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7. Neuropsychopharmacologic and neurotoxicologic effects of the combination of ethanol with mephedrone in adolescent mice

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Abstract. In the last decade, a new family of synthetic psychostimulant drugs, under the name of cathinones, broke into the market. These drugs are mainly consumed by adolescents and young adults with recreational purposes, in most cases combined with alcoholic drinks. Although a number of works about new cathinones have been recently published, none explored the consequences of such combination. Because adolescence is a crucial period in brain development, we sought to study the effects of the combination of mephedrone plus ethanol in adolescent mice, focusing on psychostimulant and conditioning effects, as well as on neurotoxicity markers. Ethanol increased both locomotor activity and conditioned place preference (CPP) induced by mephedrone. RNA microarray assays after CPP test yielded significant alterations

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in neuronal plasticity-related genes and a key role of BDNF and dopamine D3 receptors in CPP acquisition was found. Ethanol potentiated the oxidative stress as well as the decreases in dopaminergic and serotonergic markers in frontal cortex and hippocampus respectively, after a binge treatment with mephedrone. Moreover, the drug combination impaired spatial learning and memory, as well as neurogenesis to a higher extent than mephedrone alone.

Introduction

Drug abuse is a matter of concern at all life stages but its occurrence at earlier ages is especially worrisome, as it can determine the social outcome of an individual [1]. While adolescence is a crucial stage in brain maturation, experimentation with alcohol and other drugs is common; teenagers are not aware of the risks they are taking, as the regions of the brain that control impulses are still immature. Substance use during adolescence has been associated with alterations in brain structure, function, and neurocognition (reviewed by [2]). Moreover, it has been reported in studies with humans that drug consumption during adolescence increases the likelihood of drug abuse in adulthood [3]. Specifically, alterations in the prefrontal regions and limbic systems are thought to contribute to increased risk-taking and novelty/sensation seeking behaviors [4-6].

Currently, most drug use during adolescence occurs in leisure environments, such as dance clubs and parties [7]. Alcohol is omnipresent due to its legal drug status [8] while other drugs such as cannabis, cocaine, and amphetamine derivatives are often associated with it [9].

Moreover, adolescents are less sensitive than adults to the depressant effects of alcohol, as well as to the subsequent hangover (reviewed by [10]), which facilitates the intake of higher amounts. Numerous studies report neurotoxic effects of alcohol itself in consumption models using adolescent rodents (reviewed by [11]), mainly leading to impairment in memory and visual and verbal tasks [12]. Excitotoxicity and neuroinflammation seem to be involved in such deleterious effects [13].

During the last decade, a miriad of new designer drugs broke into the market. These substances structurally differred from existing banned drugs and took profit of this legal loophole to be sold through licit media such as the Internet, smart shops, gas stations, etc., always with the disclaimer “not for human use” and packaged as “bath salts”, “plant food” or “research chemical”. For this reason, these substances where generically called “legal highs”. Some of them are currently banned in many countries, but

the pace at which new substances appear exceeds that of the legal machinery to illegalize them [14].

Among these new substances, mephedrone (4-methylmethcathinone, Meph) is an increasingly consumed synthetic psychostimulant compound, which first appeared for sale on the Internet around 2007. It belongs to the β-ketoamphetamines group (Fig. 1), also known as cathinones, and is commonly taken orally or insufflated [15]. Preclinical studies have shown that mephedrone stimulates the release of dopamine (DA), serotonin (5-HT) and norepinephrine and inhibits their re-uptake in the CNS [16-19]. These actions explain the psychostimulation and the effects on perceptions reported by human consumers [20]. Experiments in rodents have demonstrated the psychostimulant (measured as hyperlocomotion) and rewarding (measured as conditioned place preference, CPP) effects of mephedrone, which are indicative of its abuse liability [18,21].

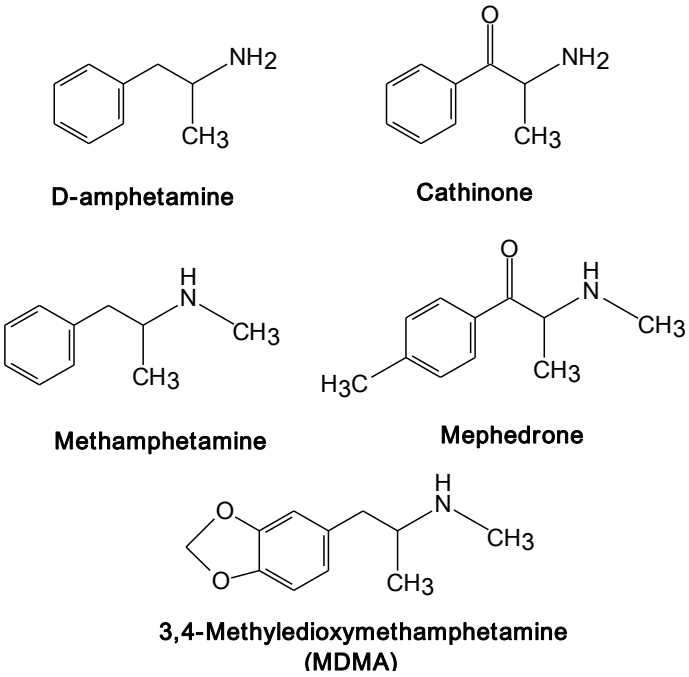

Figure 1. Chemical structures of classic amphetamine derivatives, (D-amphetamine, methamphetamine and MDMA) cathinone and mephedrone. Please notice the β-keto group in both cathinones.

Mephedrone is also commonly combined with many other drugs, but mainly alcohol [9,22] which, in turn, is the most consumed drug. The study of the interactions between drugs of abuse is of interest because potentiation of the effects could result in increased abuse liability.

In fact, ethanol can effectively potentiate the psychostimulant and rewarding effects of another widely abused amphetamine derivative, 3,4-methylenedioxy-methamphetamine (MDMA) in rodents [23] by a combination of both pharmacokinetic and pharmacodynamic interactions. Given the resemblance between mephedrone's mechanism of action and that of MDMA [9,18,24], a similar profile should be expected when combined with alcohol.

Moreover, interaction between drugs could also result in increased deleterious effects. Hernández-Rabaza *et al.* [25] described that combination of ethanol with MDMA produces cognitive impairment in adolescent rats at doses that do not when administered alone. This impairment is accompanied by a decrease in survival of neuronal precursor cells as well as a decrease in the presence of mature cells in the dentate gyrus (DG) of the hippocampus. Furthermore, Izco *et al.* [26] found that ethanol potentiates MDMA neurotoxicity through the production of hydroxyl radicals.

These antecedents justify the need for studies about the pharmacological and neurotoxicological effects of the combination of mephedrone plus ethanol. In this chapter we summarize the works we performed on this subject using adolescent mice, owing to the prevalent consumption of these drugs in this population group and the reasons explained above. Results show increased psychostimulant and rewarding effects of the combination, as well as potentiation of the neurotoxicity markers and the impaired learning and memory. Such preclinical evidences deserve to be investigated in humans in order to, if also occurred in this species, transmit a pertinent warning to the population.

1. Psychostimulant effects: Locomotor activity

In rodents, psychostimulants produce increased locomotor activity; therefore measurement of locomotion is a widely employed technique to study the acute behavioural effects of new drugs such as mephedrone. The technique consists of placing the animals in a cage equipped with infrared photocells so that ambulation produces occlusions of the photo beams, which are recorded and sent to a computerized system. The more

interruption counts (measured over blocks of 10 min during 120 min or longer), the higher locomotor activity. Previous works had demonstrated that mephedrone induces robust hyperlocomotion [16,18,21] after a single injection of 5-25 mg/kg. To assess the effect of the combination with ethanol, mice were administered with mephedrone (10 or 25 mg/kg, s.c.) alone or combined with ethanol and immediately placed in the activity box. Since ethanol, at certain doses, can modify locomotion, it was administered at doses reported to not affect basal activity (0.5 or 1 g/kg, s.c.; [27,28]).

As can be seen in Fig. 2A, the hyperlocomotion induced by mephedrone was significantly increased when administered concominantly with ethanol [29]. In order to investigate the neurotransmitter responsible for such potentiation, the experiments were repeated administering previously ketanserin (a serotonin 5-HT_2 receptor antagonist) or haloperidol (a dopaminergic antagonist with predominant D_2 activity). The result showed that, although expectedly both antagonists reduced locomotor activity, only haloperidol prevented the locomotor enhancement of the drug combination, indicating that this occurs through a dopaminergic mechanism.

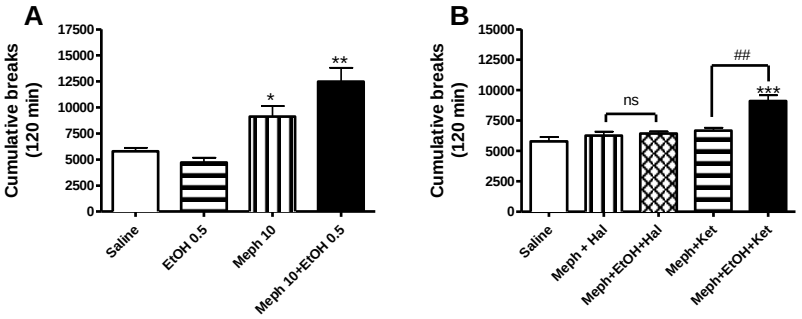

Figure 2. A. Locomotor activity of adolescent CD-1 mice treated with ethanol (EtOH, 0.5 g/kg, s.c.), mephedrone (Meph, 10 mg/kg, s.c.) or their combination, expressed as the cumulative interruptions of the infrared beams (breaks) in the activity cage during 120 min. B. Effects of the 5-HT_2 antagonist ketanserin (Ket, 1 mg/kg, i.p.) and D_2 antagonist haloperidol (Hal, 0.25 mg/kg), on the hyperlocomotion induced by mephedrone (10 mg/kg) and its combination with ethanol. Although both antagonists reduced hyperlocomotion, only haloperidol abolished the potentiation by ethanol. *P<0.05, **P<0.01, ***P<0.001 *vs.* saline; ##P<0.01 between the indicated groups; ns, non significant differences; one-way ANOVA. Graphs modified from [29].

The effects of ethanol in the brain are numerous as it easily crosses biological membranes and interacts 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 DA release in other areas of mesocortical pathways (see [30] for a review). These mechanisms are different from those of mephedrone but, in turn, would converge in increased DA release and/or desinhibition in certain brain areas, which could explain the observed increased effect.

2. Rewarding effects: conditioned place preference (CPP) and associated transcriptional changes

A rewarding stimulus is defined as a stimulus that is considered likeable and thus is worthy of being desired and pursued [31]. Rewards (both natural and exogenous) trigger two important biological processes:

- Assignment of a hedonic value. This is defined as how much the reward is "pleasurable" or "liked".
- Assignment of an incentive salience, which is defined as a motivational value or "wanting" or a given rewarding stimulus [32].

This distinction is important, since rewarding stimuli modulate behavior through an increase in dopamine in the nucleus accumbens (NAc), whereas dopamine is not a mediator of the hedonic state elicited by a rewarding stimulus [33]. Therefore, the rewarding effects can be indicative of its abuse liability.

The CPP test is performed in an apparatus composed of three distinct areas (two well distinguished compartments communicated by a central corridor) separated by manually operated doors [34]. The procedure is performed in three phases: pre-conditioning, conditioning and post-conditioning test. During the pre-conditioning phase (day 1), the mice are placed in the middle of the corridor and have free access and roam among the compartments of the apparatus. The time spent in each compartment is recorded through a zenithal camera and computerized tracking software. During the conditioning phase, mice are randomly assigned to receive the drug in one of the compartments and the vehicle in the opposite so that they receive in alternate days the drug or the vehicle (4 days each

treatment), and are confined to the assigned compartment for 30 min. In the post-conditioning day, the mice are left to freely wander through both compartments, and the time spent in each one is also registered. If the drug induced reward, the mouse will prefer to stay in the drug-matched compartment, attempting to repeat the experience produced by the drug, so an increased time (preference score) with respect to that measured in day 1 will be recorded.

Results showed that the combination of ethanol, at a dose non conditioning on its own (0.75 g/kg), increased the preference score induced by mephedrone (Fig. 3), which could, in turn, indicate increased abuse liability.

The mesolimbic pathway is involved in the acquisition of CPP, so 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 [35]. For this reason, we aimed to characterize these changes by determining major transcriptional modifications in the ventral striatum (comprising the NAc) after completing the whole conditioning process, by means of RNA microarray assays.

A number of studies using the microarray approach with psychostimulants (mainly cocaine, methamphetamine and amphetamine) in rodents have been published (reviewed by [36]). More recently, similar studies have been carried out with alcohol [37] or heroin and methamphetamine [38]. 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 timepoint 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.

Results showed significant changes in mRNAs involved in neuronal plasticity [29], which is in line with CPP acquisition. These included Syt10 and Muted, which were only significantly increased in the groups receiving mephedrone; and Arpc5, whose expression was increased in all drug-treated groups and potentiated in the Meph+EtOH group. Its product, ARPC5, plays an important role in maintaining the ARP2/3 activity (see below). Syt10 encodes synaptotagmin 10, a calcium sensor involved in the regulation of neuron size and arborization [39]. Furthermore, the Muted gene codifies for a subunit of the BLOC-1 complex, which is involved in

the activation of ARP2/3 [40], whose complex nucleating capability is essential for actin remodeling and synaptic plasticity at a pre- and post-synaptic level [41,42]. 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. 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. Similarly, Camkk1, whose codified protein plays an important role in actin dynamics, was significantly up-regulated.

Moreover, the D_3 dopamine receptor gene (Drd3) was one of the most marked and similarly increased in all drug-treated animals. D_3 dopamine receptors (D_3Rs, see [43] for a review) are a subtype of D_2-like receptors with both pre- and postsynaptic locations, negatively coupled to adenilyl-cyclase and acting as autoreceptors modulating dopamine release and/or synthesis. D_3 receptors are known to be involved in reinforcement and reward induced by many drugs, including ethanol [44], cocaine [45, 46],

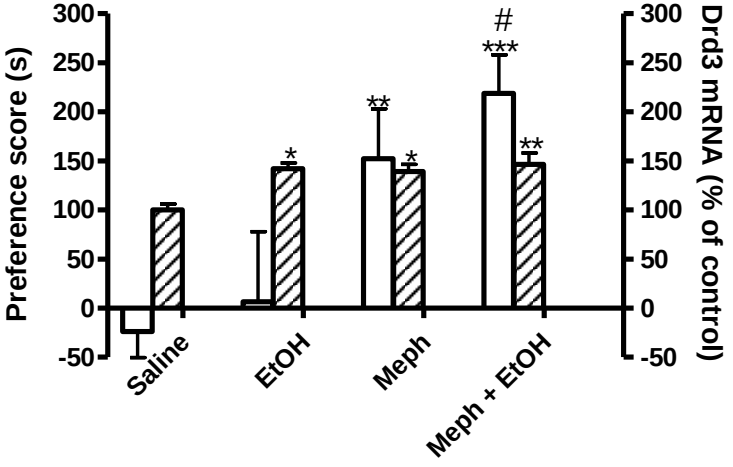

Figure 3. Place preference score (open bars, left axis) and Drd3 mRNA expression (dashed bars, right axis) in adolescent mice subjected to CPP being treated according to the schedule stated in the text with saline, ethanol (EtOH, 0.75 mg/kg) and mephedrone (Meph, 25 mg/kg) plus/minus ethanol. *P<0.05, **P<0.01, ***P<0.001 *vs*. saline; #P<0.05 *vs*. Meph.; one-way ANOVA. Data taken from [29].

morphine [47] and methamphetamine [48]. They are mainly localized in limbic brain regions, especially the nucleus accumbens [49]. Ethanol, morphine or cocaine, are also capable of upregulating Drd3 mRNA in rodents [50-52] and in human addicts [53].

Based on these antecedents, we tested whether blocking D_3 receptors with the D_3 antagonist SB-277011A affected CPP and Drd3 up-regulation induced by Meph and its combination with ethanol. The antagonist was given before the drugs each conditioning session, and was capable of completely blocking mephedrone-induced CPP and Drd3 mRNA up-regulation (Ref BJP). 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 but that other partners, probably among the other modified genes reported above, are needed.

However, due to the robust blockade obtained with the D_3 antagonist, we further explored the mechanisms involved in mephedrone-induced CPP and Drd3 up-regulation. BDNF controls dopamine D_3 receptor expression [54] and its levels are increased by psychostimulants [55]. In fact, BDNF and D_3 receptors share common pathways in their respective signalling cascades (reviewed by [56]). Furthermore, Le Foll *et al.* [51] described 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.

In order to assess a possible role of BDNF in the effects of mephedrone, we measured its mRNA in medial prefrontal cortex after an acute dose of this cathinone (25 mg/kg), finding a progressive increase along 4 h after the injection [29] up to two-fold. Further, the role of BDNF was pharmacologically confirmed by using ANA-12, a selective trkB (BDNF receptor) antagonist. When administered before the drugs each conditioning session, ANA-12 blocked both CPP and Drd3 up-regulation induced by mephedone. This confirms that D_3 receptor differential expression can be mediated by BDNF, and points to the fact that blocking their signalling can reduce the rewarding properties of mephedrone.

3. Dopaminergic and serotonergic toxicity

Neurotoxicity of amphetamine derivatives (i.e. methamphetamine, MDMA) is a matter of concern and has been subject of a great amount of

research. This led to undertake studies exploring a possible neurotoxic effect of new drugs such as mephedrone in rodents [57,58]. Reported research evidences the need to perform neurotoxicity assays under different administration schedules and controlled room temperature. Our group described mephedrone neurotoxicity using dosing schedules which agreed with mephedrone pharmacokinetics and exploring cerebral areas others than the striatum [59,60]. From these antecedents and in order to investigate the effects when combined with ethanol, we chose administering four doses of 25 mg/kg (s.c.) in one day, every two hours, given alone or combined with ethanol at changing doses calculated to cause blood ethanol concentration leveling around 1.5 g/L during the whole duration of the treatment [61]. Also, during the treatment, room temperature was set at 26 ± 2 ºC, at which mephedrone has been reported to induce signs of neurotoxicity [60], in order to reproduce the common hot conditions found in crowded dance clubs.

Decreases in the density of monoamine transporters and synthetic enzymes are characteristic markers of amphetamines' neurotoxicity. Thus, we measured the density of transporters by means of radioligand binding experiments and the enzymes by Western blotting in brain areas of mice 7 days after receiving the above treatment. The results showed that, as expected, mephedrone reduced the levels of dopamine transporters (DAT, binding of [^{3}H]WIN 35428) in the frontal cortex and of serotonin transporters (SERT, binding of [^{3}H]paroxetine) in the hippocampus (Fig. 4A, B), while DAT in the striatum and SERT in the frontal cortex were unaffected [61]. Accordingly, the levels of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) were reduced in frontal cortex and hippocampus, respectively. Moreover, the decreases were higher when mephedrone was combined with ethanol, which indicates that the combination potentiates the dopaminergic and serotonergic toxicities of these drugs.

4. Oxidative stress

Oxidative stress has been classically associated with amphetamines' neurotoxicity and previous evidence indicates that this also occurs with mephedrone. Therefore lipid peroxidation and oxidative stress-related enzymes were assessed in frontal cortex and hippocampus from mice sacrificed 24 h after receiving the above treatment.

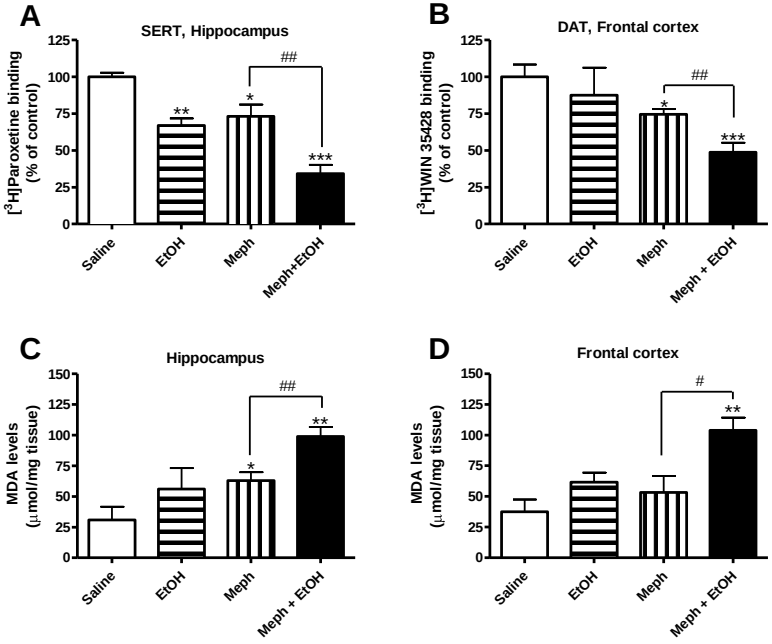

Figure 4. Assessment of the levels of serotonin (SERT) and dopamine (DAT) transporters (panels A and B) and lipid peroxidation measured as levels of malondialdehyde (MDA, panels C and D) in hippocampus and frontal cortex, respectively. Adolescent CD-1 mice were treated with either saline, ethanol, mephedrone, or their combination, following the schedule described above. Monoamine transporters were measured 7 days after the treatment, while MDA was determined in samples from mice killed 24 h after the last dose. Values represent means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline; #P < 0.05, ##P < 0.01 between the indicated groups. Data obtained from [61].

Lipid peroxidation was measured as a raise in the MDA levels, a general indicator of the decomposition of polyunsaturated fatty acids. Mephedrone alone only significantly increased MDA levels in the hippocampus. By contrast, the combination of mephedrone with ethanol caused higher and significant increases in the levels of MDA (Fig. 4B, C).

As far as antioxidant activity-related enzymes is concerned, glutathion peroxydase and catalase were significantly and similarly overexpressed (around two-fold increase) in both mephedrone-treated groups [61]. Regarding superoxide dismutase expression, there was also an increase

(around 85%), although statistically non-significant in both mephedrone-treated groups. This indicates that an antioxidant response is triggered after the treatment with mephedrone plus/minus ethanol, which is not further increased in the mephedrone+ethanol group. When comparing these with the lipid peroxidation data, it can be suggested that there is a potentiation in oxidative stress-related damage in the mephedrone+ethanol group, where the effects of the drug combination exceed the antioxidant response leading to increased effect of generated ROS.

5. Effects on learning and spatial memory

Neuronal oxidative stress and serotonergic impairment can have consequences on learning and memory, so we investigated the performance of mice in the Morris water maze seven days after receiving the treatments stated above [61]. The maze consists of a circular pool filled with water (22 ± 1º C) and rendered opaque by the addition of a non-toxic latex solution. The pool must be in an isolated room and black curtains are closed around it to suppress room cues [62]. Four positions around the edge of the tank are designated as north (N), south (S), east (E), and west (W) and also define the division of the tank into four quadrants: NE, SE, SW, and NW, providing alternative start positions. Four extra-maze distal cues are located equidistantly around the pool, labeling the N, S, E and W locations. A Plexiglas escape platform is submerged in the water so that it is not visible at the surface level.

The test measures the ability of each mouse to learn the position of the hidden platform (always the same) in relation to the distal cues, after several training sessions being placed in the water from an alternate semi-random set of start locations. The path taken by each mouse and the escape latency (the time needed by each mouse to find the platform, in s) is recorded by a zenithal video camera connected to a computer running a tracking software. Mice were trained throughout six days, receiving five trials per day [61]. A trial was started by placing the mouse in the desired start position of the pool, facing the tank wall. The mice were allowed to swim to the hidden platform, and the escape latency was determined. If an animal did not escape within 60 s, it was gently placed on the platform or guided to it. The mice were allowed to rest for 30 s (inter-trial interval) on the platform (even those that failed to locate it). Then, learning curves of the four treatment groups were plotted as the time-course evolution of the latency to reach the hidden platform. To assess reference memory at the

end of learning, a probe trial (free swimming without platform for 60 s), was given 24 h after the last training session. Different parameters of each mouse's performance are analyzed: the total time and distance spent swimming in each quadrant, entries in each quadrant and time elapsed (latency) until the mouse first reached the target zone (area where the platform was formerly located).

The learning curves of the four treatment groups (saline, mephedrone, ethanol and mephedrone+ethanol) showed that the mice pre-treated with saline or ethanol reduced the latency along the training days, which indicates learning, while those pre-treated with mephedrone and, moreover, those that received mephedrone+ethanol did not show such a progression, suggesting learning impairment [61].

As far as memory is concerned, in the probe trial, the mice that had received mephedrone and those pre-treated with the drug combination spent significantly less time than the saline and ethanol groups in the quadrant where the platform was located (Fig. 5), pointing to impaired memory. In this case, no significant differences were found between mephedrone and mephedrone+ethanol groups. As spatial memory is mainly attributable to hippocampal activity, the serotonergic impairment previously detected in the hippocampus could account for this memory affection.

6. Effects on hippocampal neurogenesis

The subgranular zone of the dentate gyrus (DG) of the hippocampus is one of the two regions in the adult brain containing neural stem cells that underlie adult neurogenesis [63,64]. It is currently accepted that hippocampal structure and function relies upon hippocampal stem cells and constitutive neurogenesis [65,66]. The thousands of new cells added daily to the DG suggest its role in hippocampal structure and/or function [67]. Neurogenesis consists of four main components: neural stem cells proliferation followed by newborn cell migration, differentiation, and survival.

It has been widely suggested that the generation of new neurons is implicated in correct learning and memory processes, including MWM performance in rodents [68]. Furthermore, neurotoxic processes are closely related to a decrease in cell proliferation and an increase in cell death. Serotonin input to the hippocampus positively regulates adult neurogenesis [69]. In this sense, serotonin reuptake inhibitors increase

hippocampal neurogenesis [70] while repeated exposure to high doses of MDMA, which produces serotonergic neurotoxicity, causes the opposite effect [71].

Neurogenesis from the granular layer of the DG is impaired following treatment with ethanol [72-74] and adolescents are more sensitive than adults to such effects [75]. Therefore, combination of alcohol with serotonergic amphetamines could account for an increased deleterious effect on neurogenesis. In fact, Hernández-Rabaza el al. [25] described that the cognitive impairment produced by the combination of MDMA with ethanol in adolescent rats was accompanied by a decrease in survival of neuronal precursor cells as well as a decrease in the presence of mature cells in the DG of the hippocampus.

With these antecedents, the effects of the combination of ethanol with mephedrone on neurogenesis deserved to be studied. For this reason, the mice that were tested in the MWM had received two injections of bromo-deoxyuridine (BrdU) 2 and 12 h after the last dose of treatment [61]. BrdU is a thymidine analog that is incorporated into cells in place of a thymine base pair as the cell undergoes DNA replication during the S phase of the mitotic cell cycle and is transmitted to the newly generated cells. BrdU can be labeled with specific antibodies so that it can be used as a measure of cell proliferation.

Twenty eight days after receiving the binge drug treatment (14 days post-MWM test) the animals were sacrificed and their sectioned brains were stained for BrdU and NeuN (a marker of neurons) and visualized under a confocal microscope. The cells colocalizing the two labels (newborn neurons) were counted and the results from the different treatment groups compared. A significant decrease in newly formed cells in the DG of mice administered with mephedrone and mephedrone+ethanol was found, with respect to saline. Furthermore, the group treated with the drug combination showed significantly less new neurons than that treated with mephedrone alone, indicating an increased deleterious effect of the combination. BrdU count in animals treated with ethanol alone was unaffected with respect to saline (Fig. 5B). Moreover, there was a good correlation between the total amount of new cells and overall MWM performance (Fig. 5).

5-HT input to the hippocampus positively regulates adult neurogenesis [69]. In this sense, 5-HT reuptake inhibitors increase hippocampal neurogenesis [70]. Furthermore, repeated exposure to high doses of MDMA causes the opposite effect [71]. Similarly to what occurs with

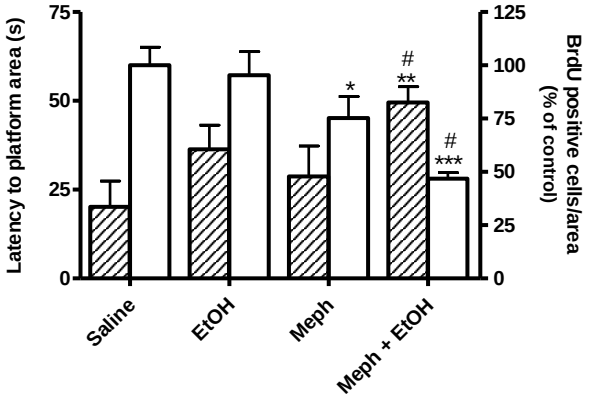

Figure 5. Effects of treatment with saline, ethanol, mephedrone or their combination on spatial memory and hippocampal neurogenesis. Adolescent mice were treated as described above and received two injections of BrdU. Seven days after, they were submitted to the Morris water maze paradigm, consisting of 6 days of training and 1 day of trial. Dashed bars represent, on the probe test day (day 7), the latency to first reach the area where the platform had been located during the training period. The animals were killed 28 days after the treatment and their brains were fixed, sliced and immunostained for BrdU (proliferating cells) and NeuN (neuronal marker). Open bars show overall quantification and means of BrdU-positive neurons per area. Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline; #P < 0.05 *vs.* mephedrone group. Data obtained from [61]

mephedrone in the present study, MDMA is known to produce a depletion of serotonergic markers in the hippocampus 7 days after repeated treatment [76]; this 5-HT depletion can, in turn, cause decreased cell survival in the dentate gyrus [77].

7. Conclusions

To sum up, the co-administration of ethanol to adolescent mice potentiates the psychostimulant and conditioning effects of mephedrone, but also its neurotoxic properties.

All this suggests an increased risk if translated to humans. On the one hand, enhancement of psychostimulant and rewarding effects could promote increased abuse liability and addiction-related disorders whereas, on the other hand, binge abuse of the drug combination could carry more severe neural damage involving dopaminergic and serotonergic impairment, decreased neurogenesis and cognitive deficits.

The preclinical studies reviewed here are the first performed on polyabuse with cathinones, which are becoming increasingly popular among adolescents. Their importance lies in that cathinones are mostly used in combination with alcoholic drinks, and are generally regarded as "safe" drugs. Thus, an experimental-based warning concerning the risks regarding the combined consumption of these drugs should be conveyed to the population at large. Nonetheless, although adolescent brains are exceptionally vulnerable, studies in adult mice would be necessary to determine whether adults could be also susceptible to these effects.

Acknowledgements

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