociation from the site (i.e. pseudo-persistent binding), thereby augmenting and extending its pharmacological effects at time points when MDPV brain levels have already become low or below threshold. This hypothesis is supported by a previous study by us (Novellas et al., 2015), which shows a counter clockwise hysteresis when plotting MDPV brain concentration vs locomotor activity. This is evidenced by equivalent locomotor activity 40 and 60 min after administration, despite brain levels being reduced by one half during this time period. In this sense, we hypothesize that, in the present study, when MDPV concentration is reduced to sub-threshold levels, DA reuptake in the synapse occurs at a higher rate, due to a low overall occupancy of this transporter type by MDPV.

Furthermore, it has been suggested that 3,4-cathecol-PV and 4-OH-3MeO-PV have little or no psychostimulant effect (Anizan et al., 2014; Novellas et al., 2015). The shift of MDPV transformation into the formation of these “inactive” metabolites (see Figure 5) could also explain the significantly lower locomotor activity in the MDPV+ethanol group, when compared to animals treated with MDPV alone, at time points when MDPV levels are equivalent in both groups. Nonetheless, it is unclear whether the metabolites resulting from other metabolic routes (i.e. M4, M5 M8 and M9) are active, which would back this hypothesis. Interestingly, M4, M5, M8 and M9 keep the methylenedioxy group on the phenyl ring, which is lost for a cathecol group in 3,4-cathecol-PV (and further methylated to 4-OH-3MeO-PV) and M3. This transformation could be responsible for the hypothetically lower psychostimulant properties of these metabolites. Further assays should be performed in this regard, although it is beyond the scope of this work.

As has been mentioned above, as MDPV doses are increased, the reduction effect of ethanol becomes less significant. At the dose of 3 mg/kg MDPV, ethanol has no perceivable effect on locomotor activity and MDPV brain and blood levels. This phenomenon fits into our working hypothesis, and points to the fact that, as MDPV concentration becomes higher, ethanol is displaced from the cytochromes it shares with MDPV, and is metabolized through other alternative routes (i.e. alcohol dehydrogenase and catalase).

Finally, it is feasible that the reduction in MDPV levels is due to changes in normal absorption. This is improbable though, as a delay in the increase in MDPV levels, as well as locomotor activity would then be expected. As shown by the pharmacokinetic profile described herein, this does not occur: MDPV concentrations of both treatment groups level off 40 minutes post-administration, and decrease in parallel thereafter. This is further backed by the time course for locomotor activity; in this sense, not only is a delay not evidenced, but AUC values are also notably lower in the combination treatment (i.e. total activity throughout the duration of the effect).

Given that MDPV showed strong conditioning properties, it became of interest to explore its ability to produce behavioral sensitization, as this phenomenon results from neuroadaptive changes associated mainly with the development of drug addiction and craving (Robinson and Becker, 1986). MDPV is used as a cheap and easily available alternative to other more classical psychostimulants, especially cocaine. Taking this into consideration, and in light of having found no potentiating effect of ethanol on MDPV-induced locomotor activity and conditioning (rather the opposite), we sought to assess the potential interdependence between MDPV and cocaine, measured as sensitization. It is known that adolescents use MDPV at a very young age; conversely, cocaine is a more expensive psychostimulant, which is generally consumed in adulthood. Therefore, it proved important to determine whether the use of MDPV could increase proneness towards cocaine consumption in future stages in life. In this sense, assessing the sensitizing potential of MDPV over cocaine proved to be good initial screening strategy to shed a light on this matter.

In agreement with its strong psychostimulant and conditioning properties, MDPV caused notable behavioral sensitization, as, on the test day, activity values were increased by 2-fold, as compared to those shown for saline-pretreated animals. Furthermore, this sensitizing MDPV treatment also strongly increased locomotor activity elicited by cocaine on the test day. As discussed above, this points to a possible interrelation between the effects of MDPV and cocaine on neuronal circuits. A possible explanation for this phenomenon is that, although these two drugs belong to entirely different families of compounds, they share their molecular

mechanism (i.e. blockade of DAT). Therefore, it was plausible that both caused similar neuroadaptive processes, that would account for the observed cross-sensitization. Accordingly, we found that MDPV elicited strong increases in CREB, p-CREB and ΔFosB, which were similar in the NAc and the striatum, pointing to an up-regulation of the cAMP pathway; there exists extensive literature on the ability of cocaine to increase these transcription factors. Moreover, a similar treatment schedule to ours conducted with cocaine yielded CREB and ΔFosB protein concentrations 21% and 9% over basal values, respectively (McClung and Nestler, 2003). Interestingly, in our study we found a much higher increase for ΔFosB (2-fold on the test day), although values for CREB remained similar. In the NAc, CREB activity (evidenced by an increase in p-CREB) is a typical phenomenon linked to hypersensitization. Furthermore, through the repeated activation of the cAMP pathway, ΔFosB is up-regulated, and protein levels remain stable; this phenomenon plays a crucial role in promoting reward and incentive salience, and serves as a key mechanism in drug sensitization and addiction, by altering gene expression and, subsequently, neural circuitry (Nestler, 2013). In sum, MDPV has a high sensitizing potential, which results in animals also becoming sensitized to an acute first time dose of cocaine after a withdrawal period. There is a clear analogy between the mechanisms through which these two drugs exert their acute rewarding effects and induce synaptic plasticity, which explains, at least in part, the strong sensitization to cocaine induced by the cathinone . Thus, an experimental-based warning concerning the risks associated to the use of MDPV should be given to the young population, both in terms of its own abuse liability as well as its potential capability to increase propensity towards cocaine abuse.

CONCLUSIONS

MDMA and its interaction with nicotinic acetylcholine receptors

1. α4β2 nAChRs partially mediate the psychostimulant, sensitizing and conditioning effects of MDMA.
2. MDMA-induced α4β2 nAChR up-regulation plays an important role in these processes. In this sense, the main areas that comprise the mesolimbic and the nigrostriatal pathway, implicated in reinforcement and movement, respectively, undergo the highest up-regulation.
3. There is a good correlation between SERT density and the degree of MDMA-induced α4β2 nAChR up-regulation in the assessed regions.
4. This α4β2 nAChR up-regulation is mediated by post-translational mechanisms, as opposed to an increase in transcription. This mechanism is shared with nicotine.

β-ketoamphetamines and their interaction with ethanol and other psychostimulants

1. Ethanol enhances the psychostimulant and conditioning properties of mephedrone, when administered concomitantly.
2. The activation of D3Rs, through a BDNF-mediated pathway, causes the up-regulation of Drd3, which is an underlying mechanism (albeit not the sole one) for the conditioning effects of mephedrone.
3. A series of other important synaptic plasticity-related genes were identified which could play a role in the conditioning effects of mephedrone and their potentiation by ethanol, namely Arpc5, Camkk1, Muted and Syt10.
4. The combination of ethanol with mephedrone notably increases the presence of biochemical markers for neurotoxicity.
5. This effect is accompanied by a reduction in neurogenesis, as well as strong learning and memory impairment.
6. Ethanol attenuates the psychostimulant and conditioning properties of low doses of MDPV.
7. This phenomenon is mediated by a reduction in brain and blood MDPV concentration; this effect could, in turn, be explained by modifications in the metabolism of MDPV.
8. MDPV elicits strong behavioral sensitization to an acute dose of MDPV or cocaine.

9. This sensitizing treatment schedule of MDPV yields a significant up-regulation of the synaptic-plasticity-related transcription factors ΔFosB, CREB, and p-CREB, a shared mechanism with cocaine, thus setting a mechanistic basis for the MDPV-induced sensitization to cocaine.

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