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ORIGINAL INVESTIGATION

Administration of neurotoxic doses of MDMA reduces sensitivity to ethanol and increases GAT-1 immunoreactivity in mice striatum

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Abstract
Rationale Mice with reduced dopamine activity following neurotoxic doses of 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') consume more ethanol (EtOH) and show greater preference for EtOH. In keeping with human studies and other animal models where alcohol consumption and preference are also high, MDMA treatment will reduce sensitivity to certain physiological effects of EtOH.
Objective We have examined the sensitivity to the acute effects of EtOH in MDMA-lesioned mice and the effects of EtOH on striatal gamma-aminobutyric acid (GABA) accumulation and expression of GABA subtype-1 transporter (GAT-1).
Methods C57BL/6J mice were injected with neurotoxic MDMA (30 mg/kg, three times, every 3 h, i.p.). Seven days later, mice were given EtOH (3 g/kg, i.p.) to determine the loss of righting response and the development of rapid tolerance to the hypothermic effect of EtOH. The effect of EtOH on the striatal accumulation of GABA after inhibiting GABA transaminase and on GAT-1 immunoreactivity was also determined.
Results Mice pre-treated with a neurotoxic dose of MDMA were less sensitive to the sedative-hypnotic effect of acute EtOH and exhibited alterations in the development of rapid tolerance to the hypothermic effect of EtOH. These animals showed an increase in striatal GAT-1 immunoreactivity. EtOH reduced GABA concentration in the striatum of non-lesioned mice, an effect not observed in MDMA-lesioned mice.
Conclusion These findings indicate that mice with a MDMA-induced dopaminergic lesion show increased expression of striatal GAT-1 that may contribute to the lower sensitivity to EtOH-induced sedative effects and the resistance to the development of rapid tolerance to hypothermia produced by EtOH.

Keywords Ethanol · Ecstasy · Neurotoxicity · Hypothermia · Sedation · GABA accumulation · GABA transporter

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is widely used as a recreational drug by young people despite having been shown to be a potent neurotoxin in the brain of rodents and non-human primates (Green et al. 2003). In mice, MDMA produces relatively selective long-term neurotoxic damage to dopaminergic pathways, having little effect on 5-HT containing neurons (Stone et al. 1987; Logan et al. 1988; O'Callaghan and Miller 1994; Colado et al. 2001). This neurotoxicity is reflected by a sustained loss in the concentration of dopamine and its metabolites and in the density of dopamine transporters principally in the striatum (Mann et al. 1997; O'Shea et al. 2001; Escobedo et al. 2005, Granado et al. 2008a, b).

Recently, it has been shown that exposure to a neurotoxic dose of MDMA critically influences the regulation of ethanol

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(EtOH) drinking behaviour. Thus, mice pre-exposed to neurotoxic doses of MDMA exhibit a higher consumption of and preference for EtOH compared with saline-treated animals (Izco et al. 2007). Administration of the full D1 agonist, SKF81297, is sufficient to reduce EtOH consumption in MDMA-lesioned mice, suggesting that the impairment is caused by the reduced D1 receptor stimulation subsequent to a deficit in dopamine neurotransmission (Izco et al. 2007). There is strong evidence indicating that EtOH intake in humans and rodents can be negatively correlated with the initial sensitivity to EtOH and that, in consequence, high levels of EtOH drinking might be associated with resistance to the physiological effects produced by this substance (Harris et al. 1995; Hodge et al. 1999; Schuckit 1986, 1988, 1994; Schuckit and Smith 1996; Thiele et al. 2000). Thus, protein kinase A mutant mice exhibit high EtOH intake and low sensitivity to EtOH-induced sedation (Thiele et al. 2000). nNOS knockout mice as well as those lacking adenosine A2A receptors were found to be less sensitive to the sedative effects of EtOH and consumed more EtOH than wild-type mice (Naassila et al. 2002; Spanagel et al. 2002). In contrast, CB1 receptor gene knockout mice show decreased EtOH self-administration and increased alcohol sensitivity and withdrawal symptoms (Naassila et al. 2004).

We have now examined whether or not mice injected with neurotoxic doses of MDMA differ in terms of sensitivity to the sedative-hypnotic effect of EtOH and/or exhibit alterations in the development of rapid tolerance to the hypothermic effect of EtOH. The GABAergic system has been reported to be involved in EtOH-induced impairments of motor function and behavioural changes, and the gamma-aminobutyric acid (GABA) subtype-1 transporter (GAT-1) plays a role in EtOH tolerance and sensitivity (Hu et al. 2004). Therefore, a second purpose of the current studies was to investigate whether GAT-1 immunoreactivity and GABA accumulation following GABA transaminase inhibition are modified in striatum of MDMA-treated mice and whether these changes are altered by EtOH administration.

Materials and methods

Animals, drug administration and experimental protocol

Adult male C57BL/6J mice (Harlan Iberica, Barcelona, Spain) initially weighing 20–25 g were housed in groups of ten in standard cages, in conditions of constant temperature (21±2°C) and a 12 h light/dark cycle (lights on at 0700 hours) and given free access to food and water. Mice (n= 155) were randomly assigned to two treatment groups. Group I was injected with saline (referred to as non-lesioned), while group II received MDMA (30 mg/kg, i.p., three times with a 3-h interval, referred to as MDMA-lesioned). Seven days later, mice were injected with EtOH or saline. The protocol of MDMA administration used in this study induces neurotoxicity in dopamine nerve terminals 7 days later (O’Shea et al. 2001; Camarero et al. 2002; Escobedo et al. 2005). Behavioural experiments were performed by an experimenter blinded to the treatment administered to mice. Separate groups of animals were used for each study.

(±)-MDMA.HCl was obtained from Lipomed (Arlesheim, Switzerland), dissolved in 0.9% *w*/*v* NaCl (saline) and injected in a volume of 10 ml/kg. Doses are quoted in terms of the base.

For injections, absolute EtOH was diluted with 0.9% saline to a 20% *w*/*v* solution. [^{3}H]-WIN 35,428 was purchased from Perkin Elmer (Spain).

All experimental procedures were carried out at a room temperature of 21±2°C and performed in accordance with the guidelines of the Animal Welfare Committee of the Universidad Complutense de Madrid (following European Council Directives 86/609/CEE and 2003/65/CE).

Measurement of sensitivity to the hypnotic effects of EtOH

The evaluation of sensitivity to the sedative/hypnotic effects of EtOH was performed by measuring the duration of the loss of the righting response following a procedure similar to that used by Harris et al. (1995). Non- and MDMA-lesioned mice received an i.p. injection of EtOH 2.5 or 3 g/kg. Once the animals had become ataxic, each mouse was placed on its back in a plastic U-shaped trough. The time (in minutes) that elapsed between the onset of EtOH-induced sedation and the moment when the mice could right themselves onto all four paws three times within a 30-s interval was used as an index of time to regain the righting reflex.

Measurement of tolerance to EtOH-induced hypothermia

The procedure followed for the measurement of rapid tolerance to EtOH-induced hypothermia was similar to that previously described by Crabbe et al. (1979) in terms of injection schedule and dosing. Briefly, non- and MDMA-lesioned mice were randomly separated into three groups. On day 1, the first two groups received saline, and the remaining group received EtOH (3 g/kg, i.p.). On day 2, basal body temperature was measured for all animals, and then group 1 received saline and groups 2 and 3 received EtOH (3 g/kg, i.p.). Rectal temperature was monitored starting 30 min after injection and up to 3 h later. A digital readout thermocouple (BAT-12 thermometer, Physitemp Instruments, Clifton, NJ, USA) with a resolution of 0.1°C

and accuracy of ± 0.1°C attached to a RET-3 rodent sensor was inserted 2.0 cm into the rectum of the mouse, the animal being lightly restrained by holding it in the hand. A steady readout was obtained within 10 s of probe insertion.

Measurement of dopamine and metabolites in the striatum

Striatal catechol concentration was evaluated 7 days after MDMA injection in order to check the long-term depletion of dopamine concentration exerted by MDMA. The mice were killed by cervical dislocation and decapitation, the brains rapidly removed and the striatum dissected out on ice. The striatum was chosen because the nigrostriatal dopaminergic pathway is selectively damaged by MDMA (Granado et al. 2008a, b).

Dopamine and the metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were measured by high-performance liquid chromatography (HPLC) and electrochemical detection. The mobile phase consisted of KH_2PO_4 (0.05 M), octanesulfonic acid (0.4 mM), EDTA (0.1 mM) and methanol (16%) and was adjusted to pH3 with phosphoric acid, filtered and degassed. The flow rate was 1 ml/min. 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; Waters, Spain) with a precolumn and a coulometric detector (Coulochem II, Esa, USA). The working electrode potential was set at 400 mV with a gain of 1 µA (for dopamine) and 500 nA (for the metabolites). The current produced was monitored by means of integration software (Unipoint, Gilson).

[^{3}H]-WIN 35,428 binding in tissue homogenates

[^{3}H]-WIN 35,428 binding was measured by modification of the method described in detail by Segal et al. (2003). The animals were killed, the brain rapidly removed and dissected on ice within 2 min. Striata from individual animals were sonicated in ice-cold sodium phosphate buffer (20 mM; pH7.4) containing sucrose (0.32 M). The homogenate was centrifuged at 30,000×*g* for 15 min at 4°C. The supernatant was discarded and the wash procedure repeated twice more. The pellet was finally resuspended in 60 vol. of homogenisation buffer. The assay solution (500 µl) contained [^{3}H]-WIN 35,428 (5 nM), desipramine (300 nM) and 100 µl tissue preparation (approximately 80 µg protein). Non-specific binding was carried out in the presence of cocaine (30 µM). The reaction mixture was incubated for 90 min at 4°C. The assay was terminated by rapid filtration, and radioactivity was counted by scintillation spectrometry. Protein concentrations were measured by the method of Lowry et al. (1951).

GABA accumulation following GABA transaminase inhibition

The ability of acute EtOH administration to alter GABA turnover was measured indirectly by determining the accumulation of GABA in the striatum following the administration of aminooxyacetic acid (AOAA), a GABA-transaminase (GABA-T) inhibitor. For this, 7 days after saline or MDMA administration, animals received AOAA (12 mg/kg, i.p.) 10 min after EtOH (3 g/kg, i.p.; Bernasconi et al. 1982; Hellewuo and Kiianmaa 1989). GABA accumulation was determined 1 h after inhibition of GABA-T by AOAA. To prevent non-specific postmortem synthesis of GABA, 3-mercaptopropionic acid (100 mg/kg, i.p.) was injected 3 min before decapitation (Gomes and Trolin 1982). GABA was measured by HPLC with electrochemical detector using a precolumn *o*-phthaldehyde/sulphite derivatisation method. The derivatisation reagent was composed of *o*-phthaldehyde (110 mg), EtOH (250 µl), 1 M Na_2SO_3 (250 µl) and 0.1 M $Na_2B_4O_7{\cdot}10H_2O$ (pH=10.4, 4.5 ml). Aliquots of samples (10 µl) were incubated with derivatization reagent (25 µl) and β-aminobutyric acid as internal standard (5 µg/ml) for 20 min at 35°C. The mobile phase consisted of KH_2PO_4 (0.1 M), octanesulfonic acid (0.4 mM), EDTA (0.5 mM) and methanol (25%) and was adjusted to pH4.5 with phosphoric acid, filtered and degassed. The flow rate was 0.3 ml/min. 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, 3 µm, 150×2.1 mm; Waters, Spain) with a precolumn and a coulometric detector (Coulochem II, Esa, USA). The working electrode potential was set at 500 mV with a gain of 2 µA. The current produced was monitored by means of integration software (Unipoint, Gilson).

Western blot analysis of GAT-1 expression

To analyse striatal GAT-1 expression following MDMA treatment and the effect of EtOH, non- and MDMA-lesioned mice were separated into two groups, were given either saline or ETOH (3 g/kg, i.p.) and killed 1 h later as described above. The brain was rapidly removed and dissected; the samples were frozen on dry ice and stored at −80°C until the assay. Striata were homogenised in ice-cold buffer (50 mM Tris–HCl, 0.32 M sucrose, 1 mM dithiothreitol, pH7.4 containing protease inhibitors) in the presence of 0.5% Nonidet P-40 and centrifuged at 27,000×*g* for 20 min at 4°C. Supernatant protein concentration was measured using a DC protein assay kit (Bio-Rad Laboratories, Madrid, Spain). Samples of total protein (50 µg) were subjected to 10% sodium dodecyl sulphate

polyacrylamide gel electrophoresis under reducing conditions and transferred to a nitrocellulose filter. Blots were blocked for 1 h with 5% non-fat powdered milk in Tris-buffered saline (TBS) buffer (10 mM Tris and 150 mM NaCl) at room temperature and then incubated overnight at 4°C with rabbit anti-GAT-1 (Chemicon International, Madrid, Spain) and mouse anti-β-actin (Sigma, Madrid, Spain) antibodies diluted 1:200 and 1:6,000, respectively, in TBST (TBS and 0.1% Tween-20) followed by donkey anti-rabbit IgG and sheep anti-mouse IgG both conjugated to horseradish peroxidase (1:2,000, GE Healthcare, Madrid, Spain) for 1 h at room temperature. Chemiluminiscence was measured using the ECL detection kit (GE Healthcare). Different film exposure times were used to ensure that bands were not saturated. Quantification of the film was performed by Quantity One software (Bio-Rad Laboratories). Equal protein sample loading was confirmed by quantification of the β-actin signal.

Statistics

Neurotoxicity data were analysed using Student's *t* test. The rest of the results were analysed using two-way ANOVA followed by Bonferroni post hoc comparisons.

Results

EtOH-induced sedation

Two-way ANOVA indicated that there was a significant effect of treatment ($F_{1,25}$=28.75, P<0.0001) and lesion ($F_{1,25}$=19.01, P=0.0002) but no interaction ($F_{1,25}$=1.08, P=0.31). Non-lesioned mice responded to the hypnotic effects of EtOH in a dose-dependent manner, regaining the righting reflex 24 and 42 min after administration of 2.5 and 3 g/kg of EtOH, respectively. MDMA-lesioned animals were less sensitive to the sedative effects of EtOH, regaining the righting reflex significantly sooner than the control mice after injection of both 2.5 and 3 g/kg EtOH doses (Fig. 1).

Tolerance to EtOH-induced hypothermia

Two-way ANOVA indicated that, in both non- and MDMA-lesioned mice, there was a significant effect of treatment ($F_{2,133}$=80.39, P<0.0001; $F_{2,119}$=158.7, P<0.0001, respectively), time ($F_{6,133}$=20.75, P<0.0001; $F_{6,119}$=27.12, P<0.0001, respectively) and interaction ($F_{12,133}$=5.58, P<0.0001; $F_{12,119}$=9.07, P<0.0001, respectively). EtOH markedly reduced rectal temperature in MDMA- and non-lesioned mice for at least 3 h. Twenty-four hours later, a second EtOH injection produced a less

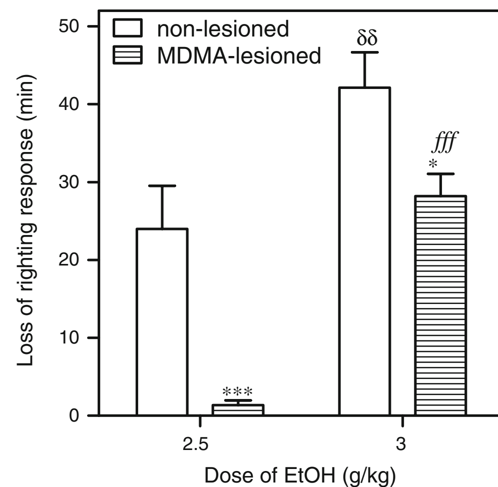

Fig. 1 Ethanol (EtOH)-induced loss of righting response in mice exposed to MDMA or saline 7 days before. Mice received MDMA (30 mg/kg, i.p.) three times with a 3-h interval. Time elapsed between the appearance of sedation following EtOH (2.5 and 3 g/kg) injection and righting of mice onto all four paws three times within a 30-s interval was used as index of time to regain the righting reflex. Results shown as mean±SEM, n=5 (MDMA + EtOH 3 g/kg), n=8 (remaining groups). Different from the corresponding non-lesioned mice, *P<0.05, ***P<0.001. Different from EtOH 2.5 g/kg in non-lesioned mice, $^{\delta\delta}P$<0.01. Different from MDMA-lesioned receiving EtOH 2.5 g/kg, ^{fff}P<0.001

pronounced hypothermic response in non-lesioned animals, which resolved by 1.5 h (rapid tolerance; Fig. 2, upper panel). However, in MDMA-lesioned mice, there was no difference between changes induced by the first and second EtOH administration since neither the extent of the hypothermic response nor the duration is altered on second exposure to EtOH (Fig. 2, lower panel).

Effects of EtOH on GABA turnover and GAT-1 expression

Two-way ANOVA indicated that there was a significant effect of treatment ($F_{1,18}$=8.84, P=0.0082) but not lesion ($F_{1,18}$=2.95, P=0.10) nor interaction ($F_{1,18}$=2.02, P=0.17). The AOAA-induced GABA accumulation in the striatum was similar in non- and MDMA-lesioned mice. EtOH reduced GABA concentration in the striatum of non-lesioned mice, an effect which was not observed in MDMA-lesioned mice (Fig. 3).

Two-way ANOVA indicated that there was a significant effect of treatment ($F_{1,26}$=6.68, P=0.02) and lesion ($F_{1,26}$=23.60, P< 0.0001) but no interaction ($F_{1,26}$=0.39, P=0.54). Striatal GAT-1 immunoreactivity was higher in MDMA-lesioned mice compared with the non-lesioned group. EtOH tended to attenuate the rise in GAT-1 expression in MDMA-lesioned animals, although the effect was not significant. EtOH did not produce any effect on GAT-1 immunoreactivity in the striatum of non-lesioned mice (Fig. 4).

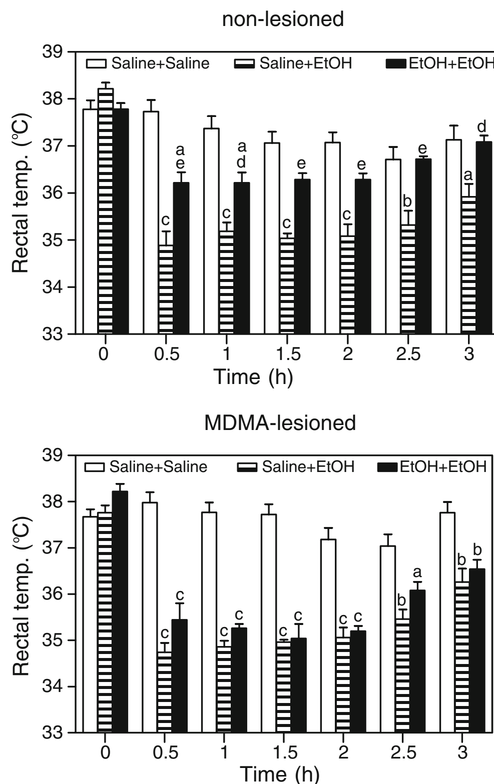

Fig. 2 Development of rapid tolerance to ethanol (EtOH)-induced hypothermia in non-lesioned but not in MDMA-lesioned mice. Mice received MDMA (30 mg/kg, i.p.) three times with a 3-h interval 7 days before EtOH. *Graphs* represent the effect of a second EtOH (3 g/kg) injection in mice that had received a first injection (3 g/kg) the day before. Results shown as mean±SEM, n=10 (in each saline+saline group). EtOH administration to mice treated with saline 24 h before produced hypothermia in both non-lesioned (*upper panel*, n=6) and MDMA-lesioned mice (*lower panel*, n=5). The hypothermic response to the second EtOH injection was attenuated compared with the first injection in non-lesioned (n=6) but not in MDMA-lesioned mice (n=5). Different from saline, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. Different from first EtOH dose, $^{d}P<0.05$, $^{e}P<0.01$

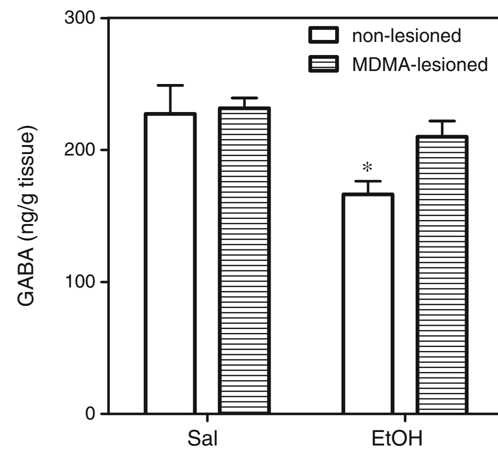

Fig. 3 Effect of ethanol (EtOH) on GABA levels in the striatum of mice treated 7 days before with MDMA or saline. Mice received MDMA (30 mg/kg, i.p.) three times with a 3-h interval. GABA was determined 1 h after inhibition of GABA transaminase (GABA-T). Mice received aminooxyacetic acid (12 mg/kg, i.p.) 10 min after ethanol (EtOH, 3 g/kg, i.p.) to inhibit GABA-T and 3-mercaptopropionic acid (100 mg/kg, i.p.) 3 min before decapitation to prevent non-specific postmortem synthesis of GABA. Results shown as mean±SEM, n=5 (in each non-lesioned group), n=6–7 (in each MDMA-lesioned group). Different from non-lesioned mice injected with saline, *$P<0.05$

Dopamine concentration and dopamine transporter density in MDMA-pretreated mice

Seven days after MDMA (30 mg/kg, three times at three hourly intervals), the time at which behavioural and neurochemical assays were performed, there was a reduction of 62% in striatal dopamine content measured by HPLC and a decrease of 50% in the density of dopamine transporters in the striatum quantified by [^{3}H]-WIN 34,428 radioligand binding. DOPAC and HVA levels were reduced about 35% (Table 1).

Discussion

This study shows for the first time that mice exposed to a neurotoxic dose of MDMA are less sensitive to the sedative-hypnotic effect of acute EtOH and exhibit alterations in the development of rapid tolerance to the hypothermic effect of EtOH. In agreement with previous data (O'Shea et al. 2001; Escobedo et al. 2005), 7 days after the acute administration of repeated doses of MDMA, the time at which EtOH was administered, mice showed a decrease in striatal dopamine concentration and in the density of dopamine uptake sites. The MDMA-induced loss in the dopaminergic markers reflects a loss of dopaminergic terminals in striatum and cell bodies in the substantia nigra of mice (Granado et al. 2008a, b).

Repeated MDMA administration makes mice more resistant to the impairing effects of acute EtOH injection, as mice are less sensitive to acute EtOH-induced sedation. Mice lesioned with MDMA recovered from the ataxic effects of EtOH much faster than control animals. This lower sensitivity to the acute effects of EtOH is also associated with resistance to the development of rapid tolerance to the hypothermic effects of EtOH. Results show that there was a clear difference between MDMA- and non-lesioned mice in hypothermic tolerance development after repeated injections of EtOH over two consecutive days. Hypothermia was less pronounced in non-lesioned animals, which had received EtOH 24 h before with no hypothermia being observed 1.5 h after the EtOH injection (rapid tolerance). However, in MDMA-lesioned mice, there was no difference between the changes induced by the first and second EtOH administration. Development of rapid tolerance to the changes in temperature induced by EtOH is not related to differences in the initial sensitivity to the

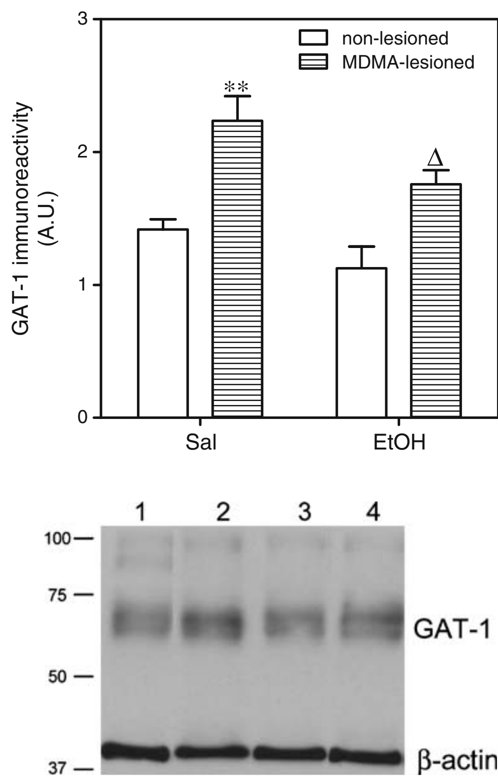

Fig. 4 Effect of ethanol (EtOH, 3 g/kg, i.p.) on GAT-1 protein levels in the striatum of mice treated with MDMA or saline 7 days before. Mice received MDMA (30 mg/kg, i.p.) three times with a 3-h interval. Data expressed as arbitrary units (AU). Results shown as mean±SEM, n=10 (in each non-lesioned group), n=5 (in each MDMA-lesioned group). Different from non-lesioned mice injected with saline, **P< 0.01. Different from non-lesioned mice injected with EtOH, $^{\Delta}P$<0.05. GAT-1 immunoreactivity in the striatum of non-lesioned mice injected with saline (*1*) or EtOH (*3*) and MDMA-lesioned mice injected with saline (*2*) or EtOH (*4*)

hypothermia by EtOH, since the initial reduction on rectal temperature induced by EtOH was similar in non-lesioned and MDMA-pretreated mice. Previous studies have shown no differences in plasma EtOH concentration during 4.5 h after 3 g/kg EtOH administration between non- and MDMA-lesioned animals (Izco et al. 2007); thus, it is unlikely that differences in clearance are an issue in the observed hypothermic and sedative effects.

The existence of a correlation between sensitivity to EtOH-induced acute effects and acquired tolerance has been shown previously (Tabakoff and Culp 1984; Crabbe 1994; Crabbe et al. 1996). Thus, the M520 strain of rats that is initially more sensitive to acute alcohol incoordinating effects was found to develop tolerance at a faster rate than those displaying greater resistance to the incoordinating effects of EtOH (Tabakoff and Culp 1984). The neurochemical mechanism underlying this altered behavioural response to EtOH is unknown.

GABA has been reported to be involved in EtOH-induced alterations of motor function (Cott et al. 1976; Frye et al. 1983) and behavioural changes (Liljequist and Engel 1982). For instance, the GABA receptor agonist muscimol potentiates the sedative properties of EtOH, while antagonists of $GABA_A$ receptors such as picrotoxin or bicuculline reduce EtOH-induced sleep time and antagonise the motor impairment induced by EtOH (Frye and Breese 1982; Liljequist and Engel 1982; Martz et al. 1983). In addition, it has been proposed that EtOH tolerance and dependence may be related to decreased sensitivity to GABA (Martz et al. 1983). GABA seems also to be involved in the regulation of body temperature (Allan and Harris 1989; Biswas and Poddar 1988; Fukuda et al. 1997; Nakamura et al. 2002), although there is no agreement on this point (Liljequist and Engel 1982; Dar and Wooles 1985). In this study, the effect of EtOH on GABA accumulation was determined by measuring the accumulation of neurotransmitter after specific inhibition of GABA transaminase. The AOAA-induced GABA accumulation in the striatum was similar in non- and MDMA-lesioned mice. EtOH reduces striatal GABA concentration in non-lesioned mice, a result that is in agreement with previous studies using similar EtOH doses and measuring GABA accumulation after AOAA (Supavilai and Karobath 1980; Wixon and Hunt 1980; Dar and Wooles 1985; Hellewuo and Kiianmaa 1989). However, EtOH does not significantly modify GABA content in the striatum of MDMA-lesioned mice. GABA is metabolised almost exclusively into succinic semialdehyde (SSA) by GABA-T, and therefore, the amount of GABA accumulated after inhibition of GABA-T should be equal to the amount of GABA synthesised (Iversen 1978). In addition, it has been shown that AOAA at a dose of around 15 mg/kg hardly inhibits glutamate decarboxylase in vivo in mouse brain and, therefore, can be

Table 1 Concentration (ng/g tissue) of dopamine, DOPAC and HVA and density (fmol/mg protein) of dopamine transporter in the striatum of mice pre-treated with MDMA or saline 1 week before

Treatment	Dopamine	DOPAC	HVA	[^{3}H]-WIN 35,428 binding
Saline	10,238±393 (10)	677±25 (9)	1,373±49 (10)	115±10 (8)
MDMA	3,882±301* (9)	418±22* (8)	904±41* (9)	57±7 (8)*

Mice received MDMA (30 mg/kg, i.p.) three times with a 3-h interval. Results shown as mean±SEM (n). Different from the corresponding saline-pretreated mice, *P<0.001

used for studies of GABA synthesis (Gomes and Trolin 1982). On this basis, the effect of EtOH in the striatum of the non-lesioned group could be interpreted as a consequence of the ability of EtOH to increase GABA release from the presynaptic GABAergic terminals and produce, on the one hand, a reduction in GABA synthesis through presynaptic interactions (Ariwodola and Weiner 2004; Roberto et al. 2004; Carta et al. 2004; Criswell and Breese 2005) and, on the other hand, to facilitate the opening of chloride channels modulated by $GABA_A$ receptors by an allosteric postsynaptic mechanism (Koob 2004). In MDMA-lesioned mice, EtOH did not modify striatal GABA accumulation, supporting the hypothesis that the regulation of GABAergic neurons in the brain of lesioned mice is not sensitive to or is, at least, less sensitive to the effects of EtOH than that of the non-lesioned mice. Similar results have been obtained in electrophysiological and in vitro slice studies, where EtOH reduces GABAergic activity more in the brain of the EtOH-sensitive long sleep than EtOH-insensitive short—sleep mice (Sorensen et al. 1980; Howerton and Collins 1984).

Although differences in the effect of EtOH on GABA neurotransmission in non- and MDMA-lesioned mice are not easily explained, it is worth pointing out that dopamine D1 receptors stimulate GABA neurotransmission in striatonigral pathways (Ferre et al. 1996; Yamamoto and Soghomonian 2008) via a cyclic AMP/protein kinase A (PKA) pathway (Arias-Montaño et al. 2007) and that MDMA-lesioned mice show an increase in the density of D1 receptors (Izco et al. 2007), an effect that can be interpreted as a compensatory up-regulation of postsynaptic D1 receptors in response to decreased dopamine content. Therefore, it does not seem unreasonable to propose that the MDMA-induced dopamine damage could result in an increased expression of proteins involved in the modulation of GABA-mediated signalling, and as a consequence, the inhibitory effect of EtOH on GABA turnover would be abolished. In addition, this would be in agreement with PKA-knockout mice being resistant to EtOH sedation (Thiele et al. 2000).

GAT-1 is the predominant GABA transporter; it is responsible for the rapid transport of synaptically released GABA as well as for regulating basal GABA levels in the extracellular space (Radian et al. 1990; Richerson and Wu 2003) and, therefore, is another important modulator in mediating the action of EtOH in vivo. Interestingly, MDMA-lesioned mice showed increased GAT-1 immunoreactivity in striatum compared with non-lesioned mice. Therefore, it seems reasonable to propose that, in MDMA-lesioned mice, the extracellular concentration of GABA following EtOH administration would be lower than that in non-lesioned mice, thus also reducing GABA accumulation in lesioned animals. Lower extracellular GABA concentrations could explain the less pronounced sedative effects of EtOH. These findings are in agreement with those showing that mice pre-injected with a competitive or a non-competitive antagonist of GAT-1 showed high sensitivity to the sedative/hypnotic effects of EtOH (Hu et al. 2004). In this line, it has been shown that transgenic mice overexpressing GAT-1 displayed low sensitivity to EtOH, as shown by the righting reflex test (Cai et al. 2006). All of these data indicate that differences in GABA accumulation and in sensitivity to the effects of EtOH seem to be related to changes in striatal GAT-1 expression.

In summary, this study indicates for the first time that mice with a long-lasting MDMA-induced dopaminergic lesion exhibit reduced sensitivity to the hypnotic effects of EtOH and a decreased development of tolerance to its hypothermic effects. Both effects could be due to a higher expression of GAT-1 in the striatum of MDMA-lesioned mice and to the inability of EtOH to reduce GABA accumulation in these mice. The resistance to the acute effects of EtOH observed in MDMA-lesioned mice probably contributes to the higher consumption of and preference for EtOH exhibited by these animals compared with non-lesioned mice (Izco et al. 2007).

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