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**Effect of the combination of mephedrone plus ethanol on serotonin and
dopamine release in nucleus accumbens and medial prefrontal cortex of awake
rats.**

Raúl López-Arnau¹, Mario Buenrostro-Jáuregui², Jorge Camarasa¹, David Pubill¹ ✉,
Elena Escubedo¹

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

²*Neuroscience Laboratory, Department of Psychology, Universidad Iberoamericana,
Ciudad de Mexico, Mexico*

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.

ORCID: 0000-0002-6627-4501

Email: d.pubill@ub.edu

Tel: +34-934024531

Fax: +34-934035982

Abstract

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3 Cathinones, such as mephedrone (Meph), are often co-abused with alcoholic drinks. In
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5 the present study we investigated the combined effects of Meph plus ethanol (EtOH)
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7 on neurotransmitter release in the nucleus accumbens (NAc) and the medial prefrontal
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9 cortex (mPFC).

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12 A guide canula was stereotaxically implanted into either the NAc or the mPFC of male
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14 Sprague-Dawley rats. 7 days after surgery a microdialysis probe was inserted and rats
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16 were administered saline, EtOH (1 g/kg, i.p.), Meph (25 mg/kg, s.c.) or their
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18 combination and dialysates were collected. Serotonin (5-HT), dopamine (DA) and their
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20 metabolites (5-HIAA, DOPAC and HVA) were determined through high pressure liquid
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22 chromatography coupled to mass spectrometry.
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26 5-HT and DA peaked 40 min after Meph administration (with or without EtOH co-
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28 treatment) in both areas. EtOH combined with Meph increased the 5-HT release
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30 compared with the rats receiving Meph alone (85% in NAc, 65% in mPFC) although
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32 the overall change in the area under the curve only reached statistical significance in
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34 the NAc. In mPFC, the increased release of 5-HT lasted longer in the combination than
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36 the NAc. In mPFC, the increased release of 5-HT lasted longer in the combination than
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38 in the Meph group. **Moreover, EtOH potentiated the psychostimulant effect of Meph**
39
40 **measured as locomotor activity.** Given that both 5-HT and DA are also related with
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42 reward and impulsivity, the observed effects point to an increased risk of abuse liability
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44 when combining Meph with EtOH compared with consuming these drugs alone.
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53 **Keywords:** Mephedrone; Ethanol; Cathinones; Microdialysis; Drug polyabuse; Bath
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55 salts.
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Introduction

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3 Mephedrone (4-methylmethcathinone, Meph) is one of the most popular new designer
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5 drugs of the cathinones' group. It is a β -keto-amphetamine which has powerful
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7 psychostimulant and entactogenic effects and has been distributed as bath salts or as
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9 a component of ecstasy tablets (Brunt et al. 2012).

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12 Most recreational drug use occurs in leisure environments, where alcoholic drinks are
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14 omnipresent, so most cathinone consumers combine them with it (O'Neill and McElrath
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16 2012) and interaction between drugs and alcohol may occur. In fact, the effects of the
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18 combination of another popular amphetamine derivative, 3,4-
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20 methylenedioxymethamphetamine (MDMA), with alcohol were broadly studied by the
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22 group of Cassel, Jones and colleagues (see Mohamed et al 2009 for a review),
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24 showing that alcohol potentiates the hyperlocomotion and conditioning (Cassel et al.
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26 2004; Jones et al. 2010) effects of MDMA, increases brain MDMA concentrations (Ben
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28 Hamida et al. 2009) and attenuates its hyperpyretic effects.

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33 Studies in animals have shown that Meph stimulates the release of serotonin (5-HT),
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35 dopamine (DA), and norepinephrine and inhibits their re-uptake in the CNS (Baumann
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37 et al. 2012; Kehr et al. 2011; López-Arnau et al. 2012). Ethyl alcohol (EtOH) also exerts
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39 complex effects on neurotransmitter release (see Clapp et al. 2008 and Siggins et al.
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41 2005 for reviews) due to its ability to cross biological membranes and to interact on
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43 several molecular targets (i.e. ligand-gated ion channels such as glutamate receptors)
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45 which can lead to uncertain interactions affecting the behavioral and toxic effects when
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47 combined with Meph, such as increased hyperlocomotion and place conditioning in
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49 mice (Ciudad-Roberts et al. 2015, 2016). EtOH is capable of increasing
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51 hyperlocomotion by inhibiting GABAergic interneurons in the substantia nigra reticulata,
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53 which leads to disinhibition and increased burst firing of dopamine neurons in the
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55 nucleus accumbens (NAc), but it also increases DA release in other areas of
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57 mesocortical pathways. Indirect mechanisms involving acetylcholine in the ventral
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1 tegmental area, and more particularly the activation of nicotinic receptors, contribute to
2 the increased DA release within the NAc. Also, activation of the opioid reward pathway
3 has been reported (Mitchell et al. 2012). On the other hand, systemic and local (Riegert
4 et al. 2008) EtOH increases the release of 5-HT in the striatum, suggesting the
5 participation of local mechanisms, and an inhibitory effect of EtOH at the serotonin
6 transporters has been reported as well (Daws et al. 2006). At the same time, 5-HT was
7 found to potentiate the EtOH-induced activation of ventral tegmental area neurons
8 (Brodie et al. 1995).

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18 Other groups have investigated the effects of Meph on neurotransmitter release
19 (Baumann et al. 2012; Golembiowska et al. 2016; Kehr et al. 2011; Wright et al. 2012)
20 but none has studied such effects in combination with ethanol. The aim of the present
21 study is to assess the effects of such drug combination on DA and 5-HT release in the
22 nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), two key areas
23 involved in drug-induced behavior (Nestler 2001; Hammerslag et al. 2014). Also, the
24 effects of such treatments on the psychostimulant effects will be measured.
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49 **Abbreviations:** 5-HIAA, 5-Hydroxyindoleacetic acid; 5-HT, serotonin; DA, dopamine;
50 DOPAC, 3,4-Dihydroxyphenylacetic acid; EtOH, alcohol/ethanol; LC-MS, liquid
51 chromatography-mass spectrometry; MDMA, 3,4-methylenedioxy-methamphetamine;
52 Meph, mephedrone; mPFC, medial prefrontal cortex; NAc, nucleus accumbens;
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Methods

Animals and drug

Male Sprague-Dawley rats (Charles River, Spain) weighing 250-300 g were used. They were housed two to three per cage at 22 ± 1 °C with an humidity of 50-55%, food and water *ad libitum* and under a normal light/dark cycle (lights on for 12 h starting at 8:00 AM). After surgery, they were individually housed in order to avoid damaging of the cannula guide implant. Pure racemic Meph hydrochloride was synthesized and characterized in our laboratory as described previously (López-Arnau et al. 2012).

Microdialysis experiments

The microdialysis experiments were carried out on awake rats ($n= 3-4$ /group) according to the protocol described by Kehr et al., (2011), with some modifications. An intracerebral guide cannula (Agntho's, Lidingö, Sweden) was surgically implanted in rats at the NAc (2.2 mm anterior to bregma (AP), 1.6 mm lateral (L) and 6.0 mm ventral to the dura surface (V)) or mPFC (3.2 mm (AP), 0.5 mm (L) and 1.6 mm (V)). Rats were allowed at least one week for recovery from surgery. On the evening before an experiment, a microdialysis probe (Agntho's, Lidingö, Sweden; 2 or 3 mm membrane length with 15000 Da cut-off) was inserted into the guide cannula and perfused overnight with artificial cerebrospinal fluid solution (148 mM NaCl, 2.2 mM CaCl₂, 0.8 mM MgCl₂, 1.2 mM Na₂HPO₄ and 0.3 mM NaH₂PO₄) at a flow rate of 0.6 µL/min. On the next day, after a stabilization period of 2 h, microdialysis samples were collected at 20 min intervals (flow 1 µL/min). The first three samples were used for estimation of basal levels of DA, 5-HT, DOPAC, HVA and 5-HIAA. Thereafter, saline, Meph (25 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 180 min and stored at -80°C before analysis. At the end of the experiments, the animals were perfused with paraformaldehyde and examined for correct placement of the probe. Only the data from those rats with correct probe

1 placements were included in the study. Of the 32 rats that underwent a successful
2 surgical process and were tested in microdialysis experiments, 4 were excluded due to
3 missed cannula placement.
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7 The dose of Meph (25 mg/kg) was chosen according to a previous work showing
8 powerful psychostimulant effects of this drug (Martinez-Clemente et al. 2013) and,
9 although it may model an acute consumption of Meph in humans (López-Arnau et al.
10 2015), previous microdialysis assays only studied the effect of lower doses (Baumann
11 et al. 2012; Golembiowska et al. 2016; Kehr et al. 2011; Wright et al. 2012). The dose
12 of EtOH (1 g/kg) is a low/moderate dose that does not produce marked behavioral
13 effects when administered once for the first time (Imperato and Di Chiara 1986;
14 Brabant et al. 2014) and when administered to rats weighing 250-300 g leads to a
15 blood ethanol concentration (BEC) of around 0.6 g/L (Bloom et al. 1982). In humans,
16 this BEC can easily be reached after a moderate recreational consumption.
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30 *LC-MS/MS determination of DA, 5-HT and metabolites in dialysate samples*

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33 An Agilent 1290 Liquid Chromatography (LC) system equipped with an autosampler
34 and coupled to AB Sciex QTRAP 6500 mass spectrometer (MS) was used to quantify
35 DA, 5-HT and metabolites. Chromatographic separation was achieved in a Discovery
36 HS F5 (150 mm x 4 mm, 3 µm, Sigma-Aldrich, St. Louis, MO, USA) pentafluorophenyl
37 column thermostated at 37 °C. The mobile phase was water (A) and methanol (B) with
38 0.1% of formic acid in both solvents. An increasing linear gradient (v/v) of B was used
39 (t(min), %B), as follows, (0, 0), (0.5, 0), (5.90, 30), (6, 100), (9, 100), (9.10, 0), (10.0, 0)
40 at a constant flow rate (500 µl/min). The flow was directed to waste for the first 2 min to
41 prevent the inorganic ions of aCSF solution to enter the mass spectrometer. The
42 microdialysate samples were refrigerated at 4 °C and 20 µL was injected, without
43 sample pretreatment, into the LC-MS/MS system. Mass spectrometric quantification in
44 positive ion mode was carried out using the following transitions: DA (m/z 154 → 137
45 and 154 → 91, collision energies (CE) of 15 and 31 V, respectively), DOPAC (m/z 123
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→ 77, CE of 24 V), 5-HT (m/z 177 → 160, CE of 13 V) and 5-HIAA (m/z 192 → 146, CE of 23 V). A negative ion mode was used in the analysis of HVA (m/z 181 → 122, CE of -20 V).

Locomotor activity recording

The locomotor responses induced by Meph (25 mg/kg, s.c.), EtOH (1 g/kg, i.p.) and their combination were assessed in black Plexiglass open field arenas (l x w x h: 45 x 45 x 40 cm) under low-light conditions. Two days before testing, the animals were handled for 10 min, administered saline (1 ml/kg) and placed in the arena for habituation. The test day, the rats were administered with the assigned treatment, placed in the arenas and their horizontal travelling were video-monitored by a zenithal video-camera coupled to a computer running a tracking software (Smart 3.0, Panlab, S.L.U., Barcelona, Spain) for 90 min. Both cumulative distances in 10-min blocks and total travelled distances were obtained.

Data analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). All statistical calculations were performed using InVivoStat software (<http://invivostat.co.uk/>). The power of the analysis was assessed for all determined monoamines and areas resulted higher than 95% for an n value of 3-4 animals per group. The temporal evolution of monoamine levels and locomotor activities were analyzed performing a 3-way ANOVA for repeated measures with between-subjects variables Meph and EtOH, and a within-subjects variable "time". Differences among AUCs of monoamine levels in dialysates and among total distances travelled were assessed through 2-way ANOVA, with Meph and EtOH as variables. The α error probability was set at 0.05. Significant differences were analyzed using a multiple comparison adjustment of p values (Bonferroni and Tukey's post hoc tests for three-way and two-way ANOVA respectively). Graphs and

calculations of AUCs were performed using GraphPAD Prism 6.00 (GraphPAD Software, La Jolla, California. USA, www.graphpad.com).

Results

Effects on 5-HT and DA release

Administration of Meph alone (25 mg/kg, s.c.) produced an increase of both 5-HT and DA in the NAc (Fig. 1a and 1b) and the mPFC (Fig. 1c and 1d) which peaked 40 min after the injections and declined until around 180 min, with the exception of mPFC, where dialysate DA levels were still increased at this time point (Fig 1d). In NAc, the release of 5-HT was much higher (around 10000-15000%) than that of DA (around 5000-6000%), whereas in mPFC the increases of both 5-HT and DA were in a similar range (3000-4000%).

As can be seen in Figs. 1a, 1c, 2a and 2c, ethanol combined with Meph provoked an increase in released 5-HT compared with the rats receiving Meph alone (85% in NAc, 65% in mPFC; % of AUC higher than Meph alone), although the overall change in AUC only reached statistical significance in the NAc ($P < 0.01$). When analyzing the 5-HT values along time, 3-way ANOVA showed significant effect of Meph treatment and interaction between Meph and time in both NAc and mPFC. Moreover, the 3-way ANOVA revealed a significant interaction between Meph and EtOH in NAc ($F_{1,10} = 9.37$, $P = 0.01$, Fig 1a). Post-hoc analyses reported that ethanol potentiated the serotonergic increase induced by Meph along the time points between 20 and 120 min in NAc (Fig. 1a) and at 60 min in the mPFC (Fig 1c). In the latter region, the significantly increased release of 5-HT with respect to saline lasted longer in the combination than in the Meph group (120 min vs. 60 min).

Similarly to 5-HT, DA time-course values in NAc showed significant effects of Meph treatment and time factors (Fig 1b). DA levels showed a trend to be potentiated by

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coadministration of EtOH in NAc. In fact, post-hoc comparisons revealed a significant difference between Meph and Meph+EtOH groups at 60 min postadministration ($3732 \pm 291\%$ release in Meph group ; $5182 \pm 532\%$ in Meph+EtOH group). However, when considering the total DA released during 180 min, analysis of AUC reported an increase of 38%, that did not reach statistical significance (Fig. 2b).

In mPFC, although 3-way ANOVA revealed effect of time and Meph treatment on DA levels in Meph and Meph+EtOH groups, no differences were observed between them (Figs 1d and 2c).

EtOH alone, at the dose we used, had no significant effect in both DA and 5-HT basal levels in the studied brain areas.

Effects on 5-HT and DA metabolites

The levels of 5-HIAA showed a slight and non-significant increase (by 10%) in the EtOH-treated rats (Fig. 2b and 2d). Conversely, 5-HIAA values were significantly decreased by 25% in the rats receiving Meph alone. The 2-way ANOVA analysis revealed a significant interaction between Meph and EtOH in both NAc and mPFC, suggesting that EtOH potentiates the decrease in 5-HIAA induced by Meph (NAc: $F_{1,10} = 5.57$, $P < 0.05$; mPFC: $F_{1,10} = 3.29$, $P < 0.05$).

Effects on locomotor activity

Meph administration significantly increased locomotor activity with respect to saline and EtOH (Fig 3). Moreover, when Meph was combined with EtOH the increase in locomotion was significantly potentiated with respect to Meph alone and 3-way ANOVA revealed a significant interaction between EtOH and Meph ($F_{1,20} = 7.78$; $P = 0.01$). The potentiation was apparent in several time points and in the total travelled distance as well.

Discussion

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3 Alcoholic drinks are frequently combined with the new psychostimulant substances
4 such as Meph (Elliott and Evans, 2014). Alcohol enhances the subjective effects of
5 other drugs of abuse such as MDMA, and studies have shown that it increases its
6 rewarding and psychostimulant effects (Ben Hamida et al. 2009). Similarly, our group
7 reported that alcohol increases Meph-induced conditioned place preference and
8 psychostimulant properties in mice (Ciudad-Roberts et al. 2015).

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16 These previous results led us to perform microdialysis studies to assess the effects of
17 such combination on the release of DA and 5-HT in two key areas involved in drug-
18 induced behavior, namely the NAc and mPFC. Release of DA in the NAc is a key
19 process related with the reinforcing and rewarding properties of a drug (Nestler 2001)
20 and this area projects to other regions which are directly related with drug-induced
21 behavior such as the mPFC. For instance, the mPFC is involved in the establishment
22 of motor (Dalley et al. 2004) and amphetamine-induced (Hammerslag et al. 2014)
23 impulsivity.

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35 We found an increased release of neurotransmitters after administration of Meph in
36 both studied brain areas, being the increases of 5-HT much higher than those of DA,
37 which is in agreement with previous reports (Golembiowska et al. 2016; Kehr et al.
38 2011; Wright et al. 2012). Nevertheless the increase in DA (peaking around 4000%)
39 was more than sufficient to account for the rewarding effects of the drug. The
40 percentages of increase in neurotransmitters release are over the double than those
41 reported by Wright et al. (2012) in rats administered with 10 mg/kg of Meph (peaks of
42 around 1000% for DA and 2200% for 5-HT in the NAc) which indicates a clear dose-
43 response relationship.

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56 The overall 3-way ANOVA of DA levels along time did not reveal a significant
57 interaction between Meph and EtOH in any of the studied brain areas. However, in
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1 NAc, a tendence to potentiation of DA release was observed in the Meph + EtOH
2 group with respect to Meph alone between 20 and 100 min post-administration. In fact,
3 the post-hoc test showed a significant difference at 60 min. This slight increase might
4 account for increased rewarding effects. Previous experiments in mice point in this
5 direction (Ciudad-Roberts et al. 2015). By contrast, we found a significant potentiation
6 of Meph-induced 5-HT release by EtOH in NAc. The increase in 5-HT was much higher
7 than that of DA and the addition of EtOH did not change this proportionality, which
8 contrasts with the work by Riegert et al. (2008) using superfused striatal slices and
9 reporting that addition of EtOH shifted the MDMA-induced monoamine overflow
10 towards higher DA release. This difference could be mainly explained by the different
11 inhibition profile of Meph and MDMA at DA and 5-HT transporters: Meph shows IC₅₀ for
12 DA and 5-HT uptake inhibition of the same order of magnitude, whereas MDMA shows
13 a much higher potency inhibiting 5-HT than DA uptake (Hadlock et al. 2011). Also the
14 examined areas (striatum vs. NAc), doses and the different techniques may partially
15 contribute to such difference. In mPFC, the overall interaction between Meph and EtOH
16 did not reach statistical significance, although the post-hoc analysis revealed a
17 significant potentiation of 5-HT release by combination with EtOH at the 60 min time
18 point.

19 The effects of EtOH are multiple and complex (reviewed by Clapp et al. 2008 and
20 Siggins et al. 2005), including increased release of DA in the NAc. However, at the
21 dose we used, EtOH alone had few or no significant effect on neurotransmitter levels,
22 suggesting that the effect in animals receiving the drug combination are due to a
23 synergistic pharmacodynamic or pharmacokinetic interaction rather than to a simple
24 addition. A number of interactions of EtOH with other drugs have been reported. For
25 example, Ben Hamida et al. (2009) demonstrated that ethanol is capable of increasing
26 the concentration of MDMA in areas with high DA transmission (striatum and frontal
27 cortex) in a much higher proportion than in the hippocampus. Although no mechanistic

1 description was found for this effect, we cannot rule out a similar pharmacokinetic
2 interaction between ethanol and Meph **in the brain areas we studied.**

3
4 **The increase in** DA and 5-HT in the rats receiving Meph was accompanied by a
5 decrease in the levels of their metabolites. This can be attributed to monoamine uptake
6 inhibition by Meph (Baumann et al., 2012, López-Arnau et al., 2012) because the key
7 enzyme in the metabolism of DA and 5-HT, namely monoaminoxidase (MAO), is
8 localized inside the nerve terminal, so that monoamines have to be taken up to be
9 metabolized. As Meph blocks reuptake, a decrease in metabolites is detected despite
10 the increase in monoamine release. A similar effect was described by Kehr et al.
11 (2011). This mechanism is backed by the fact that EtOH alone, which induces DA
12 release but does not inhibit uptake, does not produce a decrease, but rather tends to
13 increase the metabolites. **Statistical analysis revealed a significant interaction between**
14 **Meph and EtOH treatment with respect to 5-HIAA levels, suggesting that Meph exerts**
15 **a higher blockade of the transporter in presence of EtOH. A direct effect of EtOH on**
16 **serotonin transporter can be ruled out because a decrease in 5-HIAA levels should**
17 **have been shown in the EtOH group (Daws et al. 2006).** Therefore, this is in agreement
18 with the possibility of a pharmacokinetic interaction **beforehand mentioned**, leading to
19 increased interstitial levels of Meph that would further prevent 5-HT reuptake and
20 metabolism. Additional studies need to be performed to corroborate this hypothesis.
21 Similar results were described by Cassel et al. (2005) when studying the effects of
22 MDMA combined with EtOH on monoamine levels and their metabolites.

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24 **To assess whether the observed potentiation of neurotransmitter release in the**
25 **Meph+EtOH group had a measurable effect on the psychostimulant properties of**
26 **Meph, we performed locomotor activity experiments in rats using the same doses and**
27 **combinations that we did in the microdialysis assays. The results demonstrated that the**
28 **combination of EtOH with Meph potentiates the psychostimulant effects of the**
29 **cathinone. Moreover, the higher hyperlocomotion periodes coincide with the peaks in**

1 neurotransmitters. The observed increases in 5-HT-release could be responsible of
2 such potentiation of locomotor activity. In fact, previous works demonstrate a role of 5-
3 HT receptors on Meph-induced hyperlocomotion as it was reduced by administration of
4 ketanserin (López-Arnau et al. 2012; Ciudad-Roberts et al. 2015). Moreover, elevated
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6 5-HT release in the mPFC positively relates with motor impulsivity, which is related with
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8 drug relapse (Dalley et al., 2002; Hammerslag et al., 2014).
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11 To sum up, in this work we demonstrate a potentiation of the increase in monoamines
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13 release and in psychostimulant effects when combining Meph plus EtOH which might
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15 involve increased psychostimulant subjective effects and therefore increased abuse
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17 liability. Therefore an experimental-based warning concerning the risks regarding the
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19 combined consumption of these drugs could be conveyed to the population at large.
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50 **Compliance with ethical standards**

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53 All animal care and experimental protocols in this study complied with the guidelines of
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55 the European Community Council (86/609/ECC) and were approved by the Animal
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57 Ethics Committee of the University of Barcelona. All efforts were made to minimize
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59 animal suffering and to reduce the number of animals used.
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Conflict of interest

The authors declare that they have no conflict of interest.

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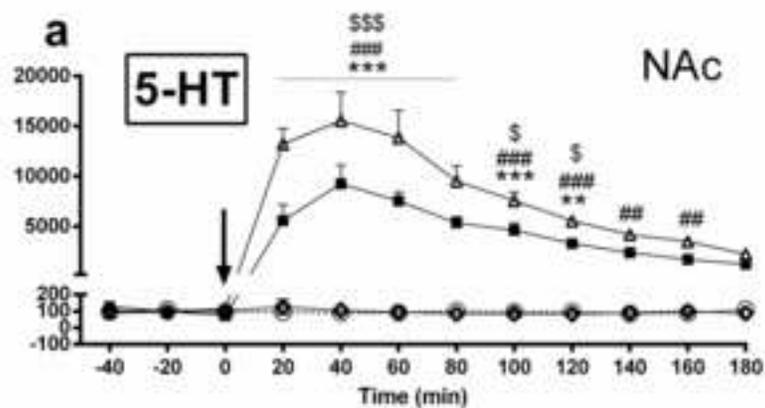
Figure captions

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3 **Fig. 1** Effects of saline, ethanol (EtOH, 1 g/kg, i.p.), mephedrone (Meph, 25 mg/kg,
4 s.c.) and their combination on NAc (a, b) and mPFC (c, d) 5-HT and DA levels,
5 expressed as a percentage of baseline neurotransmitter levels. All points show mean \pm
6 S.E.M. from 3-4 rats. Dialysate samples were collected every 20 min. The arrows show
7 when drugs were injected. The relevant results of 3-way ANOVA are included in the
8 table below each panel. Post-hoc test results are indicated on the graphs. ***P<0.001,
9 **P<0.01, *P<0.05 Meph vs. saline group; ###P<0.001, ##P<0.01, #P<0.05 Meph+EtOH
10 vs. saline group; \$\$\$P<0.001, \$\$P<0.01, \$P<0.05 Meph+EtOH vs. Meph group. The
11 tracings from saline and EtOH groups practically overlap
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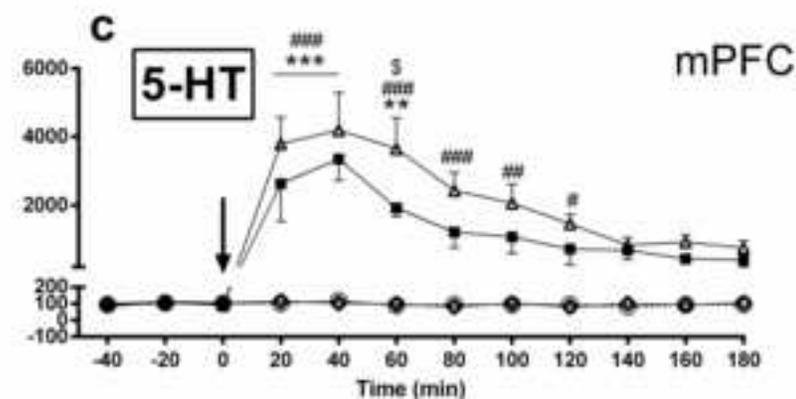
14 **Fig. 2** Effects of saline, ethanol (EtOH, 1 g/kg, i.p.), mephedrone (Meph, 25 mg/kg,
15 s.c.) and their combination on the levels of 5-HT, DA and their metabolites (5-HIAA and
16 DOPAC, respectively) in NAc (a, b) and mPFC (c, d). Levels are expressed as the area
17 under the curve comprised between the time points 0 and 180 min. All the bars show
18 mean \pm S.E.M. from 3-4 rats. The relevant results of 2-way ANOVA are included in the
19 table below each panel. Post-hoc test results are indicated on the graphs. ***P<0.001,
20 **P<0.01, *P<0.05 vs. saline group; @@@P<0.001, @@P<0.01, @P<0.05 vs. EtOH group;
21 ##P<0.01 between the indicated groups
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23

24 **Fig. 3** Effects of saline, ethanol (EtOH, 1 g/kg, i.p.), mephedrone (Meph, 25 mg/kg,
25 s.c.) and their combination on horizontal locomotor activity in rats. Travelled distances
26 were recorded in 10 min blocks and depicted in panel a, whereas total distances are
27 depicted in panel b. All points show mean \pm S.E.M. from 6 rats per group. The relevant
28 results of ANOVAs are included in the table below each panel. Post-hoc test results are
29 indicated on the graphs. **P<0.01, *P<0.05 Meph vs. saline group; ###P<0.001,
30 ##P<0.01, #P<0.05 Meph+EtOH vs. saline group; \$\$\$P<0.001, \$\$P<0.01, \$P<0.05
31 Meph+EtOH vs. Meph group.
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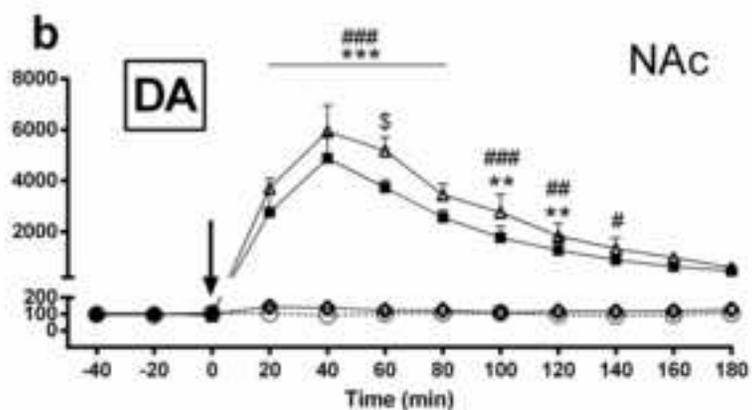
% Baseline neurotransmitter



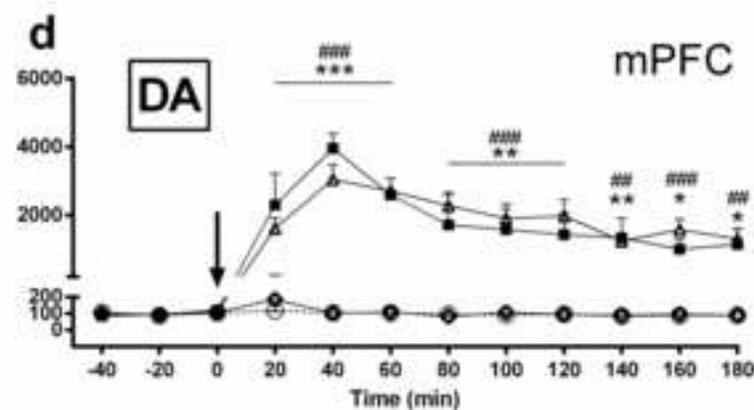
FACTOR	Meph	Meph * time	EtOH * Meph	EtOH * Meph * time
3-way ANOVA analysis	$F_{1,30} = 104.65$; $P < 0.001$	$F_{8,30} = 42.75$; $P < 0.001$	$F_{1,30} = 9.37$; $P = 0.01$	$F_{8,30} = 4.18$; $P > 0.05$



FACTOR	Meph	Meph * time	EtOH * Meph	EtOH * Meph * time
3-way ANOVA analysis	$F_{1,30} = 35.12$; $P < 0.001$	$F_{8,30} = 29.48$; $P < 0.001$	$F_{1,30} = 2.16$; $P > 0.05$	$F_{8,30} = 0.91$; $P > 0.05$



FACTOR	Meph	Meph * time	EtOH * Meph	EtOH * Meph * time
3-way ANOVA analysis	$F_{1,30} = 73.77$; $P < 0.001$	$F_{8,30} = 53.75$; $P < 0.001$	$F_{1,30} = 1.77$; $P > 0.05$	$F_{8,30} = 0.9$; $P > 0.05$



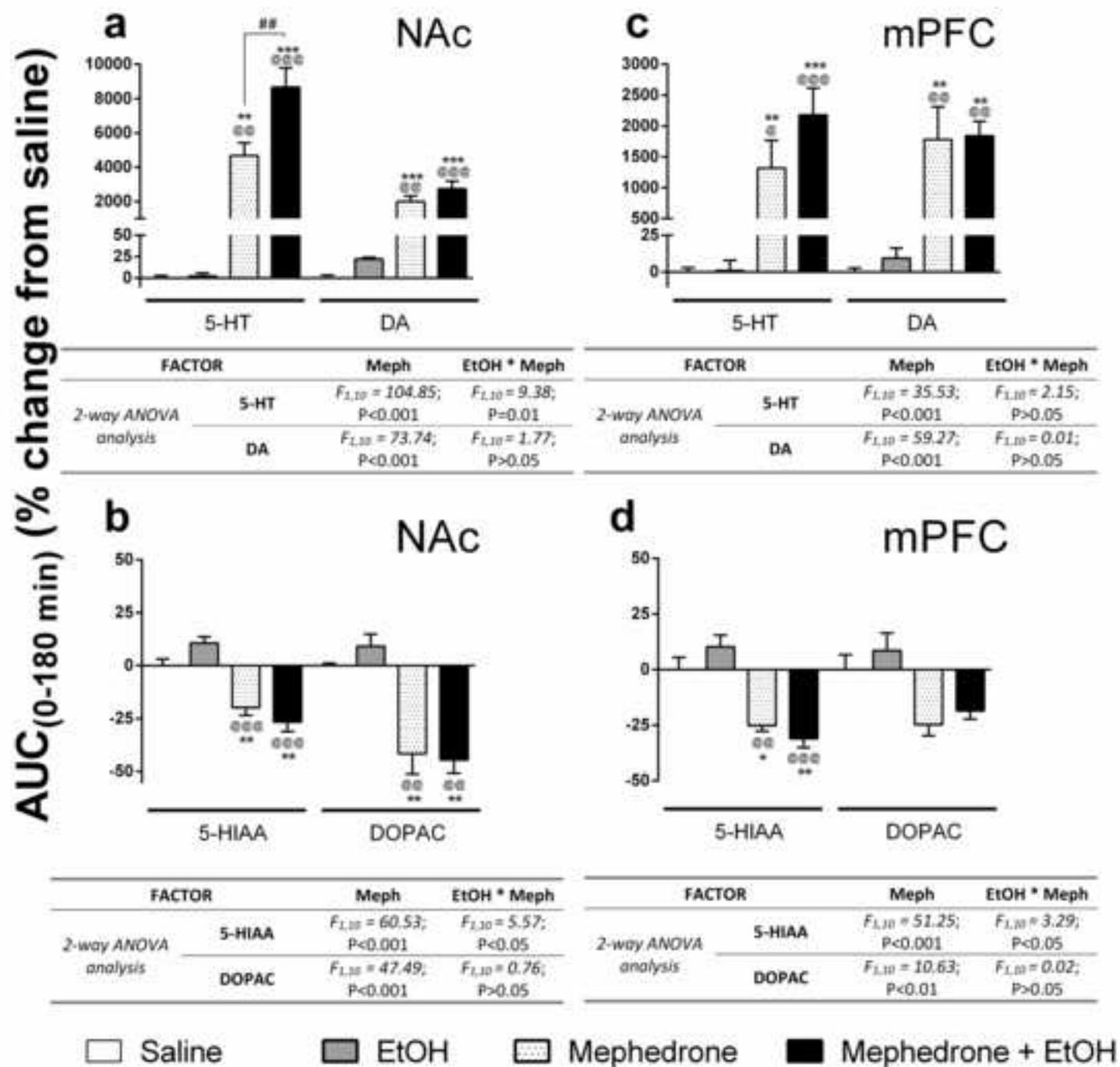
FACTOR	Meph	Meph * time	EtOH * Meph	EtOH * Meph * time
3-way ANOVA analysis	$F_{1,30} = 57.2$; $P < 0.001$	$F_{8,30} = 17.67$; $P < 0.001$	$F_{1,30} = 0.01$; $P > 0.05$	$F_{8,30} = 0.18$; $P > 0.05$

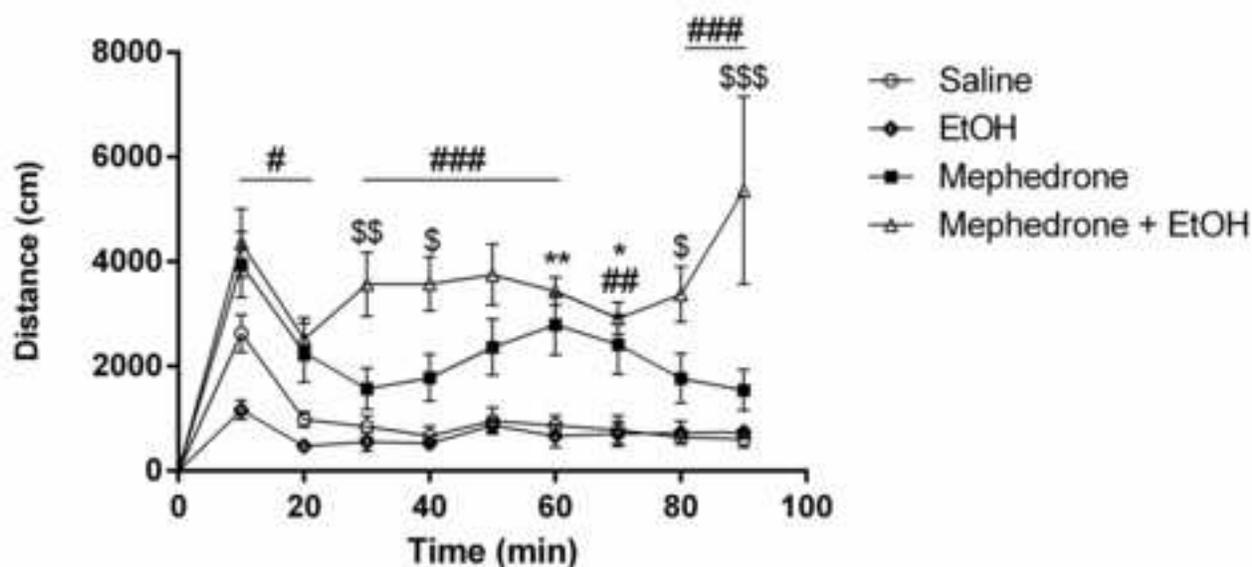
○ Saline

◆ EtOH

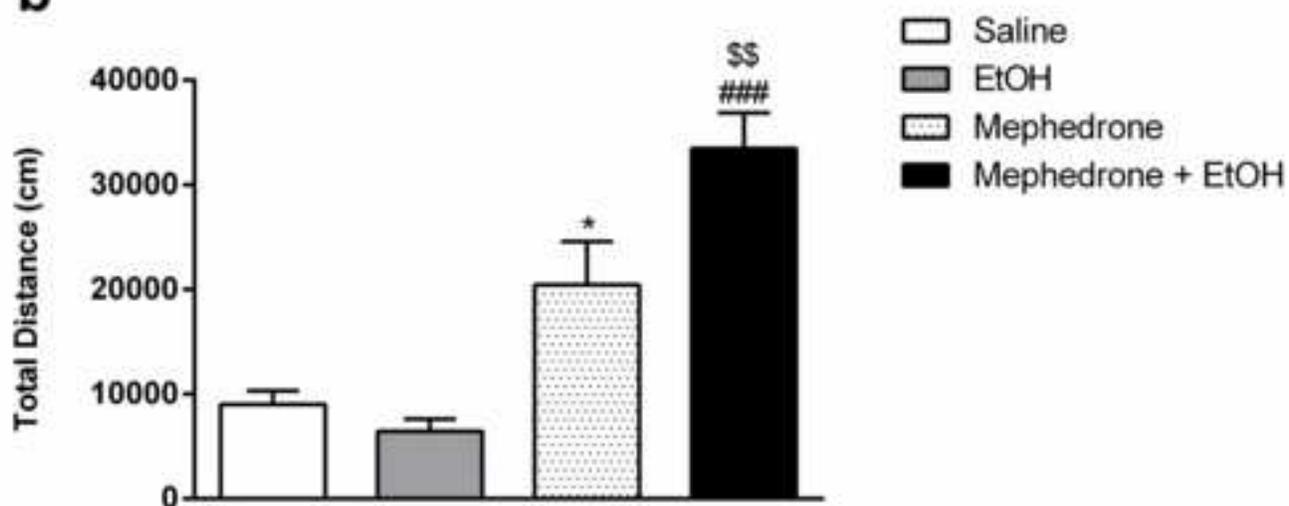
■ Mephedrone

△ Mephedrone + EtOH



a

FACTOR	Meph	Meph * time	EtOH * Meph	EtOH * Meph * time
3-way ANOVA analysis	$F_{1,20} = 49.77;$ $P < 0.001$	$F_{8,160} = 0.68;$ $P > 0.05$	$F_{1,20} = 7.78;$ $P = 0.01$	$F_{8,160} = 1.44;$ $P > 0.05$

b

FACTOR	Meph	EtOH	EtOH * Meph
2-way ANOVA analysis	$F_{1,20} = 46.69;$ $P < 0.001$	$F_{1,20} = 3.44;$ $P > 0.05$	$F_{1,20} = 7.65;$ $P = 0.01$