Binge ethanol administration enhances the MDMA-induced long-term 5-HT neurotoxicity in rat brain

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Abstract

Rationale Ecstasy abuse commonly occurs in hot, overcrowded environments in combination with alcohol. Around 90% of ecstasy users take ethanol; over 70% of these users also often drink alcohol at hazardous levels.

Objectives We wished to examine whether binge ethanol administration enhanced the long-lasting 5-HT neurotoxicity induced by 3,4-methylenedioxymethamphetamine (MDMA) in rats maintained at high ambient temperature and the role of acetaldehyde.

Materials and methods Rats were treated with a 4-day ethanol regimen leading to plasma ethanol levels of around 450 mg/dl. On day 5, rats were placed at 30°C and administered MDMA (5 mg/kg). Rectal temperature and hydroxyl radical formation were measured immediately before and up to 6 h after MDMA. 5-HT concentration and 5-HT transporter density were determined 7 days later. A group of rats received cyanamide (50 mg/kg) on days 1 and 3 of the 4-day-ethanol inhalation.

Results In ethanol treated rats, MDMA produced a hyperthermic response similar to that observed in controls but enhanced the loss of 5-HT concentration and 5-HT transporter density in the hippocampus. Cyanamide elevated the plasma acetaldehyde concentration fivefold to sevenfold, reduced the MDMA-induced hyperthermia and increased the neuronal damage with neurotoxicity also appearing in the cortex. MDMA increased hydroxyl radical production in the hippocampus, the effect being more marked in rats pre-exposed to ethanol.

Conclusions Binge ethanol administration enhances the MDMA-induced long-term 5-HT neurotoxicity by a mechanism not related to changes in acute hyperthermia but probably involving hydroxyl radical formation. The magnitude of this effect is more pronounced after increasing plasma acetaldehyde levels by aldehyde dehydrogenase inhibition.

Keywords MDMA · Neurotoxicity · Hyperthermia · 5-HT · 5-HT transporter · Ethanol · Acetaldehyde · Binge administration

Abbreviations

DHBA dihydroxybenzoic acid
DNPH dinitrophenyl hydrazine
5-HIAA 5-hydroxyindoleacetic acid
MDMA 3,4-methylenedioxymethamphetamine

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is widely used as a recreational drug by young people despite having been shown to be a neurotoxin in the brains of rodents and non-human primates (Green et al. 2003) and to produce lasting specific memory deficits in humans (Bolla et al. 1998; Verkes et al. 2001; Fox et al. 2002; Verbaten 2003; Soar et al. 2004; Wareing et al. 2000; 2004a,b; Montgomery et al. 2005; Gouzoulis-Mayfrank et al. 2003, 2005). These psychopathological disturbances are probably a consequence of the long-term neurochemical changes induced by the drug (Reneman et al. 2000; Cowan et al. 2003; Daumann et al. 2005). Ecstasy abuse usually takes place at crowded and warm dance clubs and raves in

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the context of multiple drug consumption; alcohol and cannabis being the most common drugs used in combination with ecstasy (Schifano et al. 1998; Winstock et al. 2001; Plan Nacional sobre Drogas 2004). Around 90% of ecstasy users take ethanol; over 70% of these users also often drink alcohol at hazardous levels (Winstock et al. 2001). Chronic or excessive alcohol consumption leads to permanent brain damage in both humans and experimental animals and to the impairment of cognitive functions such as learning and memory (Pfefferbaum et al. 1998; White 2003). According to Hunt (1993), binge drinkers who consume at least four or five drinks in a row are particularly susceptible to eventual brain damage. In Spain, about 40% of male college students (aged 15–34) and a high percentage (20%) of older adults (aged 35–64) reported binge drinking in a survey covering the whole of 2003 (Plan Nacional sobre Drogas 2004). The same pattern is observed in females, although percentages are lower for the same age intervals.

It was frequently reported that the administration of MDMA to rats produces an acute hyperthermic response and a relatively selective long-term neurotoxic damage to 5-HT pathways, having little effect on dopamine containing neurons (Green et al. 2003). The degeneration is reflected by a substantial decrease in the concentration of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), a reduction in the density of 5-HT uptake sites labelled with [³H]-paroxetine (Sharkey et al. 1991; Hewitt and Green 1994; Colado et al. 1997a; Sanchez et al. 2001) and a decrease in the immunoreactivity of fine 5-HT axons in the neocortex, hippocampus and striatum (O'Hearn et al. 1988).

The mechanisms involved in producing this neurodegeneration are not totally understood, but evidence indicates that a process of oxidative stress is initiated immediately after MDMA administration. Intracerebral microdialysis in vivo has shown that MDMA induces an increase in the formation of hydroxyl radicals, reflected by a rise in 2,3 dihydroxybenzoic acid (2,3-DHBA) in the hippocampal and striatal dialysates (Colado et al. 1997a; Shankaran et al. 1999). The hydroxyl radical scavenger α -phenyl-*N*-tert-butyl nitron abolishes the MDMA-induced rise in 2,3-DHBA (Colado et al. 1997a) and attenuates damage to 5-HT neurons (Colado and Green 1995; Colado et al. 1997a; Yeh 1999). Additional evidence supporting the existence of an oxidative stress process includes the observation that MDMA increases lipid peroxidation in the brain (Sprague and Nichols 1995; Colado et al. 1997b).

Episodic ethanol intoxication and withdrawal, characteristic of binge alcoholism or chronic ethanol administration, induces substantial neurodegeneration in the hippocampus and entorhinal cortex of the rat (Miki et al. 2000; Zharkovsky et al. 2003; Prendergast et al. 2004;

Baydas and Tuzcu 2005; Hamelink et al. 2005). Several mechanisms were postulated to be involved in ethanol neurodegeneration, some of them also being involved in MDMA-induced neuronal damage. Among the most relevant are excessive glutamatergic activity (Tsai et al. 1995), increased intracellular calcium (Lovinger 1993) and an oxidative stress process (Bondy et al. 1996; Crews et al. 2004) mediated by a rise in oxygen and nitrogen-derived reactive species (Lancaster 1992; Reddy et al. 1999; Huang et al. 2002; Dahchour et al. 2005), a reduction in the activity of the main antioxidant enzymes and an increase in the degree of lipid peroxidation (Somani et al. 1996; Fadda and Rossetti 1998; Reddy et al. 1999; Agar et al. 2003; Thirunavukkarasu et al. 2003).

We have now examined the effect of binge ethanol consumption on the acute hyperthermia and the long-lasting loss of 5-HT content and 5-HT transporters induced by a single MDMA administration in rats maintained at high ambient temperature to mimic human ecstasy consumption. The effect of ethanol on the changes induced by MDMA on free radical formation and the possible involvement of acetaldehyde, the main active metabolite of ethanol, in the neurochemical actions of ethanol were also evaluated.

Materials and methods

Animals and drug administration

Male Dark Agouti rats (150–175 g, Interfauna, Barcelona) were used. They were always housed in groups of six, in conditions of constant temperature (21°C±2°C) and a 12 h light/dark cycle (lights on: 07:00 h) and given free access to food and water.

For ethanol treatment rats were placed into 37 l chambers and exposed to ethanol vapour or humidified air (controls). Ethanol (absolute, Panreac, Barcelona, Spain) or water was delivered via an osmotic pump (Harvard) into an airtight flask maintained at 42°C by a water bath. A constant stream of air (10 l air/min) was passed through the flask, which carried the ethanol or water vapour into the chamber. The flow rate was varied to create different levels of ethanol vapour. This method of ethanol administration permits a control of the timing of ethanol exposure in contrast to the two-bottle free-choice paradigm and allows high blood alcohol levels to be reached that would otherwise require elevated volumes of ethanol injection by the IP route.

MDMA (NIDA, Research Triangle Park, NC, USA) and cyanamide (Sigma, Madrid, Spain) were dissolved in saline (0.9% NaCl) and given in a volume of 1 ml/kg. Doses are reported in terms of the base.

Experimental design

Study 1 Rats were placed in the inhalation chambers and exposed for 3 h to different flow rates of ethanol (0.25–1.75 ml/min). Plasma ethanol concentration was measured immediately after removal. The goal was to assess whether there was a linear relationship between the speed at which ethanol vapour was pumped into the inhalation chamber and plasma ethanol concentration. This experiment allowed us to choose flow rates producing ethanol plasma concentrations of approximately 450 mg/dl. This level of ethanol was selected to mimic the exposure received by certain sectors of society who indulge in a repeated high level consumption of ethanol in a short interval (“binge” drinking) leading to severe intoxication (Doyle et al. 1994; Parke et al. 1996; Whiteman et al. 2000).

Study 2 Rats were exposed to plasma ethanol concentrations of about 450 mg/dl before MDMA administration. Similar binge ethanol administration was previously used to mimic a single cycle of binge drinking in humans (Collins et al. 1998; Miki et al. 2000; Obernier et al. 2002a,b; Hamelink et al. 2005). Ethanol or air was pumped into the inhalation chamber at a flow of 1 ml/min for 3 h daily during 4 consecutive days. Rectal temperature was measured before placing rats in the inhalation chambers and immediately after removal. Plasma levels of ethanol were quantified daily immediately after the 3 h ethanol exposure. Then 24 h after the last ethanol exposure, rats were placed in a room maintained at an ambient temperature of 28–30°C (referred to as 30°C) for 2.5 h before MDMA (5 mg/kg, IP) injection and for 6 h after MDMA injection. Rectal temperature was measured for 1 h before and up to 6 h after MDMA injection. After that, the rats were placed at 21°C and maintained at this temperature until the end of the study. Seven days after MDMA administration, the rats were killed, their brains removed and the concentration of 5-HT and the density of 5-HT uptake sites in the hippocampus and frontal cortex were determined.

Study 3 To evaluate the involvement of free radicals on the changes induced by MDMA, the formation of hydroxyl radicals in the hippocampus of rats exposed and not exposed to ethanol was determined. For that, animals were implanted a guide cannula 3 days before ethanol exposure and dialysis probes inserted in the guide cannulae on the day of MDMA administration (24 h after chamber removal).

Study 4 To study the involvement of acetaldehyde in the changes in MDMA effects induced by ethanol exposure, a separate group of rats exposed to ethanol was injected with cyanamide (50 mg/kg, IP) or saline 30 min before placing

the rats in the inhalation chambers on days 1 and 3 of the 4 day-exposure. This dose of cyanamide increases acetaldehyde levels compared with those observed in rats receiving only ethanol (Kinoshita et al. 2002). The rest of the experimental protocol was performed in the same way as in study 2.

Plasma ethanol concentration

Blood ethanol levels were determined every day immediately after the 3 h ethanol exposure. Samples of 20 µl of blood were collected from the tail in heparanized capillary tubes, centrifuged at 1500×g for 6 min at 4°C (Microcentrifuge MK5, model 01400-00, Analox, UK) and injected in an analyser (AM1, Analox, UK). The rationale of the method consists of ethanol being oxidized by the enzyme alcohol oxidase in the presence of molecular oxygen. Therefore, the rate of oxygen consumption is directly proportional to the alcohol concentration. Plasma ethanol levels were calculated as mg/dl, using ethanol 300 mg/dl as standard.

Plasma acetaldehyde concentration

Acetaldehyde was determined by high pressure liquid chromatography (HPLC) using a modification of the method described by Kozutsumi et al. (2002). Briefly, 100 µl blood was collected in heparin tubes and deproteinized with perchloric acid (3 M) followed by the addition of sodium acetate (3 M). Blood was centrifuged at 1500×g for 10 min at 4°C. The supernatant was mixed with 500 µl of 2,4-dinitrophenyl hydrazine (5 mM, DNPH) and the mixture was allowed to react for 30 min at room temperature. A methanol solution of *n*-butylaldehyde–DNPH (20 µM) was added to the reaction as internal standard before purification by a solid-phase C18 cartridge (Sep-Pak® Vac). Columns were conditioned with 2 ml of methanol followed by 2 ml of water. The reaction mixture was loaded onto the column and after washing, the retained acetaldehyde–DNPH and internal standard were eluted with 2 ml of methanol. The recovered fraction was dried under a nitrogen stream and reconstituted in 0.1 ml of HPLC mobile phase consisting of acetonitrile and water (65:35). Both acetaldehyde–DNPH and *n*-butylaldehyde–DNPH peaks were detected at an absorbance of 365 nm with an ultraviolet–visible detector.

Measurement of rectal temperature

Rectal temperature was measured every 30 min by the use of a digital readout thermocouple (BAT12 thermometer, Physitemp, NJ, USA) with a resolution of 0.1°C and

accuracy of $\pm 0.1^\circ\text{C}$ attached to a RET-2 Rodent Sensor, which was inserted 2.5 cm into the rectum of the rat, the animal being lightly restrained by holding it in the hand. A steady readout was obtained within 10 s of probe insertion.

Measurement of monoamines and their metabolites in cerebral tissue

The rats were killed by cervical dislocation and decapitation, the brains rapidly removed and the hippocampus and frontal cortex dissected out on ice. Tissue was homogenised and 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) measured by HPLC. Briefly, the mobile phase consisted of KH_2PO_4 (0.05 M), octanesulfonic acid (0.8 mM), EDTA (0.1 mM) and methanol (16%), and was adjusted to pH 3.7 with phosphoric acid, filtered and degassed.

The HPLC system consisted of a pump (Waters 510) linked to an automatic sample injector (Loop 200 μl , Waters 717 plus Autosampler), a stainless steel reversed-phase column (Spherisorb ODS2, 5 μm , 150×4.6 mm) fitted with a precolumn, and a coulometric detector (Coulchem II, Esa, USA). The flow rate was 1 ml/min and the working electrode potential was set at 400 mV with a gain of 500 nA. The current produced was monitored by using an integration software package (Unipoint, Gilson).

[^3H]-Paroxetine binding in tissue homogenates

[^3H]-Paroxetine binding was measured by the method described in detail by Hewitt and Green (1994). The animals were killed, the brain rapidly removed and dissected on ice within 2 min. The frontal cortex and hippocampus from individual animals were homogenised in ice-cold Tris-HCl (50 mM; pH 7.4) containing NaCl (120 mM) and KCl (5 mM) using an Ultra-Turrax. The homogenate was centrifuged at $30,000 \times g$ for 10 min at 4°C . The supernatant was discarded and the wash procedure repeated twice. The pellet was finally resuspended in the Tris buffer at a concentration of 10 mg tissue/ml. To obtain an estimate of the maximal density of [^3H]-paroxetine labeled 5-HT uptake sites, the assay solution (1 ml) contained a saturating concentration of [^3H]-paroxetine (1 nM) and 800 μl tissue preparation with the addition of 5-HT (100 μM) for determination of non-specific binding. Incubation was for 90 min at room temperature. Assays were terminated by rapid filtration and counting of the radioactivity by scintillation spectrometry. Previous evidence indicates that MDMA causes a decrease in the B_{max} for paroxetine binding but does not modify K_d values (Battaglia et al. 1987; Ricaurte et al. 1992). Protein concentrations were measured by the method of Lowry et al. (1951).

Implantation of the microdialysis probe in the hippocampus

Rats were anaesthetised with sodium pentobarbital (Euta-Lender, 40 mg/kg, IP) and secured in a Kopf stereotaxic frame with the tooth bar at -3.3 mm below the interaural zero. A guide cannula was implanted in the right hippocampus according to the following coordinates: $+2.2$ mm from the interaural line, -4.3 mm lateral and -4 mm below the skull (König and Klippel 1963). Cannulae were secured to the skull as described by Baldwin et al. (1994).

On the day of the experiment, the dialysis probes (membrane length: 4.0 mm $\times$ 500 μm ; CMA/12, Sweden) were inserted in the guide cannulae such that the membrane protruded its full length from the end of the probe.

Measurement of free radical formation in vivo using microdialysis

Free radical formation in the brain in vivo was measured by the method described in detail by Colado et al. (1997a) with some modifications. The method relies on the fact that hydroxyl free radicals react with salicylic acid to generate 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid (2,3-DHBA and 2,5-DHBA). This reaction is utilised by measuring the formation of these compounds in the dialysate of a microdialysis probe implanted in the hippocampus (see above), which is being perfused with salicylic acid (see Chiueh et al. 1992; Giovanni et al. 1995). As 2,5-DHBA is also formed peripherally through salicylate hydroxylation by the cytochrome P450 system, only 2,3-DHBA concentration in the dialysate can be considered as a reliable marker of hydroxyl radical formation in the hippocampus (Halliwell et al. 1991). Probes were perfused with aCSF (KCl: 2.5 mM; NaCl: 125 mM; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$: 1.18 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 1.26 mM; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.5 mM; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 5 mM; pH=6.55) containing salicylic acid (2.5 mM) at a rate of 1 $\mu\text{l}/\text{min}$ and samples collected from the freely moving animals at 30 min intervals. The first 60 min sample was discarded and the next three 30 min baseline samples were collected.

The HPLC system consisted of a pump (Waters 510) linked to a manual sample injector (Loop 20 μL Rheodyne), a stainless steel reversed-phase column (250×4.6 mm, 5 μm C8 Ultracarb, Phenomenex) fitted with a precolumn (30×4.6 mm, 5 μm C8 Ultracarb, Phenomenex) and a coulometric detector (Coulchem 5100A) with a 5011 analytical cell. The working electrode potential was set at 400 mV with 1 μA gain. The mobile phase consisted of KH_2PO_4 (0.025 M), acetonitrile (20%) and methanol (10%) and was adjusted to pH 3.25 with phosphoric acid, filtered and degassed. The flow rate was 1 ml/min. The

current produced was monitored by using an integration software package (Unipoint, Gilson).

Statistics

Data from monoamine and binding studies were analyzed using the one-way ANOVA followed by the Newman–Keuls multiple comparison test when a significant F value was obtained. Statistical analyses of the temperature measurements and dialysis were performed using the statistical computer package BMDP/386 Dynamic (BMDP Statistical Solutions, Cork, Eire). Data were analyzed by the analysis of variance (ANOVA) with repeated measures (program 2V) or, where missing values occurred, an unbalanced repeated measure model (program 5V) was used. Both used treatment as the between-subjects factor and time as the repeated measure. Student t test was used to compare the effect on rectal temperature caused by the exposure to ethanol or air in the inhalation chamber. Differences were considered significant at $p < 0.05$. The results of the statistical comparisons are included in the figure legends.

Results

Study 1. Relationship between the flow rate of ethanol in the inhalation chamber and plasma ethanol concentrations

An initial experiment was performed to adjust the ethanol flow rate to achieve high plasma ethanol concentrations. Flow rates of 0.25, 0.5, 1, 1.5 and 1.75 ml/min led to the following plasma ethanol levels (measured at the end of the 3 h exposure period): 26 ± 6 ($n=4$), 168 ± 5 ($n=4$), 509 ± 24 ($n=4$), 675 ± 9 ($n=4$) and 778 ± 17 ($n=4$), respectively. There was a linear relationship ($R^2=0.98$) between the ethanol flow rate and plasma ethanol levels. Ethanol flow rates of 1 ml/min were used in the subsequent experiments to achieve daily plasma levels of approximately 450 mg/dl.

Study 2. Effect of intermittent exposure to ethanol on the hyperthermia and neurotoxicity induced by MDMA Table 1 shows the plasma ethanol concentration (409–480 mg/dl)

Table 1 Ethanol concentration in the plasma of rats exposed to ethanol pumped at a flow rate of 1 ml/min into the vapourization chamber

	Days of ethanol exposure			
	1	2	3	4
Plasma ethanol concentration (mg/dl)	449 ± 33	409 ± 20	471 ± 39	481 ± 47

Animals were exposed to ethanol vapour for 3 h daily on 4 consecutive days. Results are shown as the mean $\pm$ SEM ($n=12$ rats)

measured immediately after the 3 h period of exposure to ethanol pumped at a rate of 1 ml/min for 4 days. The average ethanol concentration was 453 mg/dl (referred to as 450 mg/dl).

Rats exposed to humidified air (control) in the inhalation chambers showed a reduction in rectal temperature of about 1°C at the end of each 3 h-daily exposure. Ethanol caused a significant reduction in rectal temperature of approximately 0.5°C at the end of the first day of treatment compared with the group exposed to air only (36.5 ± 0.05 vs 36.0 ± 0.09 , $p < 0.05$). In the remaining days of exposure (2 to 4 days), the rectal temperature of the rats treated with ethanol was similar to that observed in the rats exposed to air. The temperature inside the chamber ($21 \pm 2^\circ\text{C}$) did not significantly differ from the room temperature on any of the 4 treatment days.

Ethanol exposure induced a mild ataxia at the end of each of the 3 h-daily treatments compared with control rats exposed to humidified air. Animals appeared to have recovered 30 min later and did not suffer seizures 24 h after ethanol exposure (time at which MDMA was injected). These effects were observed visually, not quantified.

Administration of MDMA (5 mg/kg, IP) at a room temperature of 30°C produced a sustained hyperthermic response lasting at least 6 h, which reached a peak of 1.5°C 30 min after administration (Fig. 1). Hyperthermia was not modified by ethanol exposure during the 4 previous days (Fig. 1). Rats treated with ethanol and injected saline

Fig. 1 Hyperthermia induced by MDMA (5 mg/kg, IP) in rats pre-exposed to ethanol (EtOH) for 3 h per day on 4 consecutive days. Then 24 h after the last EtOH administration, rats were placed at an ambient temperature of 30°C for 2.5 h before and 6 h after MDMA injection. Temperature was measured for 1.5 h before and up to 6 h after MDMA injection. The arrow shows the time of MDMA administration. Results are shown as the mean $\pm$ SEM ($n=7$ –13 rats). MDMA produced a significant rise in body temperature ($F(1,23)=313.2$, $p < 0.001$) compared with the saline-injected group. Pre-treatment with EtOH did not modify the hyperthermic response induced by MDMA ($F(1,18)=0.70$, $p=0.41$) but increased the body temperature of the saline-injected rats ($F(1,22)=13.59$, $p < 0.01$)

showed a rectal temperature higher than the animals injected with saline alone (Fig. 1).

There was a significant decrease in 5-HT concentration and 5-HT transporter density in the hippocampus and frontal cortex 7 days after MDMA injection compared with the saline-injected group (Fig. 2a–d). Pre-exposure to ethanol enhanced both the loss of 5-HT (Fig. 2a) and the reduction in 5-HT uptake density in the hippocampus (Fig. 2c). No change was observed in the frontal cortex (Fig. 2b and d). Rats treated with ethanol alone did not show any change on 5-HT concentration and 5-HT transporter density (Fig. 2a–d).

Study 3. Effect of intermittent exposure to ethanol on the hydroxyl radical formation induced by MDMA MDMA administration produced a modest and significant increase

in the 2,3-DHBA concentration in the hippocampal dialysate. The rise in 2,3-DHBA was approximately 20–25% above the concentration seen in saline-injected rats (Fig. 3). Pre-exposure to ethanol significantly enhanced the MDMA-induced rise in the levels of 2,3-DHBA in the hippocampal dialysate and did not modify the content of 2,3-DHBA in saline-injected rats (Fig. 3).

Study 4. Effect of raising plasma acetaldehyde concentration on the hyperthermia and neurotoxicity induced by MDMA Rats exposed to ethanol plasma levels of 450 mg/dl showed acetaldehyde levels between 0.22 and 0.30 mg/dl immediately after the 3 h ethanol exposure on each of the 4 days of treatment (Fig. 4a). Administration of cyanamide (50 mg/kg, IP) on days 1 and 3 of ethanol exposure markedly increased plasma acetaldehyde concentration

Fig. 2 Changes induced by MDMA (5 mg/kg, IP) on 5-HT concentration (a, b) and 5-HT transporter density (c, d) in the hippocampus and frontal cortex of rats pre-exposed to ethanol (EtOH). Animals were exposed to EtOH for 3 h per day on 4 consecutive days. A group of rats was given cyanamide (50 mg/kg, IP) on days 1 and 3 of treatment. On day 5, all rats were placed at a room temperature of 30°C for 2.5 h before and 6 h after MDMA injection, animals were killed 7 days after. Results are shown as the mean±SEM ($n=6-12$ rats). Different from the corresponding

saline-injected rats: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Different from the MDMA-injected rats pre-exposed to air: $\Delta p<0.05$, $\Delta\Delta\Delta p<0.001$. Different from the MDMA-injected rats pre-exposed to EtOH: $f p<0.05$, $ff p<0.01$. Absolute values for 5-HT concentration and [³H]-paroxetine binding in the hippocampus were 432 ± 32 ng/g and 171 ± 23 fmol/mg tissue, respectively, and in the cortex were 176 ± 6 ng/g and 138 ± 15 fmol/mg protein, respectively. The basal levels of 5-HT content and 5-HT transporters were comparable in the three treatment groups for each region

Fig. 3 Changes in the levels of 2,3-dihydroxybenzoic acid (DHBA) in the hippocampal dialysate of rats injected with MDMA (5 mg/kg, IP) or saline and exposed to ethanol or humidified air 3 h per day on 4 consecutive days. The arrow shows the time of MDMA administration. MDMA produced a significant increase in the extracellular levels of 2,3 DHBA ($F(1,11)=3.32$, $p<0.05$). This effect was enhanced by pre-exposure to ethanol ($F(1,14)=3.42$, $p<0.05$). Values are expressed as a percentage of the mean of three measurements before drug administration. Each value is the mean $\pm$ SEM of five to nine experiments. The basal concentrations of 2,3-DHBA in saline-treated rats ($n=6$) were 8.69 ± 1.3 pg/ μ l

(400% on the first day and 600% on the third day) compared with animals receiving ethanol alone (Fig. 4a). No change in acetaldehyde levels was observed on days 2 and 4 of ethanol exposure (Fig. 4a). Cyanamide did not alter ethanol plasma levels on any of the 4 days of ethanol exposure (Fig. 4a).

Cyanamide significantly enhanced the ethanol-induced hypothermia with rats showing a rectal temperature 3–3.5°C below that observed in animals treated with ethanol alone (Fig. 4b).

Rats treated with cyanamide plus ethanol, 24 h after ethanol exposure, showed a hyperthermic response to MDMA that was less pronounced than that induced by MDMA in rats treated with ethanol and not given cyanamide (Fig. 5). Rats exposed to ethanol and injected with cyanamide, 7 days after MDMA administration, showed a reduction in cortical 5-HT concentration (Fig. 2b) and 5-HT transporter density in the hippocampus and cortex (Fig. 2c and d), which was greater than that observed in animals exposed to ethanol alone. Rats exposed to ethanol and injected with cyanamide, but not treated with MDMA, did not show any change on 5-HT concentration and 5-HT transporter density (Fig. 2a–d).

Discussion

This study provides the first evidence indicating that binge administration of ethanol for a restricted period of time enhances the loss of brain 5-HT terminals induced by a moderate dose of MDMA when given at an ambient

temperature of 30°C, which is often the environment in which MDMA is ingested recreationally. Data indicate that animals maintained on a 4-day binge ethanol regimen leading to plasma levels of 450 mg/dl are more vulnerable to the long-term neurotoxicity induced by MDMA, which is reflected by a more pronounced loss of 5-HT and 5-HT transporter density in the hippocampus compared with those animals inhaling air instead of ethanol vapour.

Ethanol is metabolized to acetaldehyde mainly by the alcohol dehydrogenase pathway both peripherally and in the brain (Raskin and Sokoloff 1970) and, to a lesser extent, by oxidation through the microsomal inducible isoenzyme CYP2D1 (Warner and Gustafsson 1994) and the catalase–H₂O₂ system (Aragon et al. 1992). Acetaldehyde is subsequently transformed to acetate by aldehyde dehydrogenase. There is much evidence showing that acetaldehyde mediates some of the behavioural and central effects of ethanol, such as headache, nausea, sedation and sleep-induction (Eriksson 2001; Zimatkin et al. 2001a,b). To evaluate the contribution of acetaldehyde to the enhancing effect of ethanol on MDMA-induced neuronal damage, rats were given cyanamide (an inhibitor of aldehyde dehydrogenase) on days 1 and 3 of the 4-day binge ethanol administration and MDMA was injected 24 h after the final ethanol exposure. According to previous studies, administration of cyanamide (Deitrich et al. 1976; Kinoshita et al. 2002) elevates plasma acetaldehyde concentration between fivefold and sevenfold above that observed in rats treated with ethanol alone and this was confirmed in the current study. In these rats, MDMA produced a more marked loss of 5-HT transporter density in the hippocampus than that observed in those treated with ethanol alone and induced the appearance of damage in the frontal cortex.

The ability of acetaldehyde to cross the blood–brain barrier was repeatedly questioned. From a physicochemical point of view, there is no limiting factor (Quertemont and Tambour 2004). However, it is the presence of aldehyde dehydrogenase in the microvasculature of the brain, which might limit acetaldehyde diffusion by providing a metabolic barrier (Zimatkin 1991). For this reason, the levels of acetaldehyde detected in the brain after ethanol administration are low and mainly derived from the metabolism of ethanol in the brain. However, high plasma acetaldehyde concentrations, such as those reached in the current study after aldehyde dehydrogenase inhibition in the periphery, are able to saturate the metabolic barrier afforded by the brain microvasculature, which in addition is also inhibited by cyanamide administration. In these experimental conditions, elevated brain levels of acetaldehyde with the ability to exert pharmacological activity and enhance MDMA-induced neurotoxicity in rats exposed to ethanol would be expected. Therefore, although after alcohol administration, peripherally produced acetaldehyde could

a) Plasma EtOH and AcHO levels

b) Rectal temperature

Fig. 4 a) Plasma concentration of ethanol (EtOH) and acetaldehyde (AcHO) in rats exposed to EtOH pumped at a flow rate of 1 ml/min into the vaporization chamber and treated with cyanamide. Animals were exposed to EtOH vapour for 3 h daily on 4 consecutive days. Cyanamide (50 mg/kg, IP) was given on days 1 and 3 of EtOH inhalation. **b)** Rectal temperature of rats measured for 1.5 h before EtOH treatment and immediately after removal. Results are shown as the mean $\pm$ SEM ($n=5-13$ rats). Different from rats exposed to humidified air: *** $p<0.001$. Different from rats exposed to ethanol only: $p<0.001$

be rapidly metabolized at the blood–brain barrier before penetrating into brain tissue, in aldehyde dehydrogenase deficient heavy drinkers, the risk of neuronal damage after

Fig. 5 Hyperthermia induced by MDMA (5 mg/kg, IP) in rats pre-exposed to ethanol (EtOH) for 3 h per day on 4 consecutive days and injected with cyanamide (CYAN) on days 1 and 3 of EtOH inhalation. Then 24 h after the last EtOH administration, rats were placed at a room temperature of 30°C for 2.5 h before and 6 h after MDMA injection. The temperature was measured for 1.5 h before and up to 6 h after MDMA administration. Results are shown as the mean $\pm$ SEM ($n=7-12$ rats). MDMA produced a significant rise in body temperature ($F(1,18)=268.25$, $p<0.001$) compared with the saline-injected group. This effect was not modified by EtOH exposure ($F(1,13)=0.84$, $p=0.84$, n.s.). Rats injected with CYAN on days 1 and 3 of EtOH treatment showed a less pronounced hyperthermic response than animals treated with EtOH only ($F(1,13)=4.52$, $p<0.05$). The *inset* shows the rectal temperature of rats pre-treated with EtOH and cyanamide and injected with saline instead of MDMA 24 h later. Rats pre-exposed to EtOH showed an increase in rectal temperature compared with those exposed to air ($F(1,10)=21.92$, $p<0.001$). CYAN did not modify the EtOH-induced changes in temperature ($F(1,4)=2.95$, $p<0.16$, n.s.). The *arrow* shows the time of saline administration

MDMA could be markedly increased probably due to the presence in the brain of high levels of acetaldehyde.

Cyanamide also inhibits catalase activity both in the liver and in the brain. Therefore, it could be argued that the enhanced neurotoxic effect induced by MDMA found in animals treated with ethanol and cyanamide could be attributed to higher brain levels of ethanol caused by an inhibition of its metabolism through catalase. However, the enzymatic activity of catalase in the liver is 45–60 fold higher than that observed in the brain (Yang and Lin 2002; Kaushik and Kaur 2003) and the inhibition of this activity in the periphery did not change the plasma levels of ethanol compared with those observed in animals exposed to ethanol alone (this paper). This indicates that ethanol metabolism follows other metabolic pathways. Consequently, there is no reason to assume that the more pronounced toxicity found in animals treated with ethanol and cyanamide is due to higher brain levels of ethanol caused by the inhibition of brain catalase activity.

What seems clear is that repetitive binge ethanol administration does not produce any long-term loss of the 5-HT nerve terminals of the hippocampus and frontal cortex. There is much experimental evidence indicating that binge ethanol administration produces blood alcohol levels similar to or lower than those obtained in the current study causing observable neuronal cell loss and that this effect is especially prominent in the hippocampus, entorhinal and frontal cortex (Collins et al. 1996; Obeirner et al. 2002b; Zharkovsky et al. 2003; Hamelink et al. 2005). Neuronal damage was visualized and quantified in the form of argyrophilic neurons with cupric-silver staining techniques (Collins et al. 1996; Hamelink et al. 2005), which, although methods of choice to assess irreversible neurodegeneration caused by a great variety of insults, have the disadvantage that they do not identify the nature of the damaged neuron. In this study, exposure to plasma ethanol levels of up to 450 mg/dl did not produce any long-term effect on the concentration of 5-HT or the density of 5-HT transporters in the hippocampus and cortex, which indicates an absence of neurotoxic effect on 5-HT containing neurons.

The mechanisms involved in the enhancing effect of ethanol against MDMA neurotoxicity are not totally understood at present. There is evidence that MDMA at the dose of 12.5 mg/kg given at standard ambient temperature increases the extracellular concentration of 2,3-DHBA and that this effect is involved in the long-term loss of 5-HT terminals (Colado et al. 1997a; Shankaran et al. 1999). The current study shows that this effect is also observed by administration of a lower dose of MDMA (5 mg/kg) at high ambient temperature and that ethanol treatment enhances the MDMA-induced rise in 2,3-DHBA in the hippocampal dialysate. Acute ethanol administration

produces a dose-dependent decrease in superoxide dismutase activity in all brain subcellular fractions and in catalase activity in brain mitochondria (Reddy et al. 1999). Superoxide dismutase catalyzes the dismutation of the superoxide anion to hydrogen peroxide (H_2O_2) (Freeman and Crapo 1982; Fridovich 1989) while catalase uses glutathione in the reduction of H_2O_2 to H_2O . Therefore, an increase in free radical formation induced by MDMA could be due to the impairment of the activity of the main cellular antioxidant systems caused by the previous ethanol exposure.

Rats kept in an inhalation chamber and exposed to humidified air showed a reduction in rectal temperature immediately after removal. This effect is not surprising because it is also observed in normal experimental room conditions when the body temperature is monitored throughout the day and mainly when rats are maintained without manipulation during some time (Orio et al. 2004). The exposure to binge plasma ethanol concentrations induced a more pronounced decrease in rectal temperature than that observed in rats exposed to air. This effect is only evident on the first days of treatment. After that, there appeared to be tolerance to the hypothermic effect of ethanol in such a way that rats exposed to ethanol showed a body temperature similar to that observed in rats exposed to air immediately after removal from the chamber. These data are consistent with earlier observations and confirm that the induction of tolerance can appear after one or two ethanol administrations (Crabbe et al. 1979; Maier and Pohorecky 1987; Tampier et al. 2000).

What is striking is that 24 h after ethanol exposure and when placed at high ambient temperature (30°C), rats show a body temperature higher than those exposed to air. It has been shown that the acute administration of relatively high doses of ethanol produces a condition of poikilothermia and that the maintenance of the animal at high ambient temperature immediately after ethanol injection elevates the rectal temperature of the animal during the time of heat exposure (Myers 1981). This effect reflects an alteration of the compensatory physiological responses mediating temperature control, including vasodilatation and tachypnea, which occur immediately in response to a thermal challenge. This impairment is sufficient to prevent heat loss and, consequently, to cause a marked hyperthermia if the ambient temperature is elevated.

MDMA increases rectal temperature shortly after peripheral administration. This hyperthermic response being a key factor in the long-term 5-HT neurotoxicity (Sanchez et al. 2001; Orio et al. 2004). However, the changes induced by ethanol in MDMA neurotoxicity do not seem to be related to an effect on the MDMA-induced hyperthermia. Animals exposed to ethanol developed a hyperthermic response similar to that observed in control rats treated with

MDMA alone but nevertheless the loss of 5-HT concentration and 5-HT transporter density in the hippocampus was more pronounced. In addition, when animals are treated with cyanamide and exposed to ethanol, the hyperthermia of MDMA is clearly less than that observed in animals exposed to ethanol and not given cyanamide, but the loss of 5-HT markers in the hippocampus and cortex was more pronounced. Taken together, these data indicate that the enhancing effect of ethanol on MDMA neurotoxicity is not due to an effect related to changes in body temperature.

In summary, this study demonstrates that exposure to binge plasma ethanol concentrations enhances the long-term neuronal damage induced by MDMA and points to an increase in hydroxyl radicals as a possible underlying mechanism. The magnitude of the effect is dependent on plasma acetaldehyde concentration suggesting that the MDMA-induced neurotoxicity could be more pronounced in aldehyde dehydrogenase deficient heavy ethanol drinkers.

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