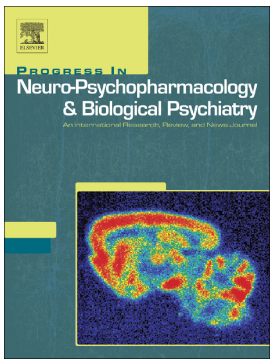

The combination of MDPV and ethanol results in decreased cathinone and increased alcohol levels. Study of such pharmacological interaction

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THE COMBINATION OF MDPV AND ETHANOL RESULTS IN DECREASED CATHINONE AND INCREASED ALCOHOL LEVELS. STUDY OF SUCH PHARMACOLOGICAL INTERACTION.

López-Arnau, R.[1], Buenrostro-Jáuregui, M.[1,2], Muñoz-Villegas, P.[1], Rodríguez-Morató, J.[3,4], Ciudad-Roberts, A.[1], Duart, L.[1], Camarasa, J.[1], De la Torre, R.[3,4,5], Pubill, D.[1,*], Escubedo, E.[1]

[1] *Department of Pharmacology, Toxicology and Therapeutic Chemistry, Pharmacology Section and Institute of Biomedicine (IBUB), Faculty of Pharmacy, University of Barcelona, Barcelona, Spain.*

[2] *Department of Psychology, Faculty of Psychology, University Enrique Díaz de León, Guadalajara, Mexico.*

[3] *Integrative Pharmacology and Systems Neuroscience, IMIM (Hospital del Mar Research Institute), Barcelona, Spain.*

[4] *Department of Experimental and Health Sciences, Universitat Pompeu Fabra (CEXS-UPF), Barcelona, Spain.*

[5] *Spanish Biomedical Research Centre in Physiopathology of Obesity and Nutrition (CIBEROBN), Instituto Salud Carlos III, Madrid, Spain.*

[*]Corresponding author:

David Pubill, Department of Pharmacology, Toxicology and Therapeutic Chemistry, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII 27-31, 08028, Barcelona, Spain.

Email: d.pubill@ub.edu

Abstract

Methylenedioxypyrovalerone (MDPV) is a new psychostimulant cathinone acting as a selective dopamine transporter blocker. Due to the concomitant consumption of ethanol (EtOH) and new psychoactive substances, it is of interest to explore a possible pharmacological interaction between MDPV and EtOH. In locomotor activity assays, EtOH (1 g/kg i.p.) elicited a reduction in the stimulant effect induced by low doses of MDPV (0.1 - 0.3 mg/kg, s.c) in rats, jointly with a decrease in blood and brain MDPV concentrations. Experiments in rat liver microsomes showed different effects depending on the [MDPV]/[EtOH] relationship, evidencing, at certain concentrations, the enhancing effect of EtOH on MDPV metabolism. These suggest that EtOH interacts with MDPV at microsomal level, increasing its metabolic rate. The interaction between both substances was also supported by results in plasma EtOH concentration, which were significantly increased by MDPV, in such a manner that EtOH elimination rate was significantly reduced. The possible toxicological impact of this phenomenon deserves further investigation. In contrast, the rewarding properties of MDPV were unaltered by EtOH. Microdialysis experiments verified that, in the NAcc, both substances could also act synergistically, in such a manner that extracellular dopamine concentrations are maintained. Finally, if the psychostimulant effect induced by MDPV decreased with EtOH, it could favor the boosting and re-dosing in search of the desired effects. However, as the rewarding effect of each dose of the substance would not decrease, the addictive liability could increase considerably. Moreover, we must warn about the increase in EtOH concentrations when consumed concomitantly with MDPV.

Keywords: 3,4-Methylenedioxypyrovalerone, Ethanol, Psychostimulant, Conditioning, Interaction

1. Introduction

The popularity of cathinones as recreational drugs has been increasing since they first appeared in the illicit drug market. 3,4-Methylenedioxypyrovalerone (MDPV) is a synthetic cathinone which shares pharmacodynamics and structural similarities with cocaine and MDMA (3,4-methylenedioxymethamphetamine). Some studies have demonstrated that MDPV is even more potent than cocaine in blocking the dopamine transporter (DAT), as well as in producing locomotor activation (Baumann et al., 2013; Cameron et al., 2013). Moreover, MDPV shows rewarding and reinforcing properties similar to those of cocaine (Baumann et al., 2013; King et al., 2015).

Recreational polydrug use is quite common (Pedersen and Skrondal, 1999). New psychoactive substances are also commonly combined with many other drugs, especially ethanol (EtOH) (Elliott and Evans, 2014). Many studies in rodents indicate that EtOH can alter the pharmacological profile of cocaine, and vice versa (Busse et al., 2005, 2004; Masur et al., 1989; Sobel and Riley, 1997). A dose-dependent attenuation of cocaine-induced hyperlocomotion when EtOH is administered prior to cocaine has been described (Dewey et al., 1997). In rats, EtOH-MDMA co-administration potentiates MDMA-induced hyperlocomotion and rewarding effect (Ben Hamida et al., 2007; Jones et al., 2010). In fact, in animal models, EtOH increases the concentrations of MDMA, and its main metabolite, in blood and brain depending on the administration regimen (Ben Hamida et al., 2007; Cassel et al., 2007). Thus, when investigating possible drug-drug interactions such as MDPV-EtOH, both pharmacokinetics and pharmacodynamics issues must be considered. Just a few studies have been published on MDPV metabolism in rats and humans (Anizan et al., 2014b; Meyer et al., 2010; Negreira et al., 2015). In previous findings from our lab, we also have demonstrated in rats a correlation between MDPV brain concentrations and enhancement of the locomotor activity (Novellas et al., 2015).

The effects of EtOH on the brain are numerous and extremely complex. EtOH potentiates GABAergic transmission, but additional mechanisms elicited by EtOH can modulate this effect, triggering different consequences in the striatum (STR) and Nucleus accumbens (NAcc). Its metabolism involves alcohol-dehydrogenase (ADH) and microsomal oxidases (for review see (Cederbaum, 2012)).

To date, there is no report describing the pharmacokinetics and/or pharmacodynamics of MDPV in the presence of EtOH. Therefore, the aim of this study was to assess whether EtOH can modify the psychostimulant and/or conditioning effects of MDPV when administered concomitantly, as well as MDPV concentrations in blood and rat brain. Moreover, it is known that dopamine (DA) and the NAcc play a key function in the neural circuitry underlying psychostimulant action and acquisition of reward. Therefore, we investigated the effects of MDPV alone and combined with EtOH on the concentration of extracellular DA and its main metabolites in NAcc. The surprising results we obtained warranted a study aimed at determining the effects of MDPV on EtOH pharmacokinetics and pharmacodynamics.

2. Experimental procedures

2.1 Subjects and Drugs

Male Sprague-Dawley rats (Charles River, Spain), weighing 250-300 g were used. All animal care and experimental protocols in this study complied with the guidelines of the European Community Council Directive (2010/63/EU) and ARRIVE, and were approved by the Animal Ethics Committee of the University of Barcelona.

MDPV and methylone were synthesized in racemic form as HCl salt in our laboratory as described previously (López-Arnau et al., 2012; Novellas et al., 2015). DA·HCl, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), NADPH, as well as chemicals for mobile phase and perfusion medium preparations were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat liver microsomes prepared from a pool of

male Sprague-Dawley rats were purchased from Life Technologies Europe. Absolute EtOH was purchased from Scharlau (Barcelona, Spain) and diluted in saline at a concentration that never exceeded 20% (v/v) to avoid tissue irritation. Animals were randomly assigned to the following treatment groups: saline+saline saline+EtOH, MDPV+saline or MDPV+EtOH, onwards saline, EtOH, MDPV or MDPV+EtOH groups.

2.2 Locomotor activity

After being assigned to the different treatment groups (N= 3–5/group) the animals received two habituation sessions (48 and 24 h before the experiment) in the activity box (LE8811, Panlab, Barcelona, Spain). On the testing day, the animals were injected with saline, MDPV (0.1, 0.3, 1 or 3 mg/kg, s.c.), EtOH (1 g/kg, i.p.) or their combination. Since EtOH, at certain doses, can impair or enhance locomotion, it was administered at doses reported to not affect basal activity (Cassel et al., 2004; Hodge et al., 2004). After drug administration, the animals were immediately placed in the activity box and horizontal locomotor activity (HLA) was monitored over a 20-min block during 60, 120 or 360 min. Occlusions of the photo beams (breaks) were recorded (SedaCom32, Panlab, Barcelona). Results are expressed as the area under the curve (AUC), which was measured as the total changes from baseline at each recording interval. This interval was taken from time 0 until hyperlocomotion vanished, that is, until activity was not significantly different from that of the saline group. Accordingly, the effect of 0.1 mg/kg MDPV ended after 60 min, that of 0.3 and 1 mg/kg after 120 min and that of 3 mg/kg after 360 min. The ANOVAs were thus performed between the values of the four groups at the same time chosen for each MDPV dose.

2.3 Conditioned place preference (CPP) test

The apparatus was composed of three distinct compartments (two compartments communicated by a central corridor) separated by manually operated doors. CPP was

performed in three phases: preconditioning, conditioning and post-conditioning test. During the pre-conditioning phase (days 0 and 1), rats were placed in the middle of the corridor and had free access and roam among the three compartments of the apparatus for 20 min. The mean time spent in each compartment was recorded (Smart 3.0, Panlab, Barcelona, Spain).

During the conditioning phase (days 2, 4, 6 and 8), rats (N= 6-10 /group) were treated with saline, MDPV (0.3 or 3.0 mg/kg s.c.), EtOH (1 g/kg i.p.) or both, and immediately confined into one of the two conditioning compartments for 30 min. We intended to use a dose of EtOH that did not produce CPP on its own (Tzschentke, 2007). On days 3, 5, 7 and 9 animals received saline and were confined to the opposite compartment. The animals were exposed to only one pairing per day and treatments were counterbalanced.

The post-conditioning test (day 10) was conducted identically to the pre-conditioning phase. A preference score was expressed in seconds and calculated for each animal as the difference between the time spent in the drug-paired compartment in the test minus the time spent in the same compartment in the pre-conditioning phase.

2.4 Surgery and microdialysis experiments

The microdialysis experiments were carried out on awake rats (N= 3-5/group) according to the protocol described by Kehr et al. (2011), with some modifications. Rats were anesthetized with ketamine (90 mg/kg i.p) plus xylazine (10 mg/kg, i.p.) and placed in a stereotaxic frame. After exposure of the skull, a hole for a guide cannula and three holes for the anchor screws were drilled. Then, an intracerebral guide cannula (AT6.14.iC, Agntho's, Lidingö, Sweden) was surgically implanted and aimed at the NAcc, according to the coordinates: 1.6 mm lateral, 2.2 mm anterior to bregma and 6.0 mm ventral to the dura surface (Paxinos and Watson, 2007), and fixed to the skull using dental cement. Rats were allowed at least one week for recovery from surgery.

On the evening before an experiment, a microdialysis probe (AT.6.14.2, Agntho's, Lidingö, Sweden; 2 mm membrane length with 15000 Da cut-off) was inserted into the guide cannula and perfused overnight with artificial cerebrospinal fluid (aCSF) solution (148 mM NaCl, 2.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 1.2 mM Na_2HPO_4 and 0.3 mM NaH_2PO_4) at a flow rate of 0.6 µL/min. On the next day, the flow was changed to 1 µL/min and after a stabilization period of 2 h, the microdialysis samples were collected at 20 min intervals in plastic vials filled with 10 µL of an antioxidant mixture (0.1 M acetic acid; 0.27 mM Na_2EDTA; 0.5 mM ascorbic acid) (Thorré et al., 1997). The first three samples were used for estimation of basal levels of DA, DOPAC and HVA. Thereafter, saline, MDPV (0.3 or 3.0 mg/kg s.c.), EtOH (1 g/kg i.p.), or both were injected to separate groups of rats and the fractions were collected for 120 or 240 min and stored at -80^{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 the data from those rats with correct probe placements were included in the study.

2.5 Liquid Chromatography/Mass Spectrometry determination of DA and metabolites in dialysate samples

An Agilent 1290 Liquid Chromatography (LC) system equipped with an autosampler and coupled to AB Sciex QTRAP 6500 mass spectrometer (MS) was used to quantify the corresponding monoamine and metabolites. Chromatographic separation was achieved on a Discovery HS F5 (150 mm 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 HCOOH in both solvents. An increasing linear gradient (v/v) of B was used (t(min), %B), as follows, (0, 0), (0.5, 0), (5.90, 30), (6, 100), (9, 100), (9.10, 0), (10.0, 0) at a constant flow rate (500 µL/min). The flow was directed to waste for the first 2 min to prevent the inorganic ions of aCSF solution to

enter the mass spectrometer. The microdialysate samples were refrigerated at 4 ^{o}C and 20 µL were injected, without sample pretreatment, into the LC-MS/MS system. Mass spectrometric quantification in positive ion mode was carried out using the following transitions: DA (*m/z 154 → 137* and *154 → 91)* and DOPAC (*m/z 123 → 77*). A negative ion mode was used in the analysis of HVA (*m/z 181 → 122*).

Six standards (from 0.1 nM to 10 nM for DA or from 10 nM to 1 µM for metabolites) were prepared daily in a solution composed by aCSF/antioxidant mixture (2:1) to obtain the calibration curve. The method showed linearity within the concentration range studied and the detection limit (signal-to-noise ratio=3) for DA was 0.05 nM and for DOPAC and HVA was 1 nM. The accuracy of the assay was 85 – 115% and the intra- and inter-assay coefficients of variation were less than 15%. Analyst v1.4.2 software was used to calculate the areas of chromatographic peaks.

2.6 MDPV concentrations in blood and the STR

Before blood sampling, rats (N= 6-8/group) were implanted with an intravenous (i.v.) jugular catheter under isoflurane anesthesia (Caine and Koob, 1993). Blood samples (150–200 µL) were collected from awake rats through the catheter at 5, 10, 20, 30, 40, 60 and 120 min after MDPV injection (0.3 mg/kg, s.c.) alone or in combination with EtOH (1 g/kg, i.p.) and transferred to 300 µL tubes with EDTA. According to the method previously described (Novellas et al., 2015), 90 µL of plasma was mixed with 10 µL of an internal standard (IS) solution (methylone, 200 ng/mL). The mixture was extracted by adding 250 µL of methanol. After centrifugation (10,000 × g, 5 min)., 250 µL of supernatant were acidified with HCOOH (50%) (Sørensen, 2011). The mixture was ultrafiltered and 100 µL of the filtrate were transferred to an autosampler vial to quantify MDPV concentrations by LC-MS/MS (Novellas et al., 2015).

A PE Sciex API3000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was used to quantify the MDPV in brain and blood

samples. Chromatographic separation was achieved on a Luna C18 (100 × 2.0 mm, 2.5 µm) column. The mobile phase was water (A) and methanol (B) with 0.1% of HCOOH in both solvents. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows, (0, 5), (20, 95), (22, 95), (22.5, 5) and (27.5, 5), at a constant flow rate (150 µL/min). 5 µL of biological samples were injected into the LC-MS/MS system.

As there is a direct correlation between brain concentrations and hyperlocomotion elicited by MDPV (Novellas et al., 2015), we quantified MDPV in rat STR after its administration alone (0.3 and 3 mg/kg s.c.) or with EtOH (1 g/kg i.p.). To minimize the number of animals used in this study and according to the data of locomotor activity, the MDPV concentrations in STR were measured only at 20 min after drug administration. Moreover, a blood sample was also collected and MDPV plasma concentrations were quantified as above in order to calculate the brain/blood ratio at this time.

This experiment was carried out as described by Novellas et al., 2015, with minor modification. Briefly, rats STR (N= 5/group) were homogenized and centrifuged (1000 × g, 20 min). The sample plus IS (methylone) was applied to a C8 Sep-Pak® SPE cartridges (Waters Corp., Milford, MA, USA). MDPV was eluted with methanol and transferred in an auto sampler vial to quantify MDPV concentrations by LC-MS/MS as above.

2.7 Blood EtOH concentration (BEC)

Blood samples (150-200 µL) from animals (N= 4/group) were collected through vein catheters at 5, 10, 20, 30, 40, 60 and 120 min after EtOH injection (1 g/kg i.p.) alone or in combination with MDPV (0.3 mg/kg, s.c.). 80 µL of plasma were transferred to sample microvials, combined with 20 µL of IS solution (1-propanol, 1 g/L) and placed in a water bath at 50ºC for 5 min. Then, 1 mL of the headspace gas was injected with a Hamilton syringe into the gas chromatograph (Agilent 7890A GC-system) equipped

with a flame ionization detector (FID). EtOH and IS were separated on a capillary column (Supelcowax-10®, 30m x 250µm x 0.25µm) using helium as a carrier gas (2.0mL/min, split ratio 20:1). The column oven temperature was isothermal at 55ºC and the injector and the FID system were selected at 150 and 200ºC, respectively. BECs were quantified from linear standard curves in EtOH-free plasma (0.1 – 2 g/L EtOH) using the peak area ratios of EtOH to the IS using Agilent Chemstation software.

2.8 MDPV metabolism in rat liver microsomes

The evaluation of MDPV metabolism was carried out at 37°C in a shaking bath. Preliminary experiments were performed to determine whether MDPV metabolism was linear with respect to time and to evaluate the percentage of MDPV metabolized. The final incubation mixtures (250 µL) contained MDPV (1 or 10 µM), EtOH (0, 0.1, 1.0, 10 or 100 mM), rat liver microsomes (0.5 mg protein/mL), and NADPH (1 mM) in 100 mM sodium phosphate buffer (pH 7.4). After a 5-min pre-incubation with NADPH, the reactions were initiated by the addition of rat liver microsomes and stopped 3 min later by the addition of 625 µL ice-cold methanol. A negative control in the absence of NADPH was employed to verify the NAPDH-dependent reaction. All the experiments were performed in triplicate. To each reaction mixture, 20 µL IS solution (methylone, 10 µg/mL) was added. The resulting mixture was centrifuged (10,000 *g*, 5 min). The supernatant was filtered (cellulose acetate membrane pore size 0.22 µm), acidified with 100 µL HCOOH (50%) and centrifuged (12,000 *g*, 10 min). The supernatants were then transferred to HPLC vials, and MDPV concentrations were analyzed by LC-MS/MS as previously described.

2.9 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences between groups were compared using a one- or two-way (repeated measures) analysis

of variance (ANOVA) or Student's t test for independent samples where appropriate. Significant differences (p<0.05) were analyzed using the Tukey's post hoc test for multiple comparison measures (InVivoStat software package). In all two-way ANOVA analysis, the variable time and the interaction treatment x time were significant. To ease reading, the statistics of these variables were not showed.

3. Results

3.1 Effects on locomotor activity

HLA was monitored for 60, 120 or 360 min depending on the MDPV dose (see Table 1). The AUC analysis of variance demonstrated an overall significant effect of treatment variable ($AUC_{0\text{-}60min}$ $F_{3,12}$= 9.73, p<0.001; $AUC_{0\text{-}120min}$ $F_{5,16}$= 30.11, p<0.001; $AUC_{0\text{-}360min}$ $F_{3,12}$= 8.33, p<0.01). The *post hoc* test revealed that MDPV increased the locomotor activity at all doses tested compared with the saline group. Moreover, at low doses of MDPV (0.1 and 0.3 mg/kg) co-administration of EtOH induced a significant decrease (p<0.01) in HLA compared with MDPV alone (See Table 1). Nevertheless, at higher doses of MDPV (1 and 3 mg/kg) co-administration of EtOH did not produce any change.

Figure 1 shows the time course of the effects on locomotor activity elicited by EtOH and MDPV (0.3 mg/kg Fig. 1A, 3 mg/kg Fig. 1B). At the dose of 0.3 mg/kg, the *post hoc* test confirmed statistical significance between 0.3 mg/kg MDPV and 0.3 mg/kg MDPV+EtOH group, suggesting a blockade of the psychostimulant effect elicited by MDPV. However, no effects of EtOH on the hyperlocomotion induced by 3 mg/kg MDPV were found at any time point.

3.2 Effect of EtOH on the place conditioning induced by MDPV

On the test day (day 10, post-conditioning), results revealed a significant effect of treatment ($F_{5,36}$= 6.94, p<0.001). MDPV treated animals acquired similar place

preference after being conditioned with both doses. The concomitant administration of EtOH did not modify the rewarding effect induced by MDPV. Moreover, as we expected, EtOH did not exert any effect on preference score by itself (Fig. 2).

3.3 Effects of MDPV alone or with EtOH on DA, DOPAC and HVA concentrations in the NAcc

Figure 3 shows the time course of the extracellular concentrations of DA in the NAcc of animals after an acute administration of MDPV 0.3 mg/kg (panel A) or 3 mg/kg (panel B), MDPV+EtOH, EtOH or saline. Two-way ANOVA revealed a significant effect of the treatment variable (panel A: $F_{3,11}$= 8.210, $p<0.01$; panel B: $F_{3,10}$= 14.58, $p<0.001$). However, the post-hoc analysis did not show any difference in DA concentrations between MDPV and MDPV+EtOH at any time point or MDPV dose tested. Administration of MDPV (0.3 and 3 mg/kg) caused a rapid increase in extracellular concentrations of DA, reaching changes in peak concentrations of 382.4±74.1% (Fig. 3A, $p<0.001$) and 1477.1±524.9% (Fig. 3B, $p<0.001$) compared to saline, at 20 and 40 min, respectively. Moreover, we observed a slight increase, although no significant, of about 50% in the extracellular concentrations of DA after EtOH (1 g/kg) administration.

The overall effects of saline, EtOH, MDPV and MDPV+EtOH on the DA concentrations, expressed as relative AUC values, are depicted in Figures 4A and 5A. MDPV (0.3 and 3 mg/kg) caused an increase in total DA of 150.0±35.1% ($p<0.01$) and 891.1±218.7% ($p<0.01$), respectively, that was unchanged when EtOH was administered concomitantly.

The same samples were also analyzed for the quantification of DOPAC and HVA concentrations. Following MDPV administration, the DOPAC and HVA concentrations were significantly reduced (21.7±0.2, and 17.4±3.5%, respectively, Fig. 4B), and this

decline was dampened by EtOH co-administration. In a similar way, when the high dose of MDPV was used, a significant reduction in the DOPAC concentrations of 23.1±6.2% was found (Fig. 5B) without being significantly modifed by EtOH. In addition, HVA concentrations remained unchanged (Fig. 5C).

3.4 Effect of EtOH on MDPV concentrations in blood and rat STR

We measured the plasma concentrations of MDPV over time after s.c. administration of 0.3 mg/kg alone or in combination with EtOH i.p. There was a significant reduction of MDPV concentrations during the first 20 min when combined with EtOH. As shown in Figure 6, the statistical test revealed a significant effect of the treatment variable ($F_{1,14}$ = 5.969, $p<0.05$). Moreover, the $AUC_{5\rightarrow120}$ analysis also showed a significant decrease in the total amount of MDPV (Fig. 6 inset).

20 min after the MDPV (0.3 mg/kg) injection, the results in STR corroborated the decrease in MDPV plasma concentrations described above, (MDPV 0.3 mg/kg= 133.6±8.5 ng MDPV/g tissue; MDPV 0.3 mg/kg + EtOH 1 g/kg= 77.4±10.4 ng MDPV/g tissue ($t=4.189$, $p<0.05$)). The assessed brain/blood ratio at 20 min was 2.2 ± 0. 5 after MDPV administration and also 2.2 ± 0.5 after MDPV+EtOH.

Because the combination of EtOH with MDPV 3 mg/kg did not modify MDPV hyperlocomotion, we tested the blood and brain concentrations of this combination only at the time point of 20 min (maximal psychostimulant effect and maximal striatal concentrations; Novellas et al., 2015). Concerning plasma concentrations, no significant changes were observed between both groups (MDPV 3 mg/kg= 446.0 ±39.5 ng/ml; MDPV 3 mg/kg + EtOH 1 g/kg= 385.8 ±25.4 ng/ml; n.s.). As expected, the combination with EtOH also resulted in no different striatal concentrations (MDPV: 1266.5±40.1 ng MDPV/g tissue vs MDPV+EtOH: 1179.8±68.8 ng MDPV/g tissue; n.s.).

3.5 MDPV metabolism in rat liver microsomes in the presence of EtOH

The metabolic rate of MDPV was determined using rat liver microsomes. The amount of MDPV decreased linearly in a time-dependent manner and at a very high velocity (63% of substrate metabolized at 15 minutes). MDPV metabolism was NADPH- and microsome-dependent. In order to avoid substrate depletion, the experiments were performed with an incubation time of 3 min (25% of MDPV metabolized). The amount of MDPV metabolized at 3 min of incubation is expressed as 100% of metabolic rate. The effects of increasing amounts of EtOH on MDPV metabolism were then assessed (Fig. 7).

When MDPV was assayed at high concentration (10 µM) (Fig. 7A), EtOH inhibited the MDPV metabolism in a concentration-dependent manner ($F_{4,10}$= 40.56; $p<0.001$). Nevertheless, when using a lower MDPV concentration (1 µM), we demonstrated a biphasic effect of EtOH on microsomal activity ($F_{4,10}$= 29.96; $p<0.001$) (Fig. 7B). EtOH 1 mM increased MDPV microsomal metabolism ($p<0.01$), but at high concentrations (100 mM) an inhibition of the microsomal activity was found. Consequently, the enhanced MDPV metabolism induced by EtOH was evidenced only when combining MDPV and EtOH at concentrations of 1 µM and 1 mM, respectively. In these conditions, the metabolic rate of MDPV was increased from 100% to 145%.

3.6 Effect of MDPV on BECs

We measured the BECs over time after i.p. administration of EtOH alone or in combination with MDPV (0.3 mg/kg, s.c.) (Fig 8). After comparing both BEC curves versus time, a significant effect of treatment ($F_{1,6}$=13.97; $p<0.001$) was observed. BECs were significantly higher during 60 min ($p<0.01$) post-administration in rats given 0.3 mg/kg MDPV compared to those given 1 g/kg EtOH alone. Furthermore, the AUC analysis also supported these differences (Fig. 8 inset; t=3.833, $p<0.01$). Additionally, both BECs time course were adequately fitted to a mono-exponential decay model,

allowing us to calculate the corresponding half-life values. MDPV increased the EtOH half-life from 23.0±2.4 min to 64.7±9.4 min (t=4.315; p<0.01).

4. Discussion

In the present study we have described a pharmacokinetic interaction between MDPV and EtOH, substances often consumed concurrently (Elliott and Evans, 2014), that could have a significant toxicological impact. The initial approach of this work was to assess the effect of EtOH on MDPV-induced changes in behavioral parameters for psychostimulant and conditioning properties. The chosen EtOH dose (1 g/kg, i.p.) yielded a BEC in the range of that observed after moderate alcohol drinking (Eckardt et al., 1998). Our initial finding was of great interest, as EtOH significantly reduced locomotor activity counts (70% and 65%) induced by low subcutaneous doses of MDPV (0.1 and 0.3 mg/kg, respectively). Being obtained the previous results on locomotor activity, our study was mainly focused on two different s.c. doses of MDPV, 0.3 and 3 mg/kg, which are equivalent to a 3 and 30 mg dose for a 60 kg person (Reagan-Shaw et al., 2008), respectively. Tentative estimations pointed towards threshold levels around 1-5 mg to “strong” effects between 10-25 mg (www.erowid.org). Interestingly, our subsequent experiments using the dose of 3 mg/kg MDPV showed that the locomotor effects of this higher dose were not significantly affected by EtOH.

Next, we assayed the effects of EtOH on the rewarding effects (CPP) induced by two different doses of MDPV (0.3 and 3 mg/kg). Both MDPV doses produced CPP, with a higher significance degree for the dose of 3 mg/kg, but not significantly different with respect to the dose of 0.3 mg/kg which showed an important SEM. This probably denotes an effect bordering on significance. By contrast, the result of the dose of 3 mg/kg is more robust and supports a high rewarding effect of this drug at this dose. In fact, DA release in the NAcc, which is directly related with rewarding effects, was about

three-fold higher after the dose of 3 mg/kg. This points to a dose-response relationship in the rewarding effect regardless the lack of significance between doses on CPP. Accordingly, other authors (i.e. King et al., 2015) reported that MDPV not always shows a clear dose-response effect in the CPP paradigm.

Conversely to the effect of EtOH on MDPV-induced hyperlocomotion, the rewarding properties of MDPV were unaffected by EtOH. If the psychostimulant effect of the cathinone decreased with alcohol, it could favor the boosting and re-dosing in search of the lost effects. These are typical behaviors followed by consumers of such substances (Ross et al., 2012). However, as the reinforcing effect of each dose of the substance would not decrease, the addictive liability could increase.

In an attempt to shed a light on the potential underlying mechanisms for this phenomenon, MDPV concentrations were determined both in plasma and STR after a single administration of MDPV alone or in combination with EtOH. It is known that MDPV crosses the blood brain barrier (BBB) through passive diffusion and active transport. This last feature is a differential trait of this compound with respect to other synthetic cathinones (Simmler et al., 2013). Thus, we initially hypothesized that EtOH could be disrupting the active transport of MDPV through the BBB. In agreement with the registered psychostimulant effect, the results revealed a significant reduction (of around 50%) in MDPV concentrations in both blood and brain when the cathinone, only at the lowest dose tested (0.3 mg/kg, s.c.), was combined with EtOH. Consequently, no blood/brain ratio disruption was observed.

With these results, taking into account the reported extensive metabolism of MDPV (Anizan et al., 2014a, 2014b; Meyer et al., 2010; Negreira et al., 2015; Uralets et al., 2014), the high liposolubility of this drug and the frequent interactions of EtOH with other drugs' metabolism, the most likely explanation for this effect could be a metabolic interaction. Consequently, we carried out the *in vitro* studies with rat liver microsomes

to test the possibility of an interaction under some specific concentrations of MDPV and EtOH. Interestingly we found two opposite effects, depending on [MDPV]/[EtOH] relationship. When MDPV was assayed at a high concentration (10 µM), EtOH inhibited the MDPV metabolism in a dose-dependent manner. When using a lower MDPV concentration (1 µM), we demonstrated a biphasic effect of EtOH. The enhancing effect of EtOH on MDPV metabolism was mainly evidenced when combining MDPV 1 µM and EtOH 1 mM.

Therefore, the stimulation of MDPV metabolism induced by EtOH is a reasonable explanation for the decrease in MDPV concentrations observed *in vivo* after concomitant administration of EtOH and low doses of MDPV. In fact, a similar interaction on microsomal metabolism had already been described for other substances. Linnoila et al. (1990) studied plasma concentrations of adinazolam and EtOH when administered jointly. They observed higher BEC when administering adinazolam after EtOH, while concentrations of the adinazolam main metabolite were higher. Moreover, Hellum and Nilsen, (2007) studied the effect of EtOH on the CYP2D6 mediated metabolism of dextromethorphan. They clearly demonstrated a biphasic effect of EtOH on CYP2D6. EtOH, at concentrations from 0.5% to 1.1%, increased CYP2D6 activity as compared to control. For EtOH concentrations exceeding 1.5%, a linear inhibition of the CYP2D6 activity was found.

The main enzymes responsible for the transformation from MDPV into its metabolites, in rats, are CYP2D1 (rat orthologue of human CYP2D6), CYP2C19 and CYP1A2 (Meyer et al., 2010; Negreira et al., 2015). CYP2C19 could be inhibited by low concentrations of EtOH (Busby et al., 1999). Therefore, CYP2D1 seems to be the main candidate to show a stimulant effect of EtOH over MDPV metabolism. However, this aspect deserves further investigation and requires extending the study also to the human liver microsomes.

Given the effect of EtOH on locomotor activity, as well as on MDPV concentrations, it proved interesting to look for an explanation to the fact that MDPV conditioning properties were not affected by the combination. For this purpose, microdialysis experiments were performed and changes in DA, DOPAC and HVA in NAcc were assessed. We found that MDPV produces a rapid dose-dependent elevation of extracellular DA, as expected (Schindler et al., 2015). As previously described for a DAT blocker (Kalivas and Duffy, 1990; Müller et al., 2004; Shimada et al., 1996), in parallel with an increase in DA concentration, a slight decrease in DA metabolites was found. This occurs because DA metabolism by MAO takes place inside the nerve terminal. The resulting DOPAC is actively transported to the synaptic cleft (Miyamoto et al., 1991). As MDPV inhibits DAT, DA metabolism is reduced. Similar effects were observed at 3 mg/kg, except for HVA, which can be explained by the high extracellular DA concentrations reached (1500%, Fig. 3B) allowing metabolism by COMT, which is localized in the plasma membrane (Trendelenburg, 1990).

Similarly, EtOH-treated animals showed a slight increase, although no significant, in DA concentrations. It is known that EtOH modulates the function of $GABA_A$ receptors, reducing DA release. In the VTA, however, EtOH decreases rather than increases GABAergic neurotransmission (Stobbs et al., 2004; Xiao et al., 2007), leading to increased mesoaccumbal DA release (Martí-Prats et al., 2015; Melendez et al., 2003; Yoshimoto et al., 1992). Thus, while in the NAcc this substance increases the release of DA, in the dorsal STR it does just the opposite (Budygin et al., 2001). This is the reason why the effect of EtOH on motor activity is more likely to vary depending on the different experimental conditions, whereas its reinforcing and rewarding effect are clear and reproducible (Acevedo et al., 2013; Cunningham et al., 2002; Durcan and Lister, 1988; Frye and Breese, 1981; Milton et al., 1995; Morales et al., 2012; Robledo et al., 1991; Wróbel, 2011).

Interestingly, the increase in DA concentrations induced by MDPV was not significantly modified by EtOH. In fact, EtOH may increase DA release. Although this increase was not statistically significant when administered alone, significant levels could be reached when combined with the DA uptake inhibition induced by MDPV. This should result in increased extracellular DA levels and potentiation of the effects of MDPV. However, the pharmacokinetic interaction with 1 g/kg EtOH observed at 0.3 mg/kg MDPV produces decreased blood and brain MDPV levels, so the effect of MDPV should be lower than expected when combined with EtOH, making a potentiation more unlikely. In our experiments, such decreased effect of MDPV appear to be compensated by the DA release induced by EtOH which may act synergistically with the uptake inhibition. At 3 mg/kg, the increase in DA concentration induced by MDPV is so high that probably could mask the enhancing effect of EtOH, which cannot be appreciated.

At that moment, we also wondered if, in addition to MDPV concentrations, those of EtOH could be modified when combining both drugs. Interestingly, BEC's were very significantly increased by MDPV, in such a manner that its elimination rate was extremely reduced (half-life increased from 23.01 ± 2.35 min to 64.69 ± 9.36, $p<0.01$). For this reason, it is really feasible that the decrease in DA (as a consequence of lower amounts of brain MDPV) was compensated by the increase in DA elicited by higher EtOH concentrations, yielding similar conditioning effects. Accordingly, although synaptic DA concentrations were similar, the amount of DA taken up into the terminal (and thus metabolized into DOPAC and HVA) was higher, as expected for a milder blockade of DAT, due to lower concentrations of MDPV.

The effects on locomotor activity must be discussed apart, as it results from increased DA not only in NAcc but also in other brain areas where EtOH inhibits DA release, so the psychostimulant effect of MDPV can be modified in a different way than the mesoaccumbal reinforcing effect.

Due to the possible toxicological impact, it would be interesting to study the exact mechanism responsible of increased BEC by the presence of MDPV. On one hand, Meyer et al. (2012) suggest that ADH has a large impact on the β-keto group reduction of cathinones (Meyer et al., 2012). On the other hand, studies carried out in urine of MDPV users demonstrate the absence of the metabolic product of this β-keto reduction, likely due to both pyrrolidine- ring and methylenedioxy- group of MDPV (Uralets et al., 2014). Therefore, MDPV could not act as a substrate of ADH but might inhibit ADH activity, impairing ethanol metabolism. However, this hypothesis requires a thorough study of the effect of MDPV on ADH.

Finally, we would like to stress that under certain conditions the combination of MDPV plus EtOH reduces the psychostimulant effect of the cathinone, whereas its reinforcing effect is maintained. This would lead to repeat or increase drug intake in search of the desired stimulation, while the accompanying extra reinforcement could induce higher abuse liability. Due to the possible toxicological impact, further research focused on finding out the exact mechanism by which MDPV increases EtOH half-life and whether this phenomenon takes also place in humans is warranted. Moreover, from the present results is mandatory to warn about the risk of EtOH intoxication when this is consumed concomitantly with MDPV.

Conclusions

In summary, EtOH, at low-moderate doses, seems to trigger a strong decrease in overall concentrations of MDPV, when low doses of cathinone are administered, which dampens the MDPV-induced hyperlocomotion. This phenomenon could be explained by a pharmacokinetic interaction in the metabolic process. Therefore, MDPV and EtOH can interact at microsomal level, increasing the metabolic rate of MDPV. The interaction between both substances was also supported by results on BEC, which were significantly increased by MDPV, in such a manner that EtOH elimination rate

was significantly lowered. In the NAcc, both substances could also act synergistically, in such a manner that although brain MPDV concentrations are lower, the synaptic DA concentration is maintained high enough by EtOH to elicit a similar reinforcing effect.

Disclosure Statement

All authors disclose any actual or potential conflict of interest including financial, personal or other relationships with other people or organizations that could inappropriately influence the present work.

Contributors

RL and MB performed microdialysis and blood kinetics experiments. PM was involved in conditioned place preference experiments. AC and LD accomplished brain kinetics. JR and RT performed microsome experiments. JC and DP performed locomotor activity experiments and analysed the data of behavioral experiments. DP wrote the first draft of the manuscript. EE designed the study, undertook statistical and non-linear regression analyses and wrote the final version of the manuscript. All authors contributed to and approved the final manuscript.

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Legends for figures:

Figure 1: Time course of locomotor activity induced by saline, EtOH, MDPV or MDPV plus EtOH administration. In panel A (MDPV 0.3 mg/kg), two-way ANOVA revealed the effect of the treatment variable ($F_{3,11}$= 24.49, p<0.001). In panel B (MDPV 3 mg/kg), two-way ANOVA also denoted the effect of treatment ($F_{3,11}$= 10.12, p<0.01). Data are expressed as the mean ± SEM. */#p<0.05, **/##p<0.01 or ***/###p<0.001 vs. saline group at the corresponding time point; &&p<0.01, &&&p<0.001 vs. MDPV+EtOH group at the corresponding time point.

Figure 2: Effect of EtOH on MDPV-induced conditioned place preference. Results are expressed as the mean ± SEM. *p<0.05 or ***p<0.001 vs. saline group.

Figure 3: Effect of saline, EtOH, MDPV alone (0.3 mg/kg, panel A, or 3 mg/kg, panel B) or in combination with EtOH on extracellular levels of DA in the NAcc of awake rats. The arrow indicates the time of drug or saline administration. Data are mean ± SEM and expressed as a percentage of preinjection baseline values (% basal). *p<0.05, **p<0.01 or ***p<0.001 MDPV vs. saline group. #p<0.05, ##p<0.01 or ###p<0.001 MDPV+EtOH vs. saline group.

Figure 4: Overall effects of saline, EtOH, MDPV alone (0.3 mg/kg) and plus EtOH on DA (panel A), DOPAC (panel B) and HVA (panel C) levels in the NAcc of awake rats. The columns represents the $AUC_{0\text{-}120min}$ values calculated as the differences in relative changes (%) in these compounds between the drug- and saline-treated groups. *p<0.05, **p<0.01 vs. saline group; &p<0.05 vs. MDPV+EtOH group.

Figure 5: Overall effects of saline, EtOH, MDPV alone (3 mg/kg) and plus EtOH on DA (panel A), DOPAC (panel B) and HVA (panel C) levels in the NAcc of awake rats. The columns represents the $AUC_{0\text{-}120min}$ values calculated as in Figure 4. *p<0.05, **p<0.01 vs. saline group.

Figure 6: Time-course of MDPV plasma levels after s.c. administration of MDPV alone (0.3 mg/kg) (filled circles) or in combination with EtOH (open circles). Data are expressed as the mean ± SEM. *p<0.05, **p<0.01 or ***p<0.001 vs. MDPV+EtOH group at the corresponding time point. **Inset:** Overall effect of EtOH on AUC values of MDPV plasma levels. *p<0.05 vs. MDPV group.

Figure 7: *In vitro* metabolism of MDPV by rat liver microsomes. MDPV 10 µM (panel A) or 1 µM (panel B) was incubated with rat liver microsomes) in the presence of different EtOH concentrations. A negative control (Ctrl -) in the absence of NADPH was included. The amount of MDPV metabolized at 3 min of incubation is expressed as 100% of metabolic rate (positive control, Ctrl +) and the rest of results are normalized by this value. Data are the mean ± SEM of the percentage of MDPV metabolic rate. **p<0.01 or ***p<0.001 vs. Ctrl +.

Figure 8: Time course of BEC's after the administration of EtOH alone (filled circles) or in combination with MDPV (open circles). Data are expressed as the mean ± SEM. **p<0.01 vs. EtOH group at the corresponding time point. **Inset:** Overall effect of MDPV on AUC values of BEC's.

Table 1: *Effect of MDPV alone or in combination with EtOH on HLA in rats. Results are expressed as mean ± SEM and represent the measurement of the area under the curve (AUC).*

Drug	AUC		
	0-60 min	***0-120 min***	***0-360 min***
Saline	10565 ± 1809	14359 ± 3233	15485 ± 3164
EtOH 1g/kg	13433 ± 2228	13827 ± 2300	15085 ± 2239
MDPV 0.1 mg/kg	30950 ± 5401 **	-	-
MDPV 0.1 mg/kg + EtOH	9050 ± 2278 ##	-	-
MDPV 0.3 mg/kg	-	88016 ± 10189 **	-
MDPV 0.3 mg/kg + EtOH	-	30506 ± 6160 #	-
MDPV 1 mg/kg	-	134596 ± 17712 ***	-
MDPV 1 mg/kg + EtOH	-	140915 ± 13917 ***	-
MDPV 3 mg/kg	-	-	202924 ± 38741 *
MDPV 3 mg/kg + EtOH	-	-	254328 ± 68569 **

**p<0.05, **p<0.01, ***p<0.001 vs.saline.*

#p<0.05, ##p<0.01 vs. the corresponding MDPV group.

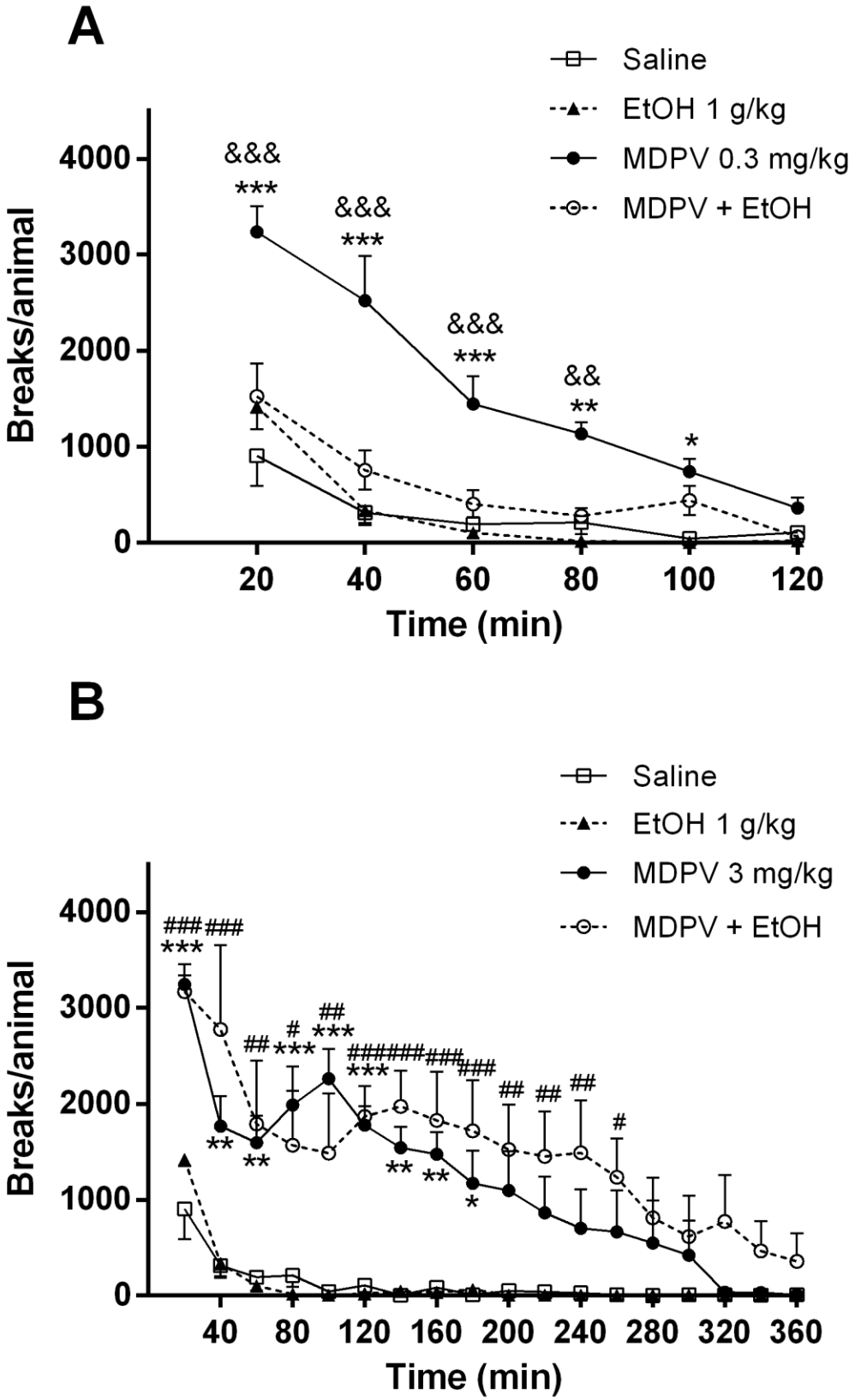


Fig. 1

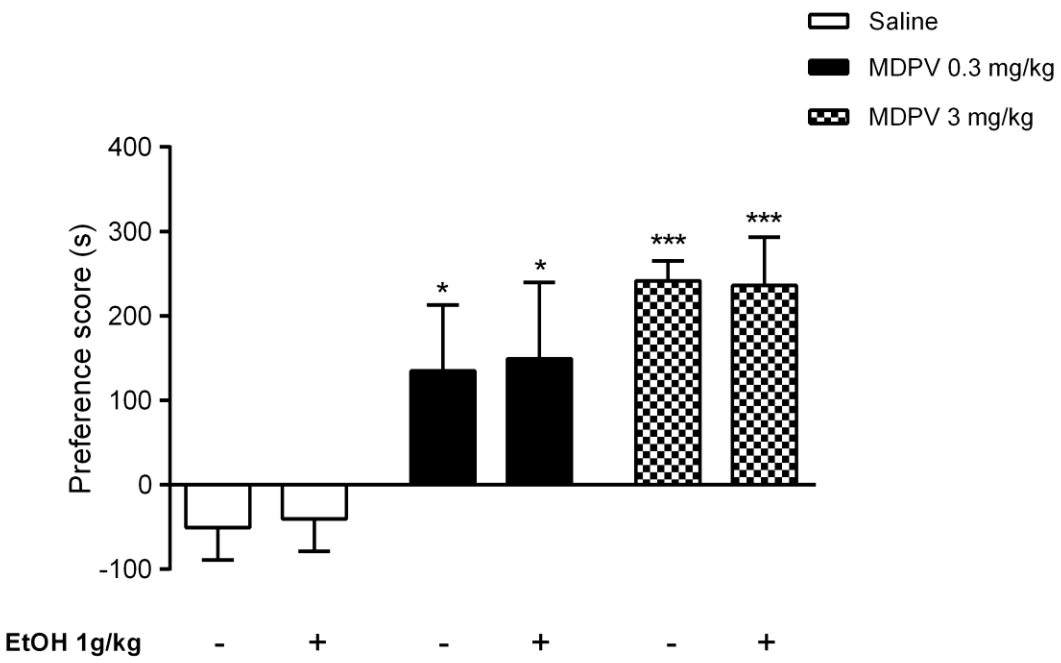


Fig. 2

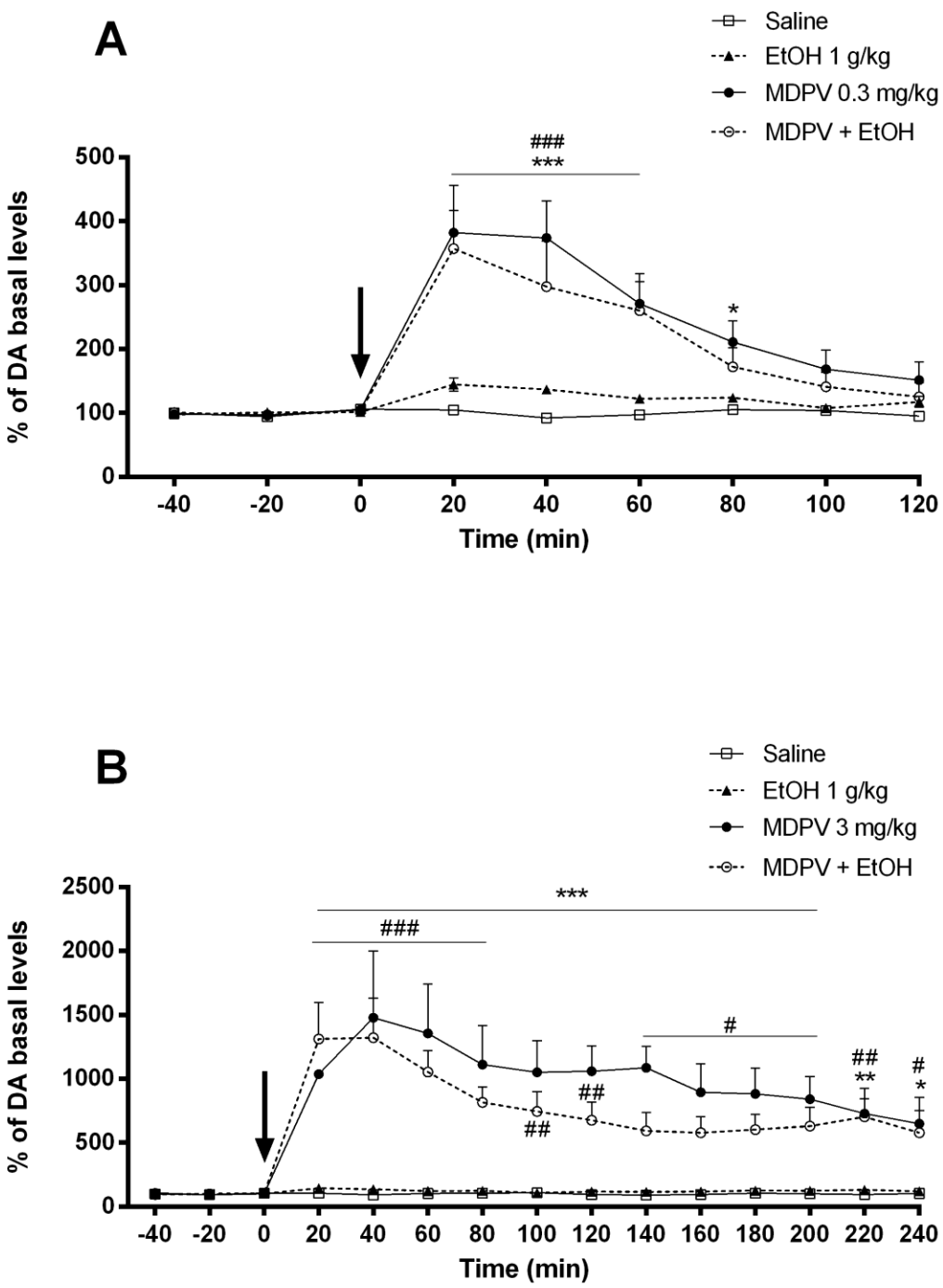


Fig. 3

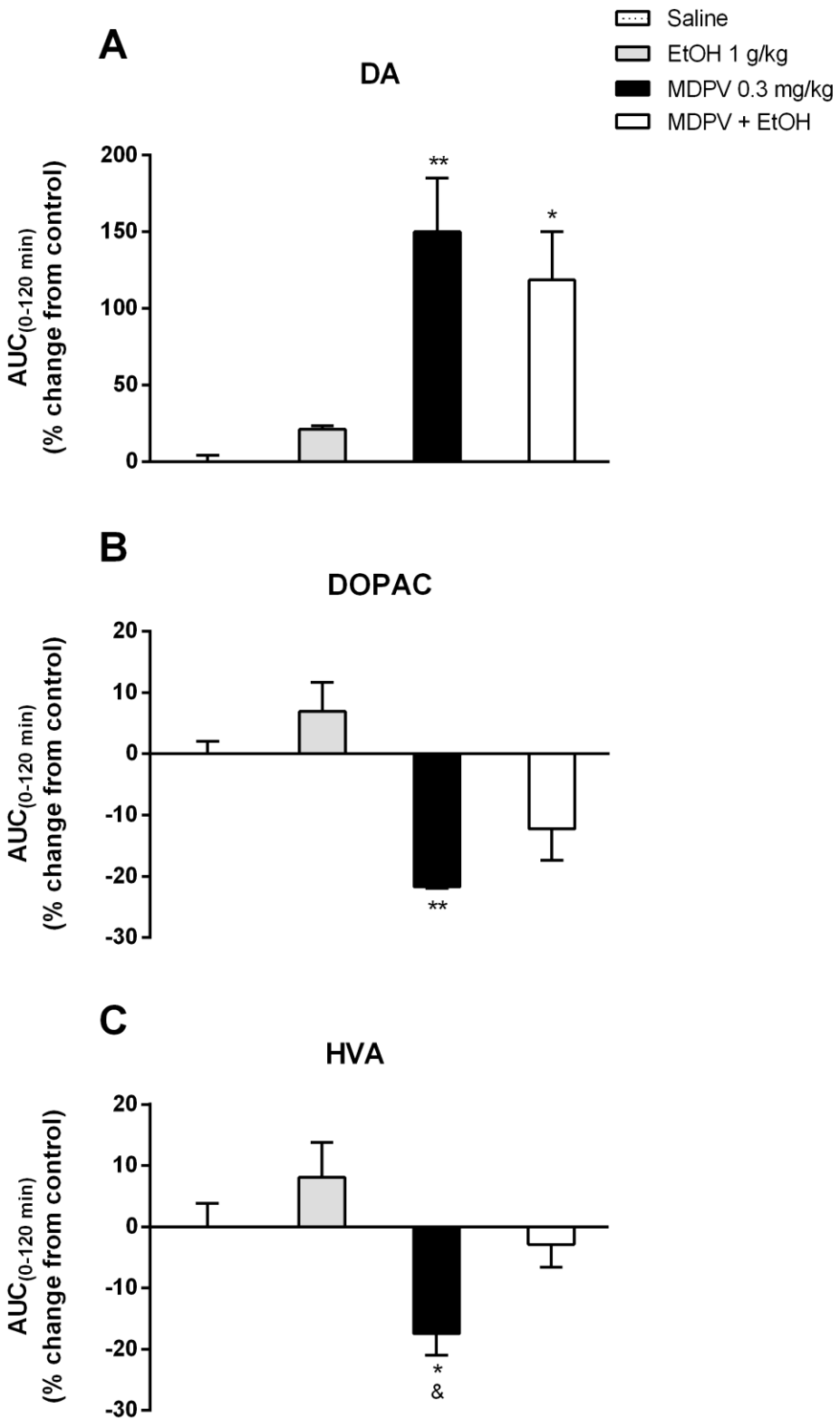


Fig. 4

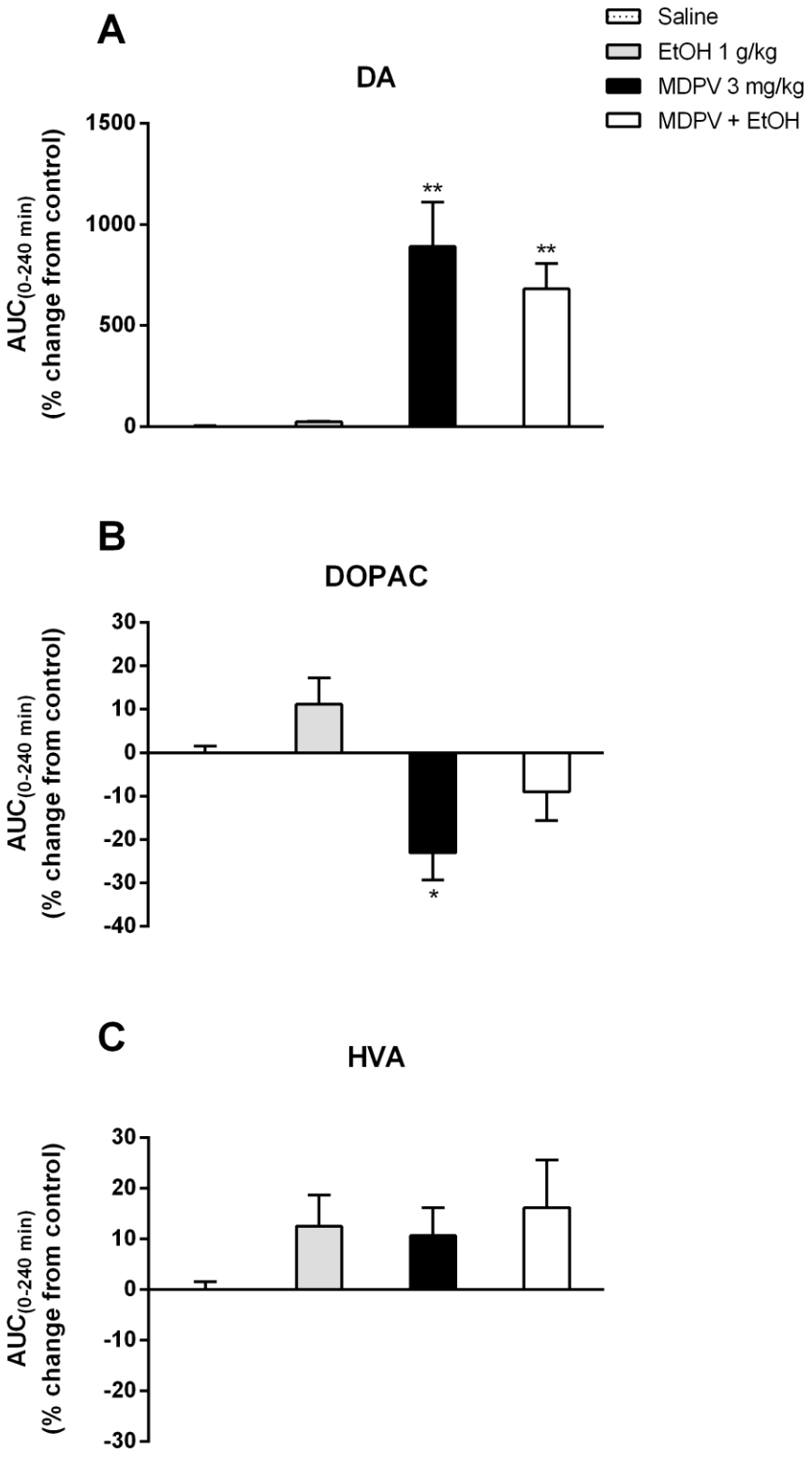


Fig. 5

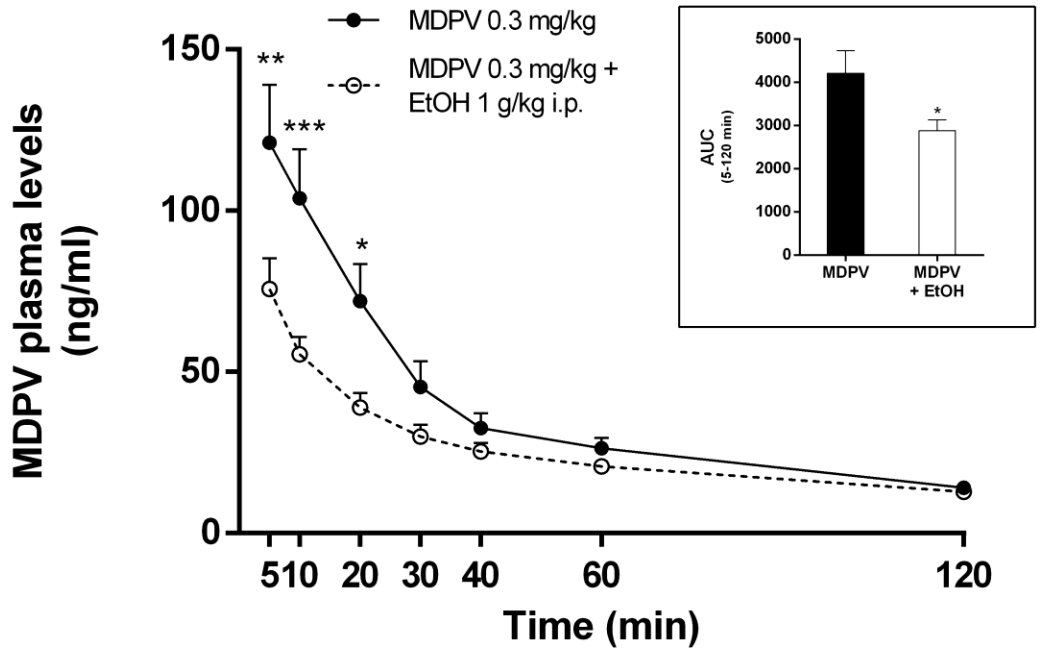


Fig. 6

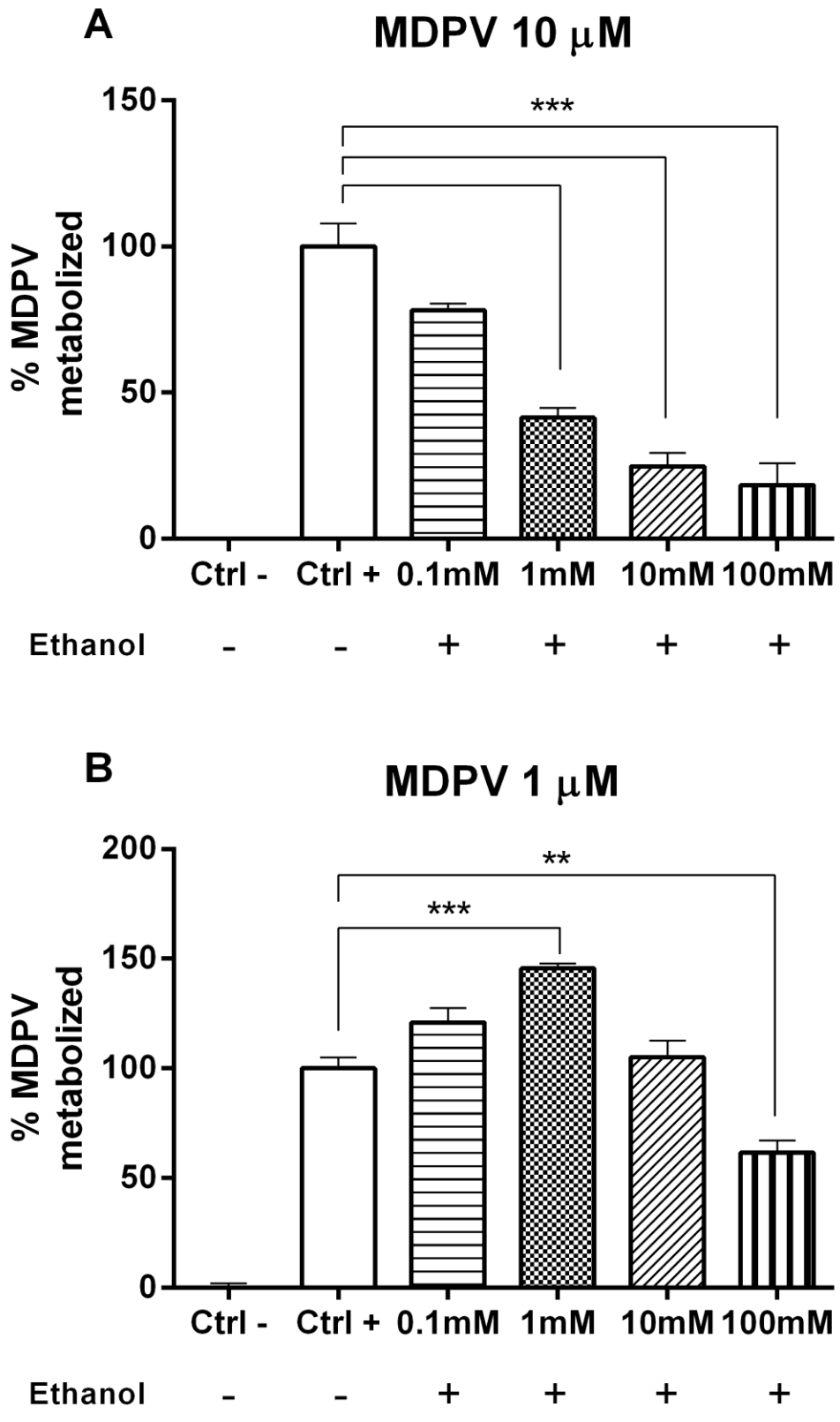


Fig. 7

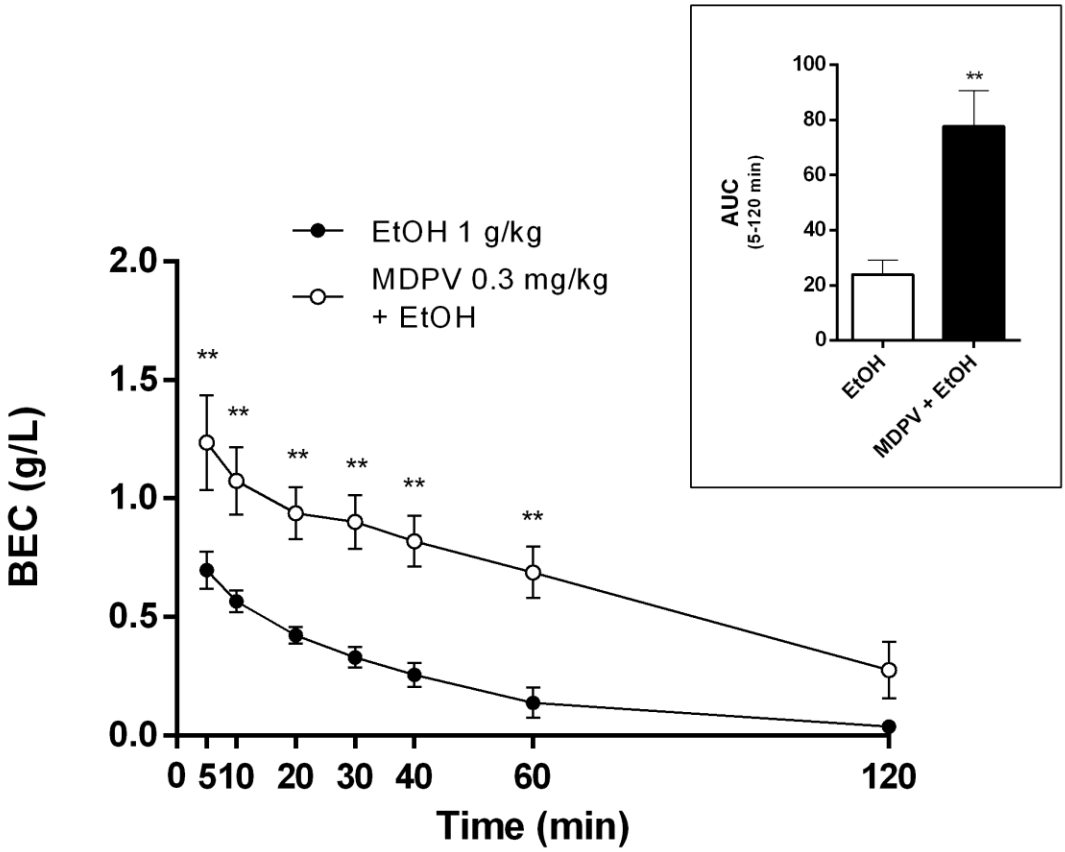
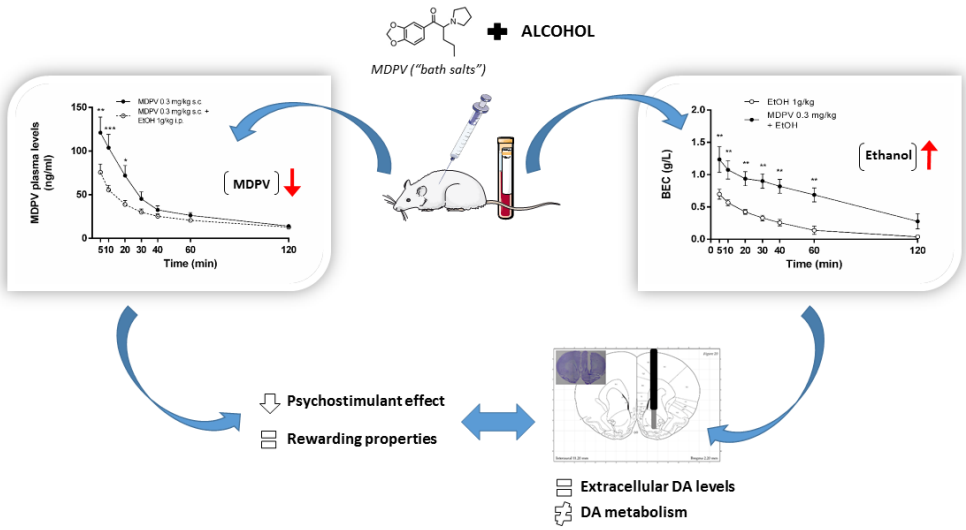


Fig. 8

Graphical abstract



Highlights

- The effects of a concomitant administration of EtOH and MDPV were studied.
- EtOH reduced the psychostimulant but not the rewarding effect induced by MDPV.
- EtOH produced a reduction in MDPV concentrations in both blood and striatum.
- EtOH half-life increased 3-fold when MDPV was coadministered.
- In vitro, the effects on MDPV metabolism depended on [MDPV]/[EtOH] relationship.