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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 $mg \cdot kg^{-1}$) increased locomotor activity, which was further enhanced by 40% when combined with EtOH (1 $g \cdot kg^{-1}$). This enhancement was blocked by haloperidol. Furthermore, mephedrone (25 $mg \cdot kg^{-1}$) induced CPP, which increased by 70% when administered with a dose of EtOH that was not conditioning by itself (0.75 $g \cdot kg^{-1}$). 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 $mg \cdot kg^{-1}$ 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

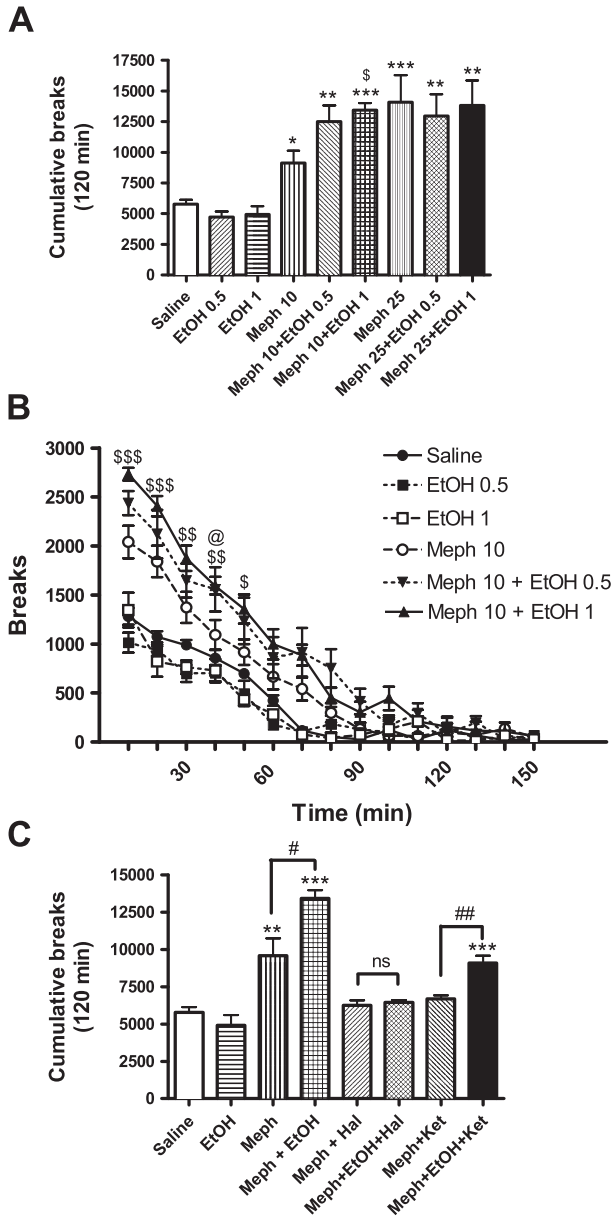

Figure 1

(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.

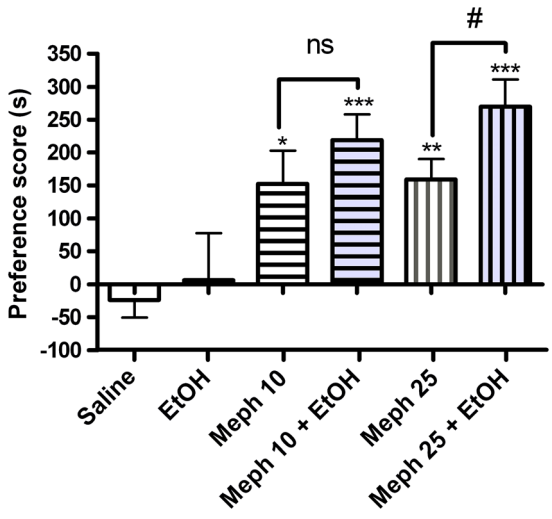

Figure 2

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

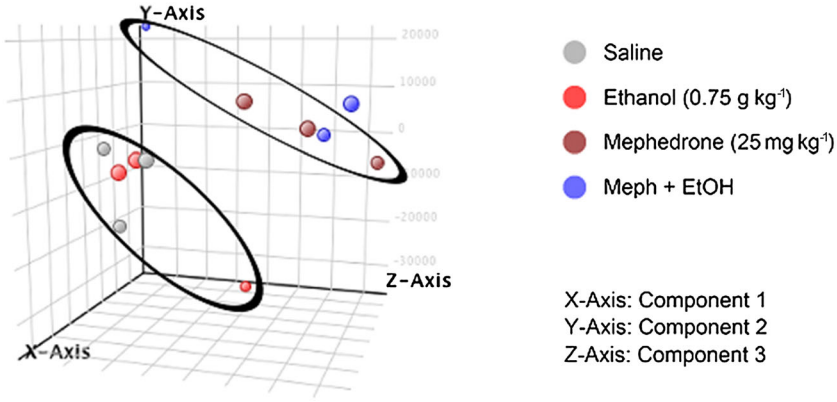

Figure 3

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.

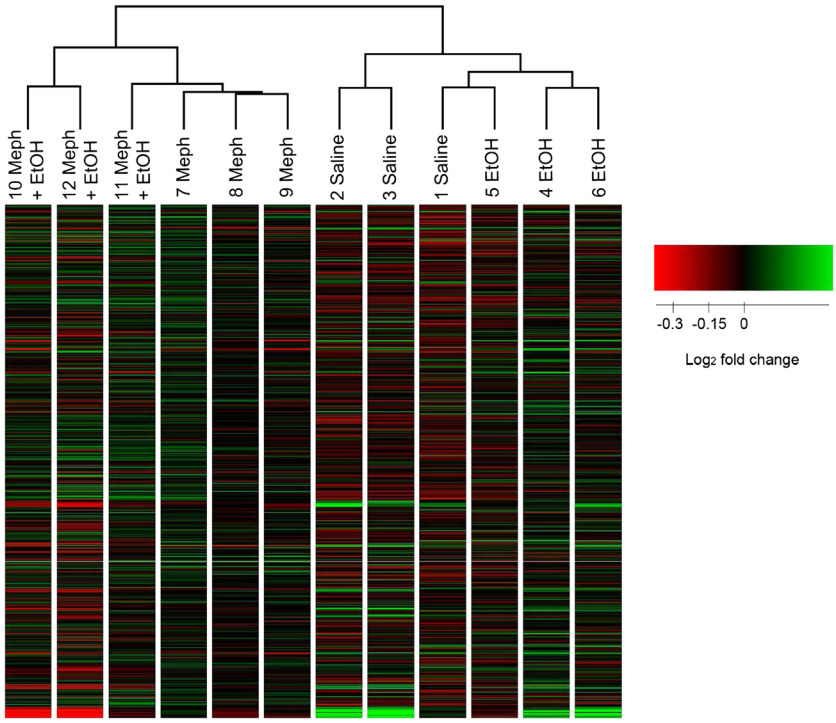

Figure 4

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.

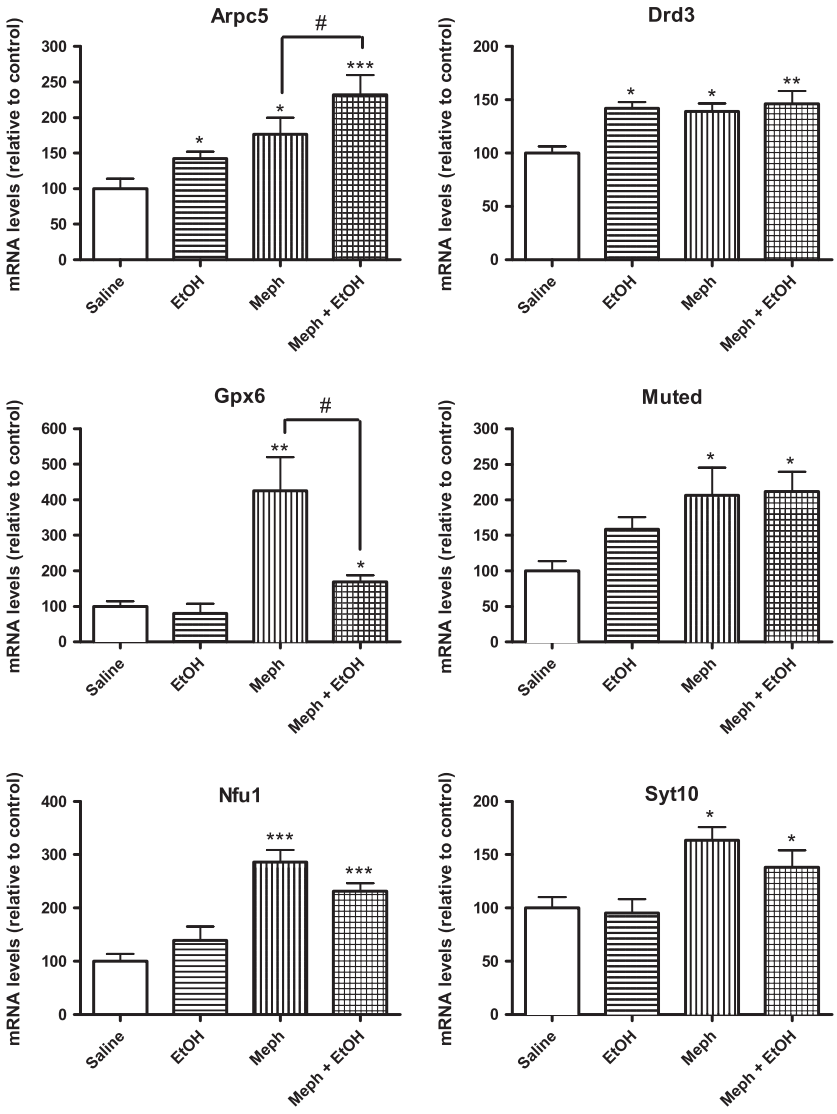

Figure 5

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.

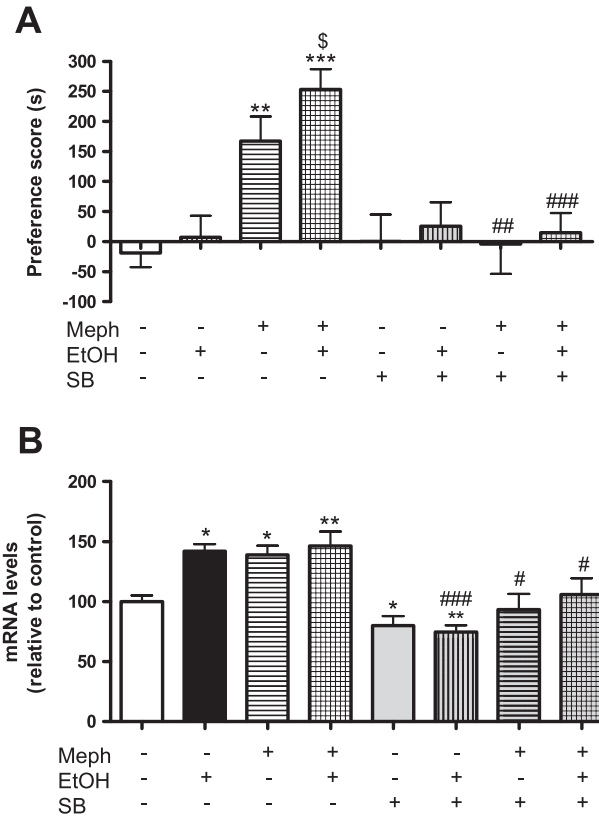

Figure 6

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).

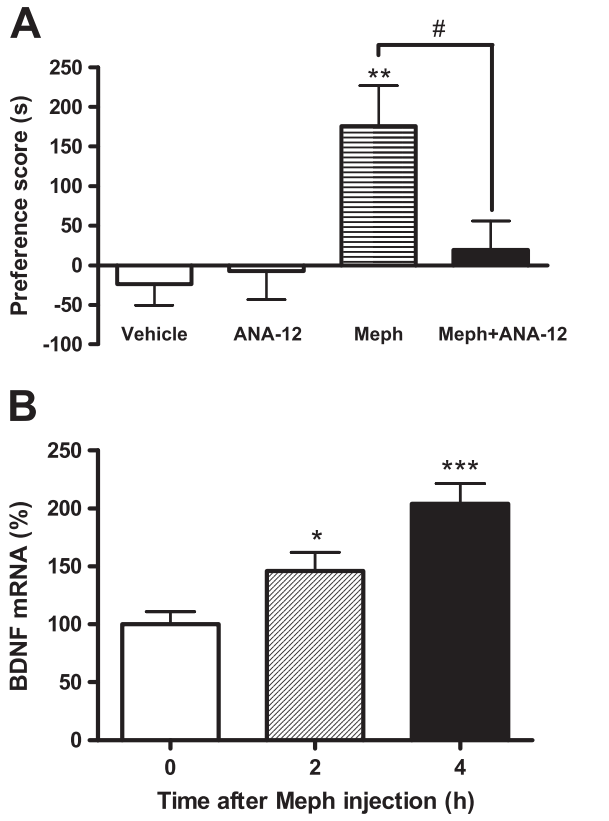

Figure 7

(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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