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ORIGINAL INVESTIGATION

Intermittent ethanol exposure increases long-lasting behavioral and neurochemical effects of MDMA in adolescent mice

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Abstract
Rationale Heavy binge drinking is increasingly frequent among adolescents, while ethanol (EtOH) is often used in combination with 3,4-methylenedioxymethamphetamine (MDMA).
Objectives The long-lasting effects of intermittent exposure to EtOH and MDMA during adolescence on motor activity, anxiety, and social behavior were evaluated in adult mice. The concentration of brain monoamines in the striatum, cortex, and hippocampus was measured following the behavioral test.
Methods Adolescent OF1 mice were exposed to ethanol (1.25 g/kg) on two consecutive days at 48-h intervals over a 14-day period (from PND 29 to 42). A total of eight injections of MDMA (10 or 20 mg/kg) were administered twice daily at 4-h intervals over two consecutive days, and this schedule was repeated 6 days later (PND 33, 34, 41, and 42). Behavioral tests and analysis of brain monoamines took place on PND 64 to 67.
Results Exposure to MDMA during adolescence increased the anxiogenic response in the elevated plus maze, with adult mice spending less time in the open arms of the maze and exhibiting lower concentrations of DA in the striatum. A pattern of ethanol administration modeling binge drinking during adolescence enhanced these effects and undermined the hyperthermic response induced by MDMA. Passive avoidance was affected only when EtOH was administered alone.
Conclusions Juvenile administration of MDMA and alcohol was found to cause a decrease in monoamine levels in adulthood, as well as changes in social interaction behaviors, locomotor activity, increase measures of anxiety in the elevated plus maze (EPM), and decrease step-through latencies in passive avoidance test.

Keywords Ethanol · MDMA · Elevated plus maze · Motor activity · Social interaction · Passive avoidance · Monoamines

The experimental protocol has been approved by an Institutional Review Committee for the use of animal subjects. Procedures involving mice and their care were conducted in conformity with national, regional, and local laws and regulations, which are in accordance with European Community Council Directives (86/609/EEC, 24 November 1986).

The authors have no possible conflict of interest in the carrying out and reporting of this research.

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Introduction

Ethanol (EtOH) is frequently used in combination with 3,4-methylenedioxymethamphetamine (MDMA) (Barrett et al. 2006; Breen et al. 2006). Riley et al. (2001) reported that 85% of those attending rave parties consumed both EtOH and MDMA. Heavy binge drinking is becoming increasingly

frequent among teenagers in the USA and Europe (e.g., Oesterle et al. 2004, 2008; Caamaño-Isorna et al. 2008). In a survey of Spanish adolescents, 49.6% of those who had consumed alcohol in the previous month reported getting drunk during binges. Among those who consumed ecstasy, 98% admitted taking it with alcohol. Similarly, use of ecstasy is more common among adolescents that drink alcohol (2.5%) (ESTUDES 2008). Research in human adolescents clearly points to the deleterious effects of alcohol abuse during the teenage years; among adolescents and fully mature adults consuming similar quantities of alcohol, neurological deficits and alcohol-related problems are more pronounced in the former group (Clark et al. 2008; Oesterle et al. 2008).

Ethanol is an allosteric modulator of many transmembrane receptors (Pohorecky and Brick 1988), but, functionally, it acts primarily as a CNS depressant, potentiating the action of GABA at the GABAA receptor (Suzdak, et al. 1988). MDMA, on the other hand, causes a rapid efflux of dopamine (DA) and serotonin (5-HT) in several brain areas, including the striatum and nucleus accumbens (NAc), immediately after it is administered (O'Shea et al. 2005).

Research has only recently begun to focus on EtOH–MDMA interactions in animal models (Cassel et al. 2004, 2005; Izco et al. 2007; Johnson et al. 2004), and studies are characterized by a great inconsistency in the treatment schedules employed and the time at which measurements were taken. It should be taken into consideration that these studies have been performed using different strains or even different species (rats or mice). EtOH was shown to increase blood concentrations of MDMA and more intensely in the striatum and cortex than in the hippocampus (Hamida et al. 2009). On the other hand, levels of alcohol dehydrogenase 2 (ADH2), which metabolizes ethanol to acetaldehyde, were found to be 35% lower in MDMA-treated rats than in controls (Upreti et al. 2009).

EtOH modifies many of the effects of MDMA, and studies suggest that the interaction between the two drugs depends on the dose, administration regimen, and ambient temperature in question (Ben Hamida et al. 2007; Cassel et al. 2007). Several studies have reported that EtOH decreases the hyperthermic response usually observed in rats following administration of MDMA (Cassel et al. 2007; Ben Hamida et al. 2006, 2008). After four daily administrations of both drugs to rats, Cassel et al. (2005) observed that ethanol inhibited MDMA-induced hyperthermia (on average −1.3°C) on the first day of treatment but not on subsequent treatment days, suggesting that this effect was subject to tolerance. However, tolerance to these interactions was not observed when a different schedule of drug administration (ethanol on four consecutive days prior to the first treatment with MDMA–ethanol) was employed (Ben Hamida et al. 2006). In mice, contrasting evidence has been obtained, with some authors reporting similar effects (decrease of the hypothermic response) as in rats (Johnson et al. 2004; Izco et al. 2010) and others observing a more pronounced hyperthermic response (Pontes et al. 2008). At the behavioral level, EtOH administration potentiates MDMA-induced hyperlocomotion in rats (Cassel, et al. 2004; Riegert, et al. 2008), and (Jones et al. 2010) have recently reported conditioned place preference in rats that had received MDMA plus ethanol but not when the drugs were administered alone.

MDMA-induced neurotoxicity is also affected by ethanol co-administration, though evidence is again contradictory. Some authors have reported that ethanol increases MDMA-induced serotonin depletion in rats (Izco et al. 2007), while others have failed to observe such an effect (Cassel et al. 2005). Fewer studies have evaluated the effect of EtOH on MDMA-induced neurotoxicity in mice, with a protector effect generally being reported (Johnson et al. 2004).

Few studies have been performed to evaluate chronic exposure to both ethanol and MDMA. Hamida et al. (2008) exposed adult rats to four drug administrations over a period of 10 days and observed that EtOH markedly increased the effect of MDMA on motor activity, an effect that represented a clear development of sensitization. On the other hand, EtOH reduced the hyperthermic effect induced by MDMA, an effect that also increased during the course of the treatment. Although hyperthermia is one of the factors most closely related with MDMA-induced neurotoxicity, other factors can influence MDMA toxicity, such as monoamine oxidase metabolism of dopamine and serotonin, nitric oxide generation, glutamate excitotoxicity, serotonin 2A receptor agonism, and the formation of MDMA neurotoxic metabolites (Sarkar and Schmued 2010).

To date, no studies have evaluated the interaction of ethanol and MDMA in adolescent animals. Clinical and experimental studies have provided evidence of the special sensitivity of the adolescent brain to some effects of ethanol, such as memory impairment (White and Swartzwelder 2005) and ethanol-induced brain damage (Crews et al. 2000). Pascual et al. (2007) applied intermittent ethanol administration during adolescence to demonstrate enhanced neural cell death in several brain regions (neocortex, hippocampus, and cerebellum) and long-lasting neurobehavioral impairments in conditional discrimination learning, motor learning, and discrimination between novel and familiar objects. On the other hand, binge administration of MDMA and cocaine to adolescent mice results in an increase of social contact and an anxiolytic response in the elevated plus maze in adulthood. However, the decrease in dopamine levels produced by administration of MDMA alone is counteracted by co-administration of cocaine (Daza-Losada et al. 2008).

Based on the above observations, we hypothesized that intermittent ethanol and/or MDMA intoxication during adolescence would cause long-lasting behavioral consequences and affect brain monoamine levels. The first aim of the present study was to investigate if ethanol affects the long-term consequences of adolescent MDMA exposure for different behaviors in adulthood. The elevated plus maze, social interaction test, and passive avoidance were studied 3 weeks after administration of the drugs. Spontaneous motor activity was also recorded. As a second objective of the study, we attempted to clarify whether or not the behavioral changes induced by drug exposure during adolescence are related with the neurotoxic damage that occurs as a result of this exposure. To do this, we determined the concentration of dopamine and serotonin and their metabolites in the striatum, hippocampus, and cortex of the animals after exposure to MDMA alone or plus ethanol. The hyperthemic response was also measured.

Material and methods

Subjects

A total of 110 male mice of the OF1 strain (CHARLES RIVER, Barcelona, Spain) were employed in the study. The mice were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of four (cage size 28×28×14.5 cm), at a constant temperature (21+2°C), with a reversed light schedule (white lights on 19:30–07:30 h) and food and water available ad libitum (except during the behavioral test). All procedures involving the mice and their care complied with national, regional, and local laws and regulations and with European Community Council Directives (86/609/EEC, 24 November 1986).

Drug treatment and experimental design

Animals were injected i.p. with volumes of 0.01 ml/g MDMA (±3,4-methylenedioxymetamphetamine hydrochloride, Laboratorios Lipomed AG, Switzerland) and ethanol in a volume of 0.02 ml/g. The control group was injected with physiological saline (NaCl 0.9%), which was also used for dissolving the drugs. For the combined groups, ethanol and MDMA were administered in two separate injections. The EtOH dose employed (1.25 g/kg) induced a blood concentration of 0.9 mg/ml in OF1 adolescent mice 5 min after administration. In an adolescent human, this dose would correspond with 33 g of ethanol, which represents two or three alcoholic drinks (taking into account that 13.7 g of ethanol is considered to be one alcoholic drink).

After an acclimatizing period of 8 days, animals were divided into six groups: two groups received physiological saline (**Sal**, $n=15$) or 1.25 g/kg of ethanol (**A1.25**, $n=15$) in a schedule in which injections (16 doses) were administered twice daily (with a 4-h interval) on two consecutive days followed by an interval of two "drug-free" days, over a 2-week period. Mice were injected twice on PND 29, 30, 33, 34, 37, 38, 41, and 42; two more groups received 10 or 20 mg/kg of MDMA (**M10**, $n=15$ and **M20**, $n=10$) in a pattern where the injections (eight doses) were given in two daily administrations (with a 4-h interval) on two consecutive days, with an interval of 6 days without injections, over a 2-week period. Adolescent animals were injected with MDMA twice daily on PND 33, 34, 41, and 42. On PND 29, 30, 37, and 38, these two groups were injected twice with saline (with a 4-h interval). Two further groups received 1.25 g/kg of ethanol and 10 or 20 mg/kg of MDMA (**A1.25+M10**, $n=15$, and **A1.25+M20**, $n=10$) in a schedule in which adolescent animals were injected with ethanol twice daily on PND 29, 30, 37, and 38 and with ethanol plus MDMA on PND 33, 34, 41, and 42. Three weeks after pretreatment had finalized, behavioral tests were performed (postnatal day 64). In this way, each mouse received eight drug administrations that simulated a binge pattern characteristic of that seen in human adolescents and young adults (Tur et al. 2003; White et al. 2006). A more detailed description of the experimental procedure is presented in Table 1. None of the animals died during the experimental procedure.

Procedure and apparatus

Spontaneous motor activity

Spontaneous locomotor activity was automatically measured by an actimeter (CIBERTEC S.A., Spain) consisting of eight cages (33×15×13 cm), each with eight infrared lights located in a frame around the cage. In this apparatus, beams are positioned on the horizontal axis 2 cm apart, at a height just above the bottom of the cage (body level of mice). The different frames are separated from each other by a distance of 4 cm and, since they are opaque, prevent animals from seeing conspecifics. Spontaneous motor activity was recorded for 6 h, without previous adaptation to the actimeter.

Passive-avoidance test

A step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed for the passive avoidance test. This cage is made of Perspex sheets and is divided into two compartments (15×9.5×16.5 cm each one). The safe compartment is white and illuminated

Table 1 Experimental procedure

PND	29	30	31–32	33	34	35–36	37	38	39–40	41	42	43–63	64	65	66	67
Sal	FS/FS	FS/FS		FS/FS	FS/FS		FS/FS	FS/FS		FS/FS	FS/FS	3 weeks without treatment	EPM and training of passive avoidance	Passive avoidance test and motoractivity	Social interaction tets	Monoamine study
M10				M10/M10	M10/M10					M10/M10	M10/M10					
M20				M20/M20	M20/M20					M20/M20	M20/M20					
A1.25	A1.25/A1.25	A1.25/A1.25		A1.25/A1.25	A1.25/A1.25		A1.25/A1.25	A1.25/A1.25		A1.25/A1.25	A1.25/A1.25					
A1.25+M10	A1.25/A1.25	A1.25/A1.25		A1.25+M10/A1.25+M10	A1.25+M10/A1.25+M10		A1.25/A1.25	A1.25/A1.25		A1.25+M10/A1.25+M10	A1.25+M10/A1.25+M10					
A1.25+M20	A1.25/A1.25	A1.25/A1.25		A1.25+M20/A1.25+M20	A1.25+M20/A1.25+M20		A1.25/A1.25	A1.25/A1.25		A1.25+M20/A1.25+M20	A1.25+M20/A1.25+M20					

by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment is dark and made of black Perspex panels. The two compartments are divided by an automatically operated sliding door at floor level. The floor is made of 48 stainless steel bars with a diameter of 0.7 mm and situated 8 mm apart.

Passive-avoidance tests were carried out following the procedure described in Aguilar et al. (2000). On the day of training, each mouse was placed in the illuminated compartment facing away from the dark compartment. After a 60-s period of habituation, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment a footshock (0.5 mA, 3 s) was delivered, and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24 h later following the same procedure but without the shock. The maximum step-through latency was 300 s.

Elevated plus maze

The EPM consisted of two open arms (30×5×0.25 cm) and two enclosed arms (30×5×15 cm). The junction of the four arms formed a central platform (5×5 cm). The floor of the maze was made of black Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. In order to help that to adapt, mice were brought into the dimly illuminated laboratory 1 h before the tests began. At the beginning of each trial, subjects were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behavior displayed by the mice was video-recorded and later analyzed by a "blind" observer using a computerized method. The measurements recorded during the test period were frequency of entries and time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. Number of open arm entries, time spent in open arms, and percentage of open arm entries are generally used to characterize the anxiolytic effects of drugs (Pellow and File 1986; Rodgers et al. 1997).

Social interaction test

This test consisted of confronting an experimental animal and a standard opponent in a neutral cage (61×30.5×36 cm) for 10 min following a 1-min adaptation period prior to the encounter. Standard opponents were rendered

temporarily anosmic by intranasal lavage with a 4% zinc sulfate solution 1 day before testing (Smoothy et al. 1986). This kind of mouse induces an attack reaction in its opponent but does not outwardly provoke or defend itself, since it cannot perceive a pheromone that is present in the urine of the experimental animals and functions as a cue for eliciting aggressive behavior in mice with a normal sense of smell (Brain et al. 1981; Mugford and Nowell 1970). Behavior was video-recorded under white illumination. The videotapes were subsequently analyzed using a custom-developed program (Brain et al. 1989) that makes it possible to estimate the time allocated to different broad functional categories of behavior—body care, digging, non-social exploration, social investigation, threat and attack—each of which is characterized by a series of different postures and elements. A more detailed description can be found in Rodriguez-Arias et al. (1998).

Measurement of rectal temperature

In a new set of animals, temperature was measured on days 33 and 34 immediately before and 30 min after each drug administration (in Table 1, there is a detailed description of the drugs administered on days 33 and 34) using a Technomed Europe thermometer (Medical Diagnosis Accessories, Netherlands) and a lubricated rectal probe (YSI-400 series). Each mouse was lightly restrained by hand for approximately 10 s while the probe was inserted into its rectum and a steady reading obtained. The room temperature during these measurements varied between 21°C and 22°C.

Analysis of biogenic amines

Twenty-five minutes after the acute injection mice were sacrificed by cervical fracture following a procedure similar to that described in Daza-Losada et al. (2007). Within 2 min, the brains were removed and placed on an ice-cold plate. The striatum, cortex (including frontal cortex), and hippocampus were removed, frozen on dry ice, and stored at −80°C. The tissue was thawed, weighed, and then homogenized in 200 μl of perchloric acid (0.1 N) using ultrasounds. The homogenate was centrifuged at 14,000 rpm for 30 min. The supernatant was divided into aliquots for the analysis of biogenic amines. Dopamine (DA), dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindole acetic acid (5-HIAA) were analyzed in a high performance liquid chromatograph (Agilent 1100 series HPLC). Samples were applied to a column (ZORBAX Eclipse XDB-C8 46 X 150 mm, 5 μm; Agilent Zorbax High Pressure Cartige Guard-column). A mobile phase consisting of 800 ml of a solution of sodium acetate (0.01 M), 500 ml of a solution of citric acid (0.01 M) ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, 148 mg), and methanol (255 ml) was passed through the column at a constant flow of 1 ml/min. The HPLC was maintained in a room at a constant temperature (21±1°C). Analytes were oxidized on a glassy carbon electrode maintained at 300 mV (450 mV for HVA detection) against an Ag/AgCl reference electrode (BAS) The complete separation of biogenic amines was achieved in 25 min. Data were collected and analyzed using the Merk-Hitachi software package (Model D-7000). Levels of 5-HT and 5-HIAA were analyzed in the striatum, cortex and hippocampus. In addition, levels of DA, DOPAC, and HVA were analyzed in the striatum.

Statistical analysis

The behaviors evaluated in the social interaction test, elevated plus maze, and monoamines were analyzed using a mixed ANOVA with two "between" subject variables—"Alcohol", with two levels (0 and 1.25 g/kg), and "dose of MDMA", with three levels (0, 10, and 20 mg/kg). Passive avoidance was analyzed using ANOVA with similar "between" variables and a "within" subject variable—"Days", with two levels (Training and Test). Bonferroni adjustment was employed for post hoc comparisons.

Motor activity data obtained during the 6 h of treatment were analyzed using a mixed ANOVA with two "between" subject variables—"Alcohol", with two levels (0 and 1.25 g/kg), and "dose of MDMA", with three levels (0, 10, and 20 mg/kg)—and a "within" subject variable—"Hours", with six levels. Bonferroni adjustment was employed for post hoc comparisons. Additionally, data from the first 10 min of recording were analyzed using a mixed ANOVA with the two between subject variables mentioned previously.

A similar ANOVA was performed for rectal temperature, with two "between" subject variables—"Alcohol", with two levels (0 and 1.25 g/kg), and "dose of MDMA", with three levels (0, 10 and 20 mg/kg)—and a "within" subject variable—"injections", with eight levels. Bonferroni adjustment was employed for post hoc comparisons.

Results

Motor activity

The ANOVA of the motor activity over 6 h (see Fig. 1a) revealed an effect of the variable dose of MDMA [F (2, 74)= 6.003; $p<0.004$], with groups treated with the highest MDMA dose (M20 and A1.25+M20) exhibiting more activity than the rest of the groups ($p<0.027$ with respect

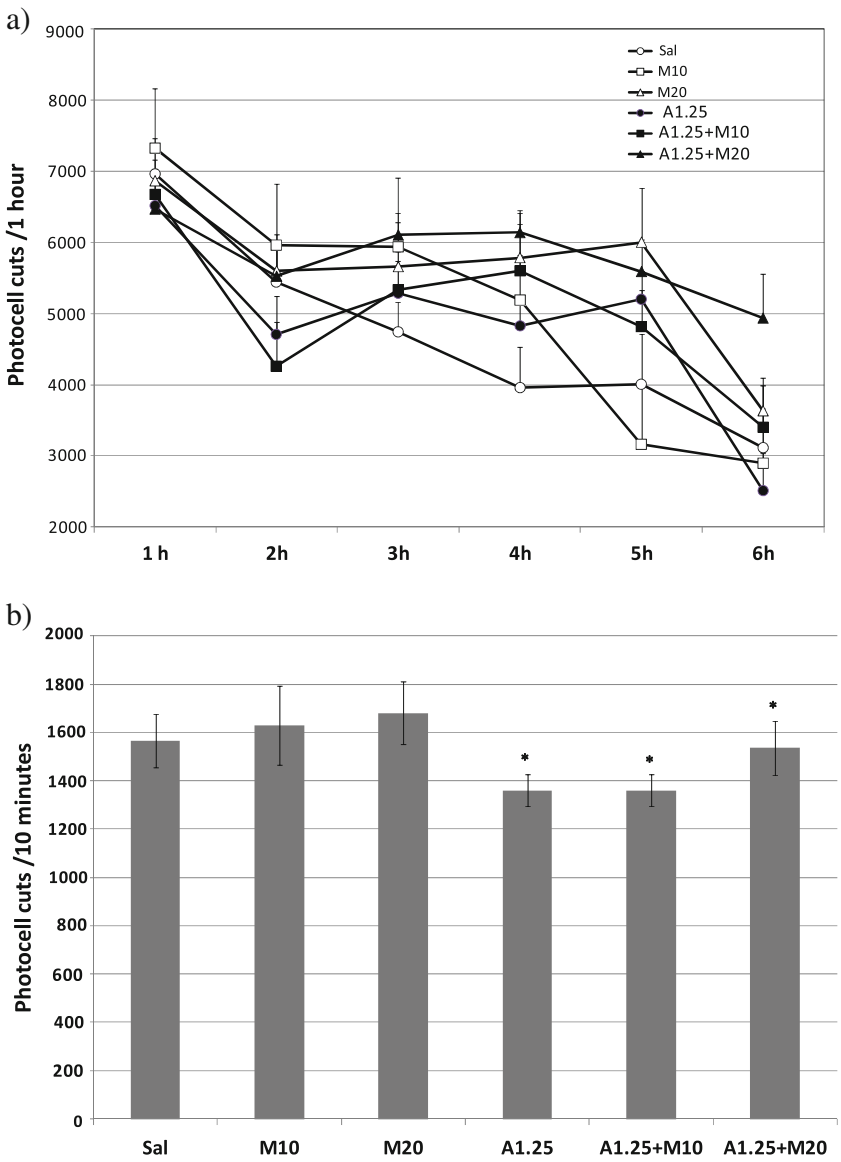

Fig. 1 Means (±S.E.M.) during (**a**) hour per hour or (**b**) the first 10 min period of locomotor activity in photocell cuts from adult mice treated during adolescence with intermittent administration of Saline (Sal), 10 mg/kg of MDMA (M10), 20 mg/kg of MDMA (M20), 1.25 g/kg of ethanol (A1.25), 1.25 g/kg of ethanol+10 mg/kg of MDMA (A1.25+M10), or 1.25 g/kg of ethanol+20 mg/kg of MDMA (A1.25+M20). Differences with respect to mice treated with saline *$p<0.05$

to Sal and A1.25 and $p<0.004$ with respect to M10 and A1.25+M10). The ANOVA of data from the first 10 min (see Fig. 1b) revealed an effect of the variable Alcohol [F (1, 74)=4.694; $p<0.033$], with animals treated with 1.25 g/kg of alcohol (A1.25, A1.25+M10 and A1.25+M20) showing less activity ($p<0.033$).

Passive avoidance

The results of the passive avoidance test are presented in Fig. 2. The ANOVA revealed a significant effect of the variable Days [F (1, 74)=251.197; $p<0.001$], as all the groups presented longer step-through latencies in the test session than in the training session ($p<0.001$). The interaction Days×Alcohol×MDMA dose [F (2, 74)= 4.699; $p<0.012$] also had a significant effect. The group treated only with alcohol (A1.25) presented shorter step-through latencies in the test session than the saline group ($p<0.02$) and took more time to enter the dark compartment in the training session than the saline, M20 and A+M20 groups ($p<0.02$ in all cases).

Elevated plus maze

The most important EPM data are presented in Fig. 3a, b. The ANOVA for the time spent and the percentage of time spent in the open arms of the maze revealed an effect of the interaction Alcohol×Dose of MDMA [F (2, 74)= 4.445; $p<0.01$], as the M20, A1.25+M10 and A1.25+M20 groups spent less time in the open arms of the maze than the saline ($p<0.05$) or M10 groups ($p<0.01$ and $p<0.004$, respectively).

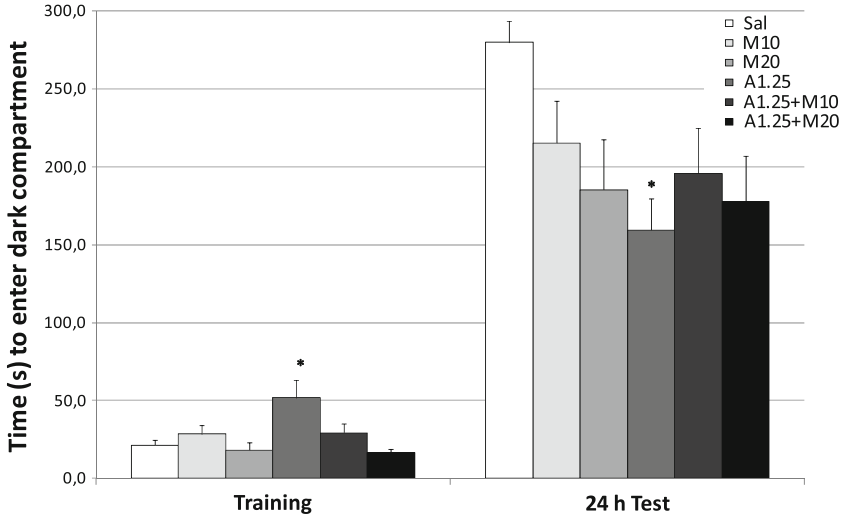

Fig. 2 Effects of intermittent ethanol and/or MDMA administration during adolescence on the time taken by the same mice to enter the dark compartment in the training and test sessions (24 h after training) of the passive avoidance test when adults. Mice were treated with Saline (Sal), 10 mg/kg of MDMA (M10), 20 mg/kg of MDMA (M20), 1.25 g/kg of ethanol (A1.25), 1.25 g/kg of ethanol+10 mg/kg of MDMA (A1.25+M10), or 1.25 g/kg of ethanol+20 mg/kg of MDMA (A1.25+M20). Data are presented as mean values±S.E.M. All the groups presented longer step-through latencies in the test session than in the training session. Differences with respect to the saline group in the Training or Test session *$p<0.02$

Social interaction

The data of the social interaction test are presented in Table 2. The ANOVA for the time spent in Social Investigation revealed that mice treated with the highest dose of MDMA [F (2, 74)=9.988; $p<0.0001$] spent less time engaged in this behavior than the other animals ($p<0.001$).

The time spent in Threat and Attack behavior showed that mice treated with the highest MDMA dose [F (2, 74)= 3.232; $p<0.045$] and [F (2, 74)=3.038; $p<0.05$] spent less time engaged in these behaviors than the other groups ($p<$ 0.05 in all cases). These same groups spent more time in Non-Social Exploration [F (2, 74)=10.719; $p<0.0001$] than the rest of the groups ($p<0.001$).

Explore from a Distance showed an effect of the interaction Alcohol×Dose of MDMA [F (2, 74)=3.833; $p<0.026$], as all the groups spent less time engaged in this behavior than saline-treated controls ($p<0.05$ for A+M10 and A+M20 and $p<0.01$ for the rest).

Rectal temperature

The ANOVA performed for rectal temperature (Fig. 4) showed a significant effect of the interaction between Injections×Alcohol×Dose MDMA [F (6, 60)=2.098; $p<$ 0.05]. With the first injection, the group treated with 10 mg/kg of MDMA plus Alcohol exhibited lower temperatures than the rest of the groups ($p<0.001$ in all cases). In the second injection, only the group treated with the highest MDMA dose presented higher temperatures than the A1.25+M10 group ($p<0.01$). With the third injection, the M20 group showed higher temperatures than the rest of the groups ($p<0.05$ for Sal, M10 and A1.25; $p<$ 0.01 for A1.25+M20 and $p<0.001$ for A1.25+M10). The A1.25+M10 group also exhibited lower temperatures than the Saline and M10 groups ($p<0.03$). With the fourth injection, the M20 group presented higher temperatures than the A1.25 group ($p<0.05$).

Brain monoamines

The brain monoamine data are presented in Table 3. The ANOVA performed for the striatal levels of DA showed a significant effect of the interaction Alcohol×Dose of MDMA [F (2, 71)=3.993; $p<0.023$], with lower levels of DA being detected in the M20, A1.25+M10 and A1.25+ M20 groups than in the saline group ($p<0.001$, in all cases). Additionally, animals treated with 20 mg/kg of MDMA alone (M20) or 10 mg/kg of MDMA plus alcohol (A1.25+M10) exhibited lower levels of DA than the M10 group ($p<0.05$).

The ANOVA performed for the striatal levels of DOPAC showed a significant effect of the interaction Alcohol×Dose of MDMA [F (2, 71)=14.777; $p<0.001$] with levels of DOPAC in all the groups being lower than those in the Saline group ($p<0.001$).

The striatal concentrations of 5-HIIA showed a significant effect of the interaction Alcohol×Dose of MDMA [F (2, 71)=5.627; $p<0.005$], with the M10, M20, and A1.25+M20 groups presenting lower concentrations of 5-HIIA than the saline group ($p<0.001$, $p<0.02$, and $p<$ 0.05, respectively).

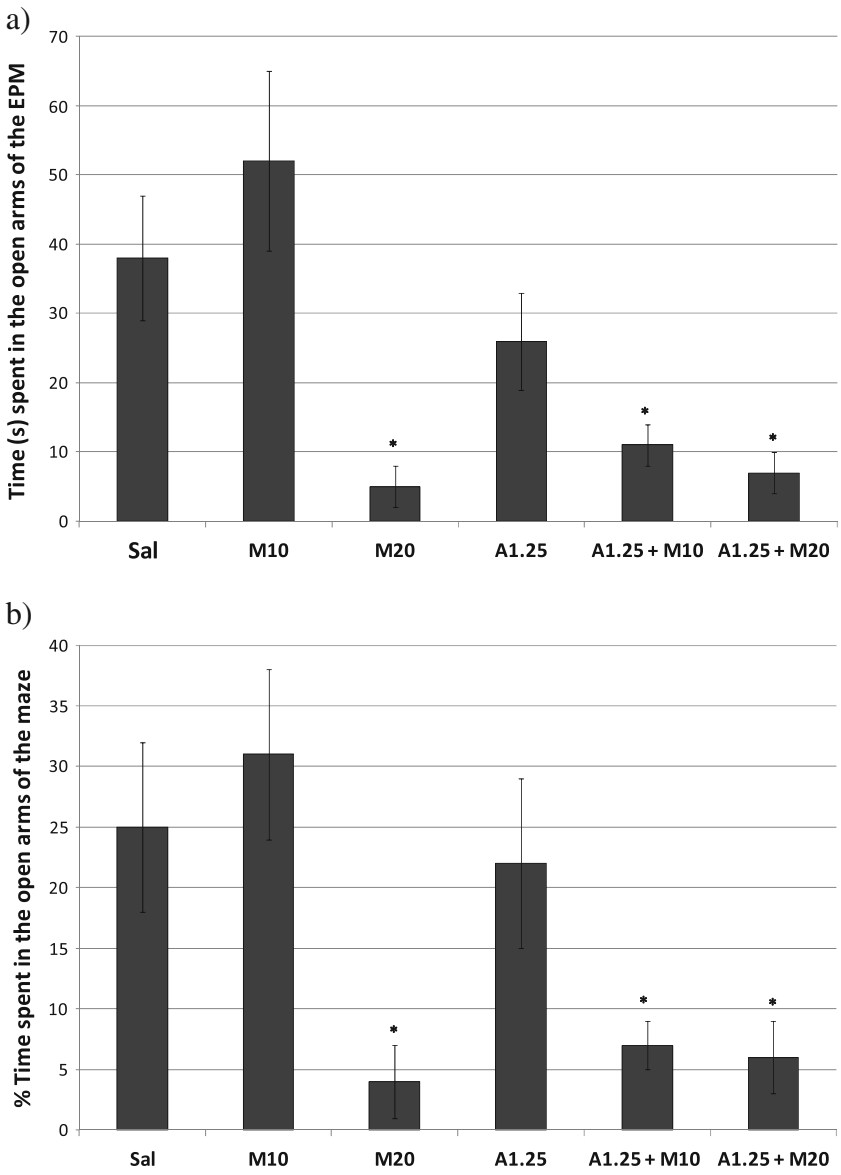

Fig. 3 Effects of intermittent ethanol and/or MDMA administration during adolescence on **a** the time and **b** the percentage of time spent in the open arms of the maze. Mice were treated with Saline (Sal), 10 mg/kg of MDMA (M10), 20 mg/kg of MDMA (M20), 1.25 g/kg of ethanol (A1.25), 1.25 g/kg of ethanol+10 mg/kg of MDMA (A1.25+M10), or 1.25 g/kg of ethanol+20 mg/kg of MDMA (A1.25+M20). Data are presented as mean values± S.E.M. Differences with respect to the saline group *$p<0.05$

Table 2 Means of accumulated times (in seconds) with ±SEM allocated to different categories of spontaneous behavior during the social interaction test in adult mice treated during adolescence with Saline (Sal), 10 mg/kg of MDMA (M10), 20 mg/kg of MDMA (M20), 1.25 g/kg of ethanol (A1.25), 1.25 g/kg of ethanol+10 mg/kg of MDMA (A1.25+M10), or 1.25 g/kg of ethanol+20 mg/kg of MDMA (A1.25+M20) (differences with respect to the saline group *$p<0.05$, **$p<0.01$, ***$p<0.001$)

	Sal	M10	M20	A1.25	A1.25+M10	A1.25+M20
Body care	25±6	20±4	37±4	25±6	36±11	28±6
Digging	45±7	34±4	25±3	32±7	41±9	32±8
Non-social exploration	418±19	438±16	504±7***	438±15	441±16	502±8***
Explore from a distance	27±2	19±3**	9±1**	14±2**	9±1*	8±1*
Social investigation	62±10	62±9	24±3***	59±8	55±7	25±5***
Threat	7±2	6.8±3	0.1±0.1*	5±2	6±3	0.1±0.1*
Attack	21±7	20±9	1±0.5*	16±8	13±7	0.5±0.1*

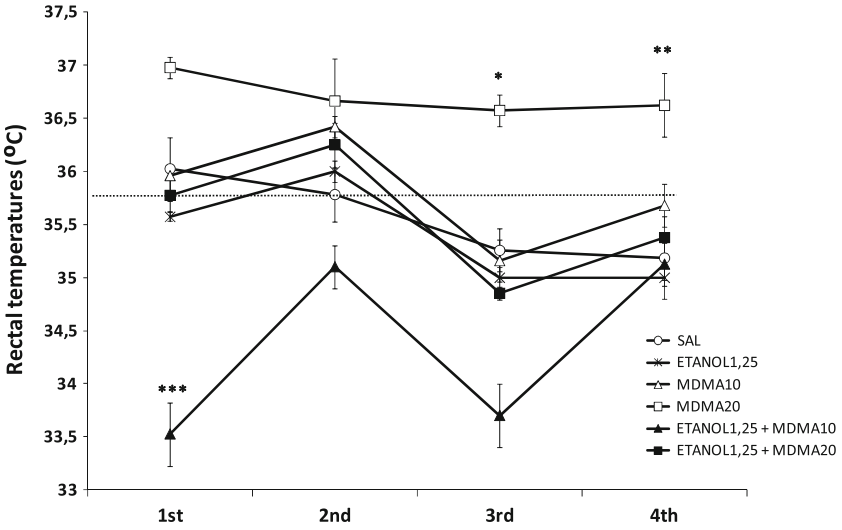

Fig. 4 Rectal temperature 30 min after administration of an injection of Saline (Sal), 10 mg/kg of MDMA (M10), 20 mg/kg of MDMA (M20), 1.25 g/kg of ethanol (A1.25), 1.25 g/kg of ethanol+10 mg/kg of MDMA (A1.25+M10), or 1.25 g/kg of ethanol+20 mg/kg of MDMA (A1.25+M20). The mean basal temperature was 35.9±0.01°C (*dotted line*). Data are presented as mean values±S.E.M. Differences with respect to mice treated with saline *$p<0.05$; **$p<0.01$, ***$p<0.001$

The ANOVA performed for 5-HIIA levels in the hippocampus revealed an effect of the interaction Alcohol×Dose of MDMA [F (2, 80)=5.474; $p<0.004$], as levels of this metabolite were higher in the groups treated with 10 mg/kg of MDMA than in the saline group ($p<0.04$).

Discussion

Cumulative evidence has revealed the special susceptibility of the adolescent brain to drug-induced neurotoxicity and cognitive deficits. The present results confirm that the adolescent brain is highly sensitive to EtOH and MDMA and that the effects of these drugs are manifested in adulthood. This is the first study to evaluate MDMA plus intermittent EtOH administration, a model of binge drinking, in adolescent animals. This schedule of EtOH administration during adolescence has been shown to enhance neural cell death in several brain regions and to cause long-lasting neurobehavioral impairments (Pascual et al. 2007). Our results show that, in addition to these effects, this pattern of drinking during adolescence

Table 3 Long-term effects of administration of EtOh and MDMA during adolescence on the concentration of brain monoamines in the striatum, cortex, and hippocampus in mice

	Sal	M10	M20	A1.25	A1.25+M10	A1.25+M20
Striatum						
DA	14,271±911	12,125±708	8,119±765***	11,116±726	8,559±761***	9,300±597***
DOPAC	1,824±125	777±74***	698±31***	1,163±105***	1,093±7***	744±62***
HVA	119±12	73±4	89±3	88±7	73±6	83±7
5-HT	1,048±59	1,000±67	1,068±126	1,178±65	1,075±72	1,259±132
5-HIIA	763±79	354±22***	457±42**	633±93	692±91	396±45*
Cortex						
5-HT	444±18	342±24	550±31	515±71	299±17	616±53
5-HIIA	352±30	272±31	575±45	419±73	275±26	699±63
Hippocampus						
5-HT	597±36	743±67	565±97	762±81	624±37	461±46
5-HIIA	475±50	870±118*	656±53	730±138	496±28	527±53

Animals were treated during adolescence with Saline (Sal), 10 mg/kg of MDMA (M10), 20 mg/kg of MDMA (M20), 1.25 g/kg of ethanol (A1.25), 1.25 g/kg of ethanol+10 mg/kg of MDMA (A1.25+M10), or 1.25 g/kg of ethanol+20 mg/kg of MDMA (A1.25+M20). Data are presented in means with ±SEM

*$p<0.05$, **$p<0.02$, ***$p<0.001$; differences with respect to the saline group

increases the long-lasting effects of MDMA on anxiety and the loss of brain DA levels in adult mice. In accordance with most previous reports, EtOH also reduced the hyperthermic response induced by MDMA. However, no interaction was detected between the two drugs in the social interaction test or with respect to spontaneous motor activity. The data in the literature regarding the biochemical and behavioral interactions between ethanol and MDMA are inconsistent. Discrepant results have been published concerning the protective or deleterious effect of EtOH on MDMA-induced neurotoxicity and of the action of EtOH on the behavioral changes produced by ecstasy. The present work is set apart from previous studies by two distinctive characteristics. Firstly, we employed a schedule of intermittent and repetitive EtOH and MDMA consumption that models a common pattern of drinking and drug consumption among adolescents. Secondly, both behavioral and neurochemical measurements were taken 3 weeks after the last drug administration, which allowed reliable comparisons to be made.

Neurochemical and pyretic findings of drug combination

As we observed in a previous report, chronic administration of MDMA induced a decrease in the striatal levels of DA in mice (Daza-Losada et al. 2007, 2008) (Table 3). The group treated with the highest dose of MDMA (20 mg/kg), alone or plus EtOH, presented 44% and 35% lower striatal DA concentrations, respectively. The lowest MDMA dose (10 mg/kg), which by itself did not have a substantial effect on DA concentration (only 15% less than controls) induced a significant decrease of DA (41% lower striatal DA levels than controls) when administered with alcohol (group A1.25+M10). The decrease observed in striatal DOPAC levels was less specific, as it was observed in all of the treatment groups. Serotonin or its metabolite, 5-HIAA, were practically unaffected by the treatments employed.

A contrary effect of EtOH on the neurotoxicity induced by MDMA in mice was previously reported by Miller and O'Callaghan (1994). They observed a neuroprotective role of EtOH, although it must be said that the schedule of drug administration, use of adult mice and, in particular, the moment at which analysis was carried out (only 2 h after the last injection) distinguish their study from ours considerably. In addition to a neuroprotective effect, a more recent study observed that EtOH caused hypothermia and induced a four- to sevenfold increase in striatal D-MDMA levels (Johnson et al. 2004). Similarly, we found that EtOH decreased the hyperthermic response induced by MDMA, and a hypothermic response was even observed in the group treated with the lowest dose of MDMA (Fig. 4). On the other hand, similar results to ours have been obtained in adult rats submitted to a 4-day ethanol regimen and administered MDMA (5 mg/kg) at 30°C; although the hyperthermic response induced by MDMA was not altered, a more pronounced loss of 5-HT concentration and 5-HT transporter density was detected in the hippocampus 7 days after the last administration (Izco et al. 2007).

Most of the available evidence suggests that merely preventing MDMA-induced hyperthermia is enough to produce significant neuroprotection (Colado et al. 2001); however, this is not supported by the present results. Although EtOH efficiently decreased the hyperthermic response induced by MDMA, it did not protect mice treated with 20 mg/kg of MDMA and actually increased the toxic effect in those treated with 10 mg/kg of MDMA, in which a hypothermic response was observed. As Izco et al. (2007) have previously suggested, this effect could be a result of binge pattern ethanol administration enhancing MDMA-induced long-term neurotoxicity through a mechanism that is unrelated to changes in acute hyperthermia and which appears to involve hydroxyl radical formation.

Cassel et al. (2005) administered 1.5 g/kg ethanol and/or 10 mg/kg MDMA over four consecutive days to adult male Long–Evans rats and measured brain monoamine levels 20 days after the last administration, a period comparable to that of our study. Similarly, they observed that ethanol attenuated MDMA-induced hyperthermia, but found that the behavioral and neurochemical effects of the combination of ethanol and MDMA were comparable to those of MDMA alone. There are important differences between our work and that of Cassel et al. (2005), including the use of different species, the employment of adult instead of adolescent animals, and, most importantly, the number of drug administrations and treatment period. The use of intermittent administrations in our study could have been responsible for the manifestation of the potentiating effects of EtOH.

Behavioral effects

The results obtained for time and percentage of time spent in the open arms of the maze showed that pretreatment with the highest dose of MDMA induced a long-lasting anxiogenic effect evident in a decrease in the time spent in these arms (Fig. 3). Alcohol exposure potentiated this effect. Mice treated with the lowest dose of MDMA (10 mg/kg) spent the same time in the open arms of the maze than those treated with saline, but those treated with same dose plus alcohol spent less time in the open arms, as occurred with the animals treated with 20 mg/kg of MDMA. Similar results were observed when the number and percentage of open entries were evaluated.

Although we have not previously observed any changes in the behavior of mice in the EPM after exposure to MDMA during adolescence, the schedule of MDMA

administration and the age of the animals in the present study are not comparable with those of earlier work (Daza-Losada et al. 2008). Our results are in accordance with those of Faria et al. (2006), who reported an increased anxiety-like behavior (decreased number of entries in the open arms) 10 days after adolescent rats were exposed to three administrations of 10 mg/kg of MDMA.

With respect to the rest of the behavioral tests, changes in social behaviors were only observed in the groups treated with the highest dose of MDMA, and EtOH did not modify these effects (Table 2). Mice treated with 20 mg/kg of MDMA were more active, engaged in fewer social contacts, and were less aggressive than the rest of the animals. Exposure to the highest dose of MDMA increased motor activity in adult animals, thereby confirming the results obtained in the social test (Fig. 1). Specifically, locomotion did not decrease over time in these animals, as occurred in the rest of the groups. However, when activity was analyzed during the first 10 min, a time period in which the level of investigation of a novel environment can be measured, we observed that pre-exposure to alcohol, either alone or plus MDMA, undermined this exploration.

In the passive avoidance test, all groups presented longer step-through latencies in the test session than in the training session, which shows that the animals remembered the test (Fig. 2). The lack of an effect in the MDMA-treated groups confirms the results of previous reports (Moyano et al. 2005). However, animals that received alcohol during adolescence took significantly more time than those in the saline-treated group to enter the dark compartment in the training session. This may have been related to the lower level of activity observed in the alcohol-treated groups during the first minutes of motor activity recording and suggests an increase in anxiety levels in the novel environment. This result may also have been due to a delayed initiation of action or recognition of the entrance upon initial exposure to the bright chamber (Sakata et al. 2010). This group also displayed shorter step-through latencies in the test session than those treated with saline. One interpretation of the deficit of aversively motivated learning in this test is that it reflected an impairment of the inhibitory control of impulsivity due to adolescent exposure to ethanol (Qin et al. 2011). It is important to note that the passive avoidance test cannot differentiate between other behavioral processes that can alter the results and be misinterpreted as changes in learning and memory, such as pain perception, motivation, anxiety-related behaviors, and locomotor activity (Sarter et al. 1992).

The present results confirm that ethanol modifies the behavioral and neurochemical actions of MDMA. A pattern of ethanol administration that models binge drinking increases the anxiogenic effects and the loss of striatal DA produced by MDMA, despite the fact that ethanol undermines the hyperthermic response induced by this drug. All the groups that presented loss of striatal DA showed an increase in anxiety. We have previously observed that adolescent mice treated with a schedule of MDMA that provoked a similar decrease in DA concentration without changes in DOPAC levels, behave normally in the EPM (Daza-Losada et al. 2008). However, in the present study, the decrease in DA was accompanied by a considerable decrease in DOPAC levels, which may have been responsible for the behavioral differences observed. The presence of EtOH can increase the availability of MDMA in the plasma or/and brain of mice. An increase in the levels of MDMA has been reported in the brain of mice (Johnson et al. 2004) and in the plasma of humans (Hernandez-Lopez et al. 2002). A recent study in rats showed that EtOH increases delivery of MDMA to the brain, especially in the striatum and cortex, which may increase the risk of drug neurotoxicity (Hamida et al. 2009). Additionally, there may be an additive synergism between the effects of MDMA and EtOH on the release of monoamines, particularly of dopamine and 5-HT. In this context, a local synergistic interaction of EtOH and MDMA on the spontaneous outflow and electrically evoked release of striatal DA and 5-HT has been reported (Riegert et al. 2008).

The study of the effects of drugs of abuse in animals enables researchers to overcome the limitations that are inherent to human studies. However, animal studies have their own drawbacks that make the translation of data to humans difficult. For example, acute cardiovascular responses and temperature alterations after MDMA administration are similar in rats, mice, and humans, but there are physiological variations between these species. The results obtained in studies of polydrug abuse, especially that including MDMA, are difficult to translate to humans and should always take into consideration dosing regimen, strain, species, sex, age, and experimental conditions (such as ambient temperature). Recreational users of ecstasy often claim the adverse effects of MDMA obtained in experimental animals do not reflect human use. This assumption is based on the use of much higher doses and different routes of administration to those of recreational human use, leading to the suggestion that animal data reflect "heavy" use of MDMA (Easton and Marsden 2006). A recreational user can be defined as "a person who ingests a standard dose (80–150 mg) of MDMA (Schifano 1991; Henry 1992) occasionally". When extrapolated to humans, the doses and pattern of MDMA administration employed in this study represent a higher amount of ecstasy, but the marked metabolic differences between rodents and humans need to be taken into consideration in this context (Green et al. 2009). Although mice have been described to have a more rapid and efficient metabolism than humans, no auto-

inhibition of MDMA metabolism has been described in these animals. Based on these differences, we chose a consecutive pattern of MDMA administration, since we calculated that four doses would induce lower levels of MDMA in mice than in humans. In addition, we administered a moderate dose of ethanol. Therefore, we believe that our model mimics the pattern of use of adolescents who takes a medium (10 mg/kg) or high (20 mg/kg) number of MDMA pills with only two or three alcoholic drinks.

The risks associated with multi-drug exposure during adolescence are currently unknown. The developing brain is highly vulnerable to the damaging effects of ethanol, and these effects are usually irreversible (for a review, see Guerri 2002). Our study has confirmed this vulnerability, since mice treated during adolescence with EtOH were less active and exhibited an impaired learning and memory in adulthood. On the other hand, and in the line of previous data produced by our group, repeated exposure to MDMA during adolescence decreased DA levels in the striatum of adult mice and induced several behavioral alterations, including an increase in spontaneous motor activity, an anxiogenic response in the EPM, and a lower number of social contacts. Therefore, MDMA consumption or binge drinking during adolescence leads to long-lasting behavioral and neurochemical effects; indeed, in our animals, alterations continued to be detected 3 weeks after treatment had been discontinued, when they had entered adulthood. Our study also showed that adult animals exposed to a pattern of polydrug consumption during their adolescence were more affected with respect to their anxiety response in the EPM and the MDMA-induced decrease in their striatal DA levels than control animals. Alterations in memory capacity were dependent on the test used; although alcohol affected all the tests, MDMA counteracted these effects in the two tests that involved an aversive stimulus. The present results confirm that the interaction of MDMA and ethanol varies depending on the parameter evaluated but in all cases induces alterations that persist long after the last exposure.

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