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ARTICLE

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Effects of ethanol and 3,4-methylenedioxymethamphetamine (MDMA) alone or in combination on spontaneous and evoked overflow of dopamine, serotonin and acetylcholine in striatal slices of the rat brain

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Abstract

Ethanol (EtOH) potentiates the locomotor effects of 3,4-methylenedioxymetamphetamine (MDMA) in rats. This potentiation might involve pharmacokinetic and/or pharmacodynamic mechanisms. We explored whether the latter could be local. Using a slice superfusion approach, we assessed the effects of MDMA (0.3, 3 μM) and/or EtOH (2‰, corresponding to 34.3 mM) on the spontaneous outflow and electrically evoked release of serotonin (5-HT), dopamine (DA) and acetylcholine (ACh) in the striatum, and for comparison, on 5-HT release in hippocampal and neocortical tissue. MDMA and less effectively EtOH, augmented the outflow of 5-HT in all regions. The electrically evoked 5-HT release was increased by MDMA at 3 μM in striatal slices only. With nomifensine throughout, EtOH significantly potentiated the 0.3 μM MDMA-induced outflow of 5-HT, but only in striatal slices. EtOH or MDMA also enhanced the spontaneous outflow of DA, but MDMA reduced the electrically evoked DA release. With fluvoxamine throughout superfusion, EtOH potentiated the effect of MDMA on the spontaneous outflow of DA. Finally, 3 μM MDMA diminished the electrically evoked release of ACh, an effect involving several receptors (D_2, 5-HT_2, NMDA, nicotinic, NK_1), with some interactions with EtOH. Among other results, we show for the first time a local synergistic interaction of EtOH and MDMA on the spontaneous outflow of striatal DA and 5-HT, which could be relevant to the EtOH-induced potentiation of hyperlocomotion in MDMA-treated rats. These data do not preclude the contribution of other pharmacodynamic and/or pharmacokinetic mechanisms in vivo but support the hypothesis that EtOH may affect the abuse liability of MDMA.

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Key words: EtOH, hippocampus, MDMA, neocortex, neurotransmitter release, striatum.

Introduction

The amphetamine derivative (±)-3,4-methylenedioxymethamphetamine (MDMA, ecstasy) is a popular recreational drug of young people (Green et al., 1995, 2003; Schifano, 2004). In humans and rats, MDMA causes sustained release of serotonin (5-HT) and, to a lesser extent, of dopamine (DA) in various brain regions, moreover it has psychostimulant effects, produces hyperthermia and, depending on dose and environmental conditions, may be toxic for serotonergic neurons in the long term (Green et al., 2003). In humans, MDMA is frequently taken with

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other drugs, e.g. amphetamine, cocaine, cannabis, or ethanol (EtOH) (Lora-Tamayo et al., 2004; Pedersen and Skrondal, 1999; Schifano, 2004; Topp et al., 1999). Taken with EtOH the euphoric effects of MDMA were shown to be longer lasting and a dissociation between subjective and objective sedation was observed (Hernandez-Lopez et al., 2002). Both pharmacodynamic and pharmacokinetic mechanisms may contribute to these effects of EtOH (Oesterheld et al., 2004). For example, in mice EtOH was shown to increase the brain concentration of MDMA (Johnson et al., 2004).

We recently showed that co-administration of EtOH and MDMA in rats potentiated the locomotor effects of MDMA (Cassel et al., 2004; Hamida et al., 2006, 2007). The bases for this potentiation are still unknown, although the concurrent addition of EtOH did not increase cortical, striatal or hippocampal MDMA levels at a post-administration delay of 45 min (Hamida et al., 2007), an observation which appears to preclude a pharmacokinetic explanation. Because the release of DA in the nucleus accumbens is thought to participate in the hyperlocomotor effects of psychostimulants, and because 5-HT may synergistically contribute to this response – if not being essential in the case of MDMA (Callaway et al., 1990) – the mesolimbic dopaminergic system becomes a potential target where EtOH taken in addition to MDMA could enhance activity levels of monoaminergic terminals. This hypothesis seems all the more reliable because EtOH alone, when given systemically, increases the release of both DA and 5-HT in the nucleus accumbens (Di Chiara and Imperato, 1988; Yoshimoto et al., 1992). There is evidence showing that a local action of EtOH may contribute to (but certainly does not exclusively account for) enhanced DA release (Brodie et al., 1990; Löf et al., 2007; Wozniak et al., 1991; Yim et al., 1998). Interestingly, cocaine-induced excitation of ventral tegmental area (VTA) dopaminergic neurons is also enhanced by EtOH (Bunney et al., 2000), perhaps with participation of the psychoactive metabolite cocaethylene (Pan and Hedaya, 1999). The EtOH-induced enhancement of DA release is not necessarily direct. Indeed, according to a recent study by Löf et al. (2007), indirect mechanisms involving acetylcholine (ACh) in the VTA, and more particularly the activation of nicotinic receptors, contribute to the increased DA release within the nucleus accumbens. In that context, it is also noteworthy that DA release, as shown in vitro (Cao et al., 2005; Grady et al., 1992a, 1994, 2002; Quik et al., 2003; Wonnacott et al., 2000), can be elicited by activation of nicotinic heteroreceptors located on dopaminergic terminals.

Although local application of EtOH augments the release of 5-HT (Yan et al., 1996), this effect is neither calcium-dependent nor tetrodotoxin-sensitive, suggesting that it is not exocytotic and not related to action potential propagation. Given systemically, EtOH decreases the firing rate of 5-HT neurons in the dorsal raphe, but increases the release of 5-HT in the striatum, suggesting that the EtOH-driven effect on 5-HT release involves local mechanisms (Thielen et al., 2001). Other studies support this possibility, at least for the VTA (e.g. Yoshimoto et al., 1992). Finally, 5-HT was found to potentiate the EtOH-induced activation of VTA neurons (Brodie et al., 1995).

All of these data indicate that under some conditions, the effects of EtOH on the release of DA and/or 5-HT may partly rely upon local mechanisms. Hence, it seems worthwhile to explore whether locally acting mechanisms could contribute to the EtOH-induced potentiation of the hyperlocomotion induced by MDMA (Cassel et al., 2004; Hamida et al., 2006, 2007). We therefore investigated the effects of MDMA, EtOH and their combination on the spontaneous and electrically evoked release of 5-HT, DA and ACh in *striatal* slices of the rat brain, a region particularly involved in the regulation of locomotion. Moreover, for comparison, the effects of these treatments were also characterized on 5-HT release in hippocampal and neocortical slices from the same animals.

Materials and methods

Subjects

Male Long–Evans rats (age 3–4 months; Centre d'élevage R. Janvier, Le Genest-St-Isles, France) were used for the main part of this study. Wistar rats of the same age (from the animal house of the University of Freiburg) were used for pilot experiments, where indicated. The latter experiments aimed at setting up the release protocols. All rats were housed individually in transparent Makrolon cages ($42 \times 26 \times 15$ cm) under controlled temperature (23 °C) and a 12 h light/dark cycle (lights on at 07:00 hours). Food and water were provided ad libitum. After arrival in the laboratory, the animals were allowed to acclimatize for 1 wk before the experiments were started. All procedures involving animal care and experimentation were conducted in accordance with institutional guidelines that comply with German law for animal protection (DtTSchG, 25.5.1998, last modification: 21.6.2005) and the European Communities Council Directive of 24 November 1986 (86/609/EEC); reference of the official licence to J-C.C. is no. 67-215).

Chemicals and drugs

Chemicals and drugs were obtained from the following sources: [^{3}H]DA (dihydroxyphenylethylamine 3,4-[ring-2,5,6-^{3}H]-(DA), 60.0 Ci/mmol) and [^{3}H]5-HT (5-[1,2-^{3}H(N)] hydroxytryptamine creatinine sulphate, 30.0 Ci/mmol) from PerkinElmer, Rodgau, Germany; [^{3}H]choline ([methyl-^{3}H]choline, 82 Ci/mmol) from GE Healthcare GmbH, Freiburg, Germany; ethanol (HPLC gradient grade) from Carl Roth, Karlsruhe, Germany; 3,4-methylenedioxymethamphetamine (MDMA), olanzapine, selegeline from Sigma-Aldrich, Taufkirchen, Germany; bromocriptine methane sulfonate from RBI, Natick, MA, USA; mecamylamine HCl from Merck, Darmstadt, Germany; *N*-acetyl-L-tryptophan 3,5-bis(trifluoro-methyl)benzyl ester (L-732,138); MK-801 maleate, D,L-2-amino-5-phosphonopentanoic acid (DL-AP5), ritanserin and tropanyl-3,5-dimethylbenzoate from BioTrend (Tocris), Köln, Germany.

The following drugs were kindly donated: entacapone (Orion Pharma, Turku, Finland); fluvoxamine-maleate (Duphar, Veesp, The Netherlands); nomifensine hydrogen maleate (Hoechst, Germany); L-sulpiride (Ravizza, Milan, Italy).

Locomotor activity

Activity measures for the rats were obtained in their home cages by automated recording devices, and all rats were tested as previously described (Cassel et al., 2004). Because previous studies showed the EtOH-induced potentiation of the psychostimulant effects of MDMA to be maximal during the first post-injection hour, the rats' activity was monitored only during this interval. All animals were left undisturbed during recording. As in our previous studies, activity was monitored on the first and last days of acclimatization to the test room. Then, over an additional 3 d, all rats were familiarized with being intraperitoneally (i.p.) injected with 0.9% NaCl at 12:00 hours. Activity was recorded during 2 h before and 4 h after each injection. Subsequently, activity was recorded 2 h before and over 1 h after injections of MDMA (3.3, 6.6, 10 and 20 mg/kg i.p.), in combination or not with 1.5 g/kg EtOH (Hamida et al., 2007). The drugs were administered in a volume of 7.5 ml/kg. In case of MDMA + EtOH treatment, MDMA was diluted in the EtOH solution as in our previous experiments. Control groups were given NaCl (dose 0 of MDMA) or EtOH alone. The ambient temperature of the room during testing was 23 ± 1 °C and rats had free access to food and water during monitoring.

Electrically evoked release of [^{3}H]DA and [^{3}H]5-HT

Rats that had never been treated with MDMA or EtOH before were anaesthetized in a carbon dioxide chamber and then decapitated. The brain was quickly removed and transferred into ice-cold modified Krebs–Henseleit (KH) buffer containing (in mM): 118 NaCl, 4.8 KCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 10 glucose, 0.6 ascorbic acid, 0.03 Na_2EDTA; the buffer was saturated with carbogen (95% O_2/5% CO_2) and the pH adjusted to 7.4. Both striata were dissected out from the brain and cut along the antero-posterior axis in 300-μm-thick slices using a McIlwain tissue chopper. The resulting slices were washed three times with ice-cold KH buffer and incubated for 30 min at 37 °C under carbogen in 2 ml KH buffer containing either [^{3}H]DA (0.1 μmol/l, in the presence of 1 μM fluvoxamine), or [^{3}H]5-HT (0.1 μmol/l, in the presence of 1 μM nomifensine), respectively. Fluvoxamine or nomifensine were added to the respective superfusion media to avoid false labelling (i.e. [^{3}H]DA in serotonergic terminals or [^{3}H]5-HT in dopaminergic ones). After incubation, all brain slices were washed three times with KH buffer (37 °C), transferred into superfusion chambers (one slice per chamber) and superfused continuously with oxygenated KH buffer (37 °C) at a flow rate of 0.6 ml/min. The superfusion medium was supplemented with 1 μM fluvoxamine for [^{3}H]DA release and with 1 μM nomifensine for [^{3}H]5-HT release, if not indicated otherwise. After 25 min of superfusion, brain slices were electrically pre-stimulated (18 rectangular pulses at 3 Hz, 2 ms, 4 V/chamber, 26–28 mA), as done routinely to speed up the release of excessive tritium and reach a stable baseline level. After 49 min of superfusion, the collection of 4-min fractions was started. The release of [^{3}H]DA or [^{3}H]5-HT was induced at two occasions by electrical field stimulation (360 rectangular pulses at 3 Hz, 2 ms, 4 V/chamber, 26–28 mA) after 57 min (S_1) and 101 min (S_2) of superfusion. Drugs to be tested [2‰ EtOH (v/v, which corresponds to 34.3 mM), 0.3 or 3 μM MDMA, or 0.3 or 3 μM MDMA + 2‰ EtOH] were added to the superfusion buffer from 24 min before S_2 onwards (i.e. after 77 min of superfusion). At the end of the experiment (i.e. after 125 min of superfusion), the radioactivity of the superfusate samples and the residual radioactivity in the slices (dissolved in 300 μl Solvable 350®, PerkinElmer) were determined by liquid scintillation counting.

Experiments on the spontaneous and evoked overflow of [^{3}H] in rat hippocampal and neocortical slices preincubated with [^{3}H]5-HT were performed as

described above. Hippocampal and neocortical slices (300-μm thick) were prepared as described previously from each brain used for the preparation of striatal slices (Birthelmer et al., 2003a,b).

Electrically evoked release of [^{3}H]ACh

These experiments were performed as described above with the following modifications: striatal slices were preincubated in the presence of [^{3}H]choline (0.1 μM, in the presence of 1 μM fluvoxamine) and superfused in KH buffer containing routinely 1 μM fluvoxamine; collection of 4-min fractions started after 34 min. The slices were stimulated electrically twice after 42 (S_1) and 66 min (S_2) of superfusion using the following stimulation conditions: 36 pulses at 3 Hz (2 ms, 25–28 mA). EtOH, MDMA or their combination were added 4 min before S_2 only, i.e. 62 min after the start of the superfusion. In some experiments (as indicated), additional drugs were present throughout the entire superfusion period or even during the preincubation period.

Calculations and statistics

For the analysis of activity scores, we used a MDMA (0, 3.3, 6.6, 10, 20 mg/kg) × EtOH (0, 1.5 g/kg) ANOVA followed, where appropriate, by the Newman–Keuls test for multiple comparisons (Winer, 1971). All individual scores were subjected to a square-root transformation to get more homogeneous variances. For the release data, the *fractional rate of tritium outflow* (in percent of tissue tritium per 4 min) was calculated as:

$$\frac{(\text{moles tritium outflow per 4 min}) \times 100}{\text{pmol tritium in septal slices at start of respective 4-min period}}.$$

The '*basal tritium outflow*' (b_1 value) represents the 'fractional rate of tritium outflow per 4 min' in the fraction preceding S_1 (i.e. from 53 to 57 min of superfusion). The *stimulation-evoked overflow of tritium* at S_1 or S_2 was expressed as a percentage of the tritium content of the striatal slices just before the onset of the respective stimulation period (see Figures 2, 4 and 6). It was calculated following subtraction of the basal tritium outflow; the latter was assumed to decline linearly from the 4-min fraction immediately *before* the onset of the stimulation to the 4-min fraction 12–16 min *after* the onset of the stimulation. *Effects of drugs* added before S_2 on the evoked overflow of [^{3}H] were estimated as the ratio of the overflow evoked by the corresponding stimulation periods (S_2/S_1) and then compared to the appropriate control ratios (no drug addition before S_2). *Effects of drugs* added before S_2 on the basal outflow of [^{3}H] were determined as the ratio (b_2/b_1) of the fractional rates of [^{3}H]outflow of the fractions preceding the corresponding stimulation periods and then compared to the appropriate control ratios (no drug addition before S_2).

In brain slices preincubated with [^{3}H]DA or [^{3}H]5-HT, respectively, the effects of EtOH, MDMA or their combination added before S_2 on the spontaneous outflow of [^{3}H] ('*drug-induced overflow of [^{3}H]*') were calculated as the '*area under the curves*' from 77 to 97 min of superfusion, using the corresponding fractional rate values and after subtraction of the basal tritium outflow (see shaded areas in Figures 2 and 4).

Given the homogeneity of the variances and normal distribution of the values, all neuropharmacological data were analysed using analyses of variance (ANOVA) and, when appropriate, ANOVA was followed by multiple comparisons using the Newman–Keuls test (Winer, 1971). All values shown in the Figures and Tables are means ± S.E.M. (n representing the total number of slices taken into account under each experimental condition).

Results

Locomotor activity

The activity scores recorded during the first post-injection hour are shown in Figure 1. The ANOVA showed a significant overall MDMA effect, as well as a significant overall effect of EtOH. In fact, MDMA induced hyperactivity that was significant at doses of 6.6, 10 and 20 mg/kg ($p < 0.05$), and this hyperactivity was significantly potentiated by EtOH at the same doses.

[^{3}H]5-HT release in striatal slices

Preincubation of striatal slices with [^{3}H]5-HT [0.1 μM; in the presence of the DA transporter (DAT) blocker nomifensine, 1 μM] led to the accumulation of 1.10 ± 0.03 pmol/slice of [^{3}H]5-HT ($n = 103$). Subsequently the slices were superfused in the presence of 1 μM nomifensine. The basal outflow of [^{3}H] in the fraction (b_1) before the first electrical field stimulation period (S_1) reached 3.98 ± 0.04% of tissue-^{3}H ($n = 106$), whereas the electrically evoked [^{3}H] overflow at S_1 amounted to 3.10 ± 0.08% of tissue-[^{3}H] ($n = 103$). Figure 2 illustrates the time-course of the tritium outflow from striatal slices preincubated with [^{3}H]5-HT [given as fractional rates of [^{3}H] outflow (mean ± S.E.M.,

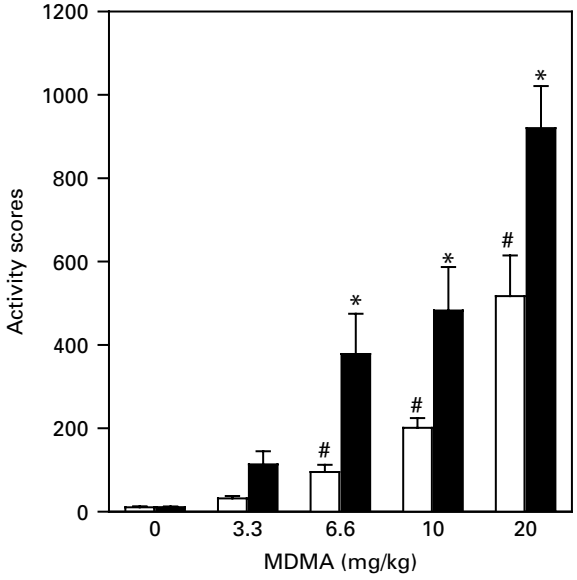

Figure 1. Locomotor activity in the home cage. Average (±S.E.M.) locomotor activity scores recorded during the first hour after the administration of MDMA (3.3, 6.6, 10 and 20 mg/kg vs. 0) in combination (■) or not (□) with 1.5 g/kg EtOH. Statistical analyses: # significant MDMA-induced increase vs. NaCl ($p<0.05$); * significant potentiation by co-administration of EtOH ($p<0.05$). All injections were made intraperitoneally at a volume of 7.5 ml/kg.

$n=15$–16)] under the influence of drugs added 24 min before the second stimulation period (S_2).

Figure 2 suggests that 2‰ EtOH alone had only a small effect on basal and electrically evoked [^{3}H] outflow, whereas 3 μM MDMA, both alone and in combination with EtOH, significantly enhanced the basal [^{3}H] efflux.

In order to quantify the effect of drugs on the spontaneous outflow of [^{3}H] from striatal slices pre-incubated with [^{3}H]5-HT, the shaded areas under the curves (see Figure 2) of all experiments were calculated and analysed using ANOVA, followed by a Newman–Keuls multiple comparisons test. This 'drug-induced [^{3}H] outflow' is depicted in Figure 3.

Figure 3 shows that the addition of EtOH (2‰) and MDMA (0.3 and 3 μM) or of both drugs to the superfusion buffer significantly increased the spontaneous outflow of tritium (given as a percentage of the [^{3}H] content of the slices just before addition of drugs). The magnitude of this drug-induced [^{3}H] outflow was smallest with EtOH alone, but much higher with 0.3 μM and 3 μM MDMA. The combination of 0.3 μM MDMA + 2‰ EtOH led to a further significant enhancement of the MDMA-induced [^{3}H] outflow.

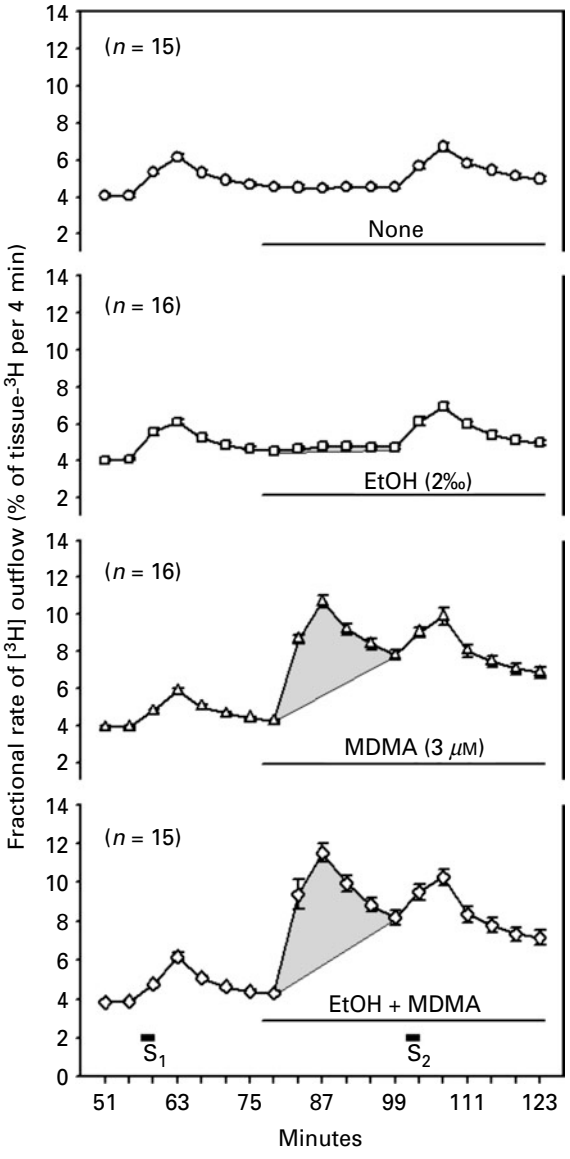

Figure 2. Rat striatum (5-HT release), superfusion in the presence of nomifensine. Time-course of [^{3}H] outflow from rat striatal slices preincubated with [^{3}H]5-HT. Following preincubation with [^{3}H]5-HT (0.1 μmol/l, in the presence of 1 μM nomifensine), the striatal slices were continuously superfused in the presence of 1 μM nomifensine and stimulated twice electrically, as indicated by the short horizontal bars: S_1, S_2 (360 rectangular pulses at 3 Hz, 2 ms, 4 V/chamber, 26–28 mA). The outflow of [^{3}H] from the slices is shown as the fractional rate of [^{3}H] outflow (in percent of tissue-^{3}H per 4-min fraction). Ethanol (EtOH, 2‰), MDMA (3 μM), or the combination of both drugs were added to the medium from 24 min before S_2 onwards as indicated by the longer horizontal bars; the outflow of [^{3}H] induced by the latter drugs is represented by the shaded areas under the curves (see also Figure 3). For further details see text (means ± S.E.M., n = number of slices).

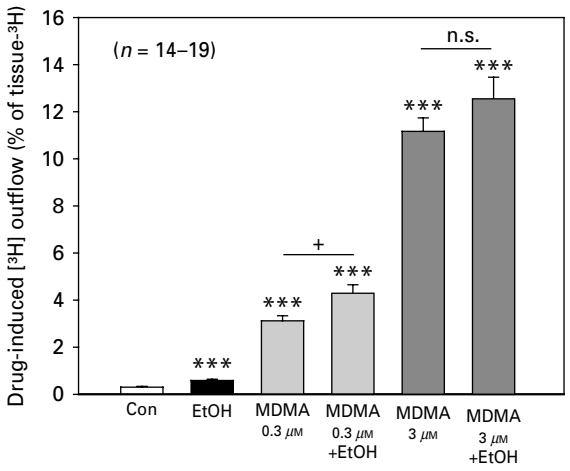

Figure 3. Rat striatum (5-HT release), superfusion in the presence of nomifensine. Effects of EtOH and MDMA (alone, or in combination) on the spontaneous outflow of [^{3}H] in rat striatal slices preincubated with [^{3}H]5-HT. Following preincubation with [^{3}H]5-HT (0.1 μmol/l, in the presence of 1 μM nomifensine), the striatal slices were continuously superfused in the presence of 1 μM nomifensine and stimulated twice electrically as shown in Figure 2. Ethanol (EtOH, 2‰), MDMA (3 μM), or the combination of both drugs were added to the medium from 24 min before S_2 onwards. Their effects on the spontaneous outflow of [^{3}H] ('drug-induced [^{3}H] outflow') were calculated as the shaded areas under the curves (see Figure 2) and are shown as a percentage of the tissue-^{3}H content before drug addition. Statistics: means $\pm$ S.E.M., n = number of slices; *** $p < 0.001$ vs. controls (Con, no drug addition); $^+$ $p < 0.05$, n.s., not significant, vs. corresponding MDMA concentration alone.

From the [^{3}H] outflow curves depicted in Figure 2 the question arises whether MDMA alone and/or in combination with EtOH also changed the size and the time-course of the electrically evoked [^{3}H] overflow at S_2 (although the still increased basal [^{3}H] outflow makes it difficult to appreciate such effects). Evaluation of these effects showed, however, that only the high concentration of MDMA (3 μM) significantly enhanced the electrically evoked overflow of [^{3}H] [S_2/S_1 ratios in percent of corresponding drug-free controls: 2‰ EtOH (106.68 ± 4.42; $n = 18$, n.s. vs. control); 0.3 μM MDMA (79.86 ± 2.90; $n = 14$, n.s. vs. control); 0.3 μM MDMA + EtOH (84.19 ± 5.02; $n = 15$, n.s. vs. control); 3 μM MDMA (139.23 ± 7.67; $n = 20$, $p < 0.001$ vs. control); 3 μM MDMA + EtOH (128.45 ± 7.85; $n = 20$, $p < 0.05$ vs. control)].

In order to check for a possible participation of endogenously released DA on the effects of MDMA and/or EtOH on the basal outflow and the electrically evoked outflow of [^{3}H]5-HT from striatal slices, experiments on striatal slices were also performed, in which the DAT inhibitor nomifensine (1 μM) was present only during tissue accumulation of [^{3}H]5-HT, not during superfusion (Table 1).

Statistical comparison of the data of Table 1 with those of Figure 3 showed that the absence of nomifensine throughout superfusion did not significantly change the MDMA- (or EtOH-) induced enhancement of the [^{3}H] outflow. The electrically evoked [^{3}H] overflow at S_1 was significantly ($p < 0.001$) smaller in the absence, compared to the presence, of nomifensine, whereas the effects of MDMA on the electrically evoked [^{3}H] overflow at S_2 were similar.

[^{3}*H*]*5-HT release in hippocampal and cortical slices*

MDMA and EtOH alone or in combination also affected in a similar manner [^{3}H] outflow in slices of the rat hippocampus and neocortex, especially by markedly increasing basal [^{3}H] outflow. Data from these experiments were evaluated in a similar manner as above and are summarized in Table 2 (hippocampus) and Table 3 (neocortex). However, the following experimental differences to the investigations on striatal slices (see above) should be noted. (1) Hippocampal and neocortical slices of the rats were preincubated with [^{3}H]5-HT in the absence of reuptake inhibitors. (2) Reuptake inhibitors were also absent during superfusion. (3) Lower concentrations of MDMA (0.1 or 1 μM) were added to the superfusion medium 24 min before S_2.

ANOVA of the data in Tables 2 and 3 show that also in rat hippocampal or neocortical slices, respectively, EtOH and especially MDMA increased the outflow of [^{3}H] (i.e. led to a 'drug-induced [^{3}H] outflow'); the combination of EtOH + MDMA, however, produced no additional effects. The electrically evoked overflow of [^{3}H] was completely unaffected by MDMA, EtOH or their combination.

[^{3}*H*]*DA release in striatal slices*

Preincubation of striatal slices with [^{3}H]DA [0.1 μM, in the presence of serotonin transporter (SERT) blocker fluvoxamine, 1 μM] led to the accumulation of 2.83 ± 0.06 pmol/slice [^{3}H]DA ($n = 99$). Subsequently the slices were superfused in the presence of 1 μM fluvoxamine. The basal outflow of [^{3}H] in the fraction (b_1) before the first electrical field stimulation period (S_1) corresponded to 1.77 ± 0.03% of tissue-^{3}H ($n = 99$), whereas the electrically evoked [^{3}H] overflow at S_1 amounted to 6.08 ± 0.16% of tissue-[^{3}H] ($n = 99$). Figure 4 illustrates the time-course of tritium outflow

Table 1. Rat striatum (5-HT release); superfusion in the absence of nomifensine: effects of EtOH and MDMA alone, or in combination on spontaneous and electrically evoked [^{3}H] outflow in rat striatal slices preincubated with [^{3}H]5-HT

Drug added 24 min before S_2	Conc.	Drug-induced [^{3}H] outflow (% of tissue-^{3}H)	Electrically evoked [^{3}H] overflow (S_2/S_1; % of controls)	*n*
None (controls)	–	0.31 ± 0.05	100.00 ± 3.54	20
EtOH	2‰	0.73 ± 0.06	102.19 ± 4.59	20
MDMA	0.3 μM	3.06 ± 0.24***	102.87 ± 3.74	18
EtOH + MDMA	2‰ + 0.3 μM	3.87 ± 0.29***	101.82 ± 4.43	19
MDMA	3 μM	11.26 ± 0.62***	144.30 ± 5.84***	17
EtOH + MDMA	2‰ + 3 μM	10.71 ± 0.91***	133.40 ± 10.12*	19

Following preincubation with [^{3}H]5-HT (0.1 μmol/l, in the presence of 1 μM nomifensine), the slices were continuously superfused in the absence of nomifensine and stimulated twice electrically as shown in Figure 2. Tissue accumulation of [^{3}H]5-HT amounted to 1.01 ± 0.02 pmol/slice (n = 114), the basal [^{3}H] outflow b_1 to 3.90 ± 0.04% of tissue-^{3}H (n = 114) and the electrically evoked overflow at S_1 to 2.45 ± 0.07% of tissue-^{3}H (n = 114). EtOH and MDMA, alone or in combination were added to the medium from 24 min before S_2 onwards (see Figure 2). Their effects on the spontaneous outflow of [^{3}H] ('drug-induced [^{3}H] outflow') are given as a percentage of the tissue-^{3}H content before drug addition. Their effects on the electrically evoked overflow at S_2 are shown as S_2/S_1 ratios in percent of the corresponding controls (no drug addition before S_2).
Values are means ± S.E.M.; n = number of slices.
* $p < 0.05$, *** $p < 0.001$ vs. controls.

Table 2. Rat hippocampus (5-HT release): effects of EtOH and MDMA (alone, or in combination) on spontaneous and electrically evoked [^{3}H] outflow in rat hippocampal slices preincubated with [^{3}H]5-HT

Drug added 24 min before S_2	Conc.	Drug-induced [^{3}H] outflow (% of tissue-^{3}H)	Electrically evoked [^{3}H] overflow (S_2/S_1; % of controls)	*n*
None (controls)	–	0.58 ± 0.06	100.00 ± 2.41	16
EtOH	2‰	1.30 ± 0.08***	102.21 ± 3.48	17
MDMA	0.1 μM	2.89 ± 0.24***	108.62 ± 3.34	18
EtOH + MDMA	2‰ + 0.1 μM	3.33 ± 0.23***	97.94 ± 3.89	20
MDMA	1 μM	16.87 ± 1.04***	96.59 ± 3.76	13
EtOH + MDMA	2‰ + 1 μM	18.35 ± 1.10***	100.89 ± 5.92	16

Following preincubation with [^{3}H]5-HT (0.1 μmol/l), the slices were continuously superfused in the absence of further drugs and stimulated twice electrically as shown in Figure 2. Tissue accumulation of [^{3}H]5-HT amounted to 0.58 ± 0.01 pmol/slice (n = 105), the basal [^{3}H] outflow b_1 to 3.80 ± 0.04% of tissue-^{3}H (n = 105) and the electrically evoked overflow at S_1 to 3.62 ± 0.07% of tissue-^{3}H (n = 105). EtOH and MDMA, alone or in combination were added to the medium from 24 min before S_2 onwards (see Figure 2). Their effects on the spontaneous outflow of [^{3}H] ('drug-induced [^{3}H] outflow') are given as a percentage of the tissue-^{3}H content before drug addition. Their effects on the electrically evoked overflow at S_2 are shown as S_2/S_1 ratios in percent of the corresponding controls (no drug addition before S_2).
Values are means ± S.E.M.; n = number of slices.
*** $p < 0.001$ vs. controls.

from striatal slices preincubated with [^{3}H]DA [given as fractional rates of [^{3}H] outflow (mean ± S.E.M., n = 8–16)] under the influence of drugs added 24 min before the second stimulation period (S_2).

Evidently, 2‰ EtOH alone had only a small effect on basal [^{3}H] outflow, whereas 3 μM MDMA, both alone and in combination with EtOH, significantly enhanced the basal [^{3}H] efflux. Moreover, MDMA

Table 3. Rat neocortex (5-HT release): effects of EtOH and MDMA (alone, or in combination) on spontaneous and electrically evoked [^{3}H] outflow in rat neocortical slices preincubated with [^{3}H]5-HT

Drug added 24 min before S_2	Conc.	Drug-induced [^{3}H] outflow (% of tissue-^{3}H)	Electrically evoked [^{3}H] overflow (S_2/S_1; % of controls)	n
None (controls)	–	1.03 ± 0.09	100.00 ± 3.21	18
EtOH	2‰	1.68 ± 0.13***	91.64 ± 4.09	17
MDMA	0.1 μM	4.25 ± 0.22***	101.26 ± 4.09	18
EtOH + MDMA	2‰ + 0.1 μM	4.65 ± 0.22***	97.66 ± 3.95	19
MDMA	1 μM	23.90 ± 1.06***	95.11 ± 7.23	15
EtOH + MDMA	2‰ + 1 μM	21.14 ± 1.03***	84.92 ± 5.02	16

Following preincubation with [^{3}H]5-HT (0.1 μmol/l), the slices were continuously superfused in the absence of further drugs and stimulated twice electrically as shown in Figure 2. Tissue accumulation of [^{3}H]5-HT amounted to 0.51 ± 0.01 pmol/slice (n = 107), the basal [^{3}H]outflow b_1 to 4.11 ± 0.05% of tissue-^{3}H (n = 107) and the electrically evoked overflow at S_1 to 3.18 ± 0.06% of tissue-^{3}H (n = 107). EtOH and MDMA, alone or in combination were added to the medium from 24 min before S_2 onwards (see Figure 2). Their effects on the spontaneous outflow of [^{3}H] ('drug-induced [^{3}H] outflow') are given as a percentage of the tissue-^{3}H content before drug addition. Their effects on the electrically evoked overflow at S_2 are shown as S_2/S_1 ratios in percent of the corresponding controls (no drug addition before S_2).
Values are means ± S.E.M.; n = number of slices.
*** $p < 0.001$ vs. controls.

alone and in combination also visibly changed the size and the time-course of the stimulation-evoked [^{3}H] outflow at S_2 (see below).

In order to quantify the effect of drugs on the spontaneous outflow of [^{3}H] from striatal slices, the shaded areas under the curves (see Figure 4, i.e. the 'drug-induced [^{3}H] outflow') of all experiments were calculated and analysed using ANOVA, followed by a Newman–Keuls multiple comparisons test. This 'drug-induced [^{3}H] outflow' is depicted in Figure 5.

It is evident from Figure 5 that the addition of EtOH (2‰) and MDMA (0.3 and 3 μM) or of both drugs to the superfusion buffer significantly increased the spontaneous outflow of tritium (shown as a percentage of the [^{3}H] content of the slices just before addition of drugs). The magnitude of this 'drug-induced [^{3}H] outflow' was smallest with EtOH alone or with 0.3 μM MDMA and much higher following 3 μM MDMA. Interestingly, the combination of MDMA (both 0.3 and 3 μM) with EtOH (2‰) led to a further significant enhancement of the MDMA-induced [^{3}H] outflow.

The [^{3}H] outflow curves depicted in Figure 4 suggest that MDMA alone and in combination with EtOH not only affected the basal outflow of [^{3}H], but also changed the size and the time-course of the electrically evoked [^{3}H] overflow at S_2 (although the still increased basal [^{3}H] outflow makes it difficult to appreciate such effects). In fact, MDMA both alone and in combination with EtOH significantly reduced the electrically evoked [^{3}H] overflow in rat striatal slices preincubated with [^{3}H]DA. The following data (given as S_2/S_1 ratios in percent of corresponding drug-free controls) were observed: 0.3 μM MDMA (82.42 ± 3.89; n = 18, $p < 0.05$ vs. controls); 0.3 μM MDMA + EtOH (77.64 ± 3.24; n = 20, $p < 0.001$ vs. controls); 3 μM MDMA (59.89 ± 3.69; n = 15, $p < 0.001$ vs. controls); 3 μM MDMA + EtOH (55.86 ± 5.07; n = 16, $p < 0.001$ vs. controls). Addition of 2‰ EtOH alone did not significantly change the electrically evoked overflow at S_2 (95.71 ± 4.82; n = 18, n.s. vs. control).

In order to check for a participation of endogenously released 5-HT on the effects of MDMA and/or EtOH on the basal and electrically evoked outflow of [^{3}H]DA from rat striatal slices, the experiments described above were also performed on striatal slices, in which the 5-HT reuptake inhibitor fluvoxamine (1 μM) was only present during tissue accumulation of [^{3}H]DA, but not during superfusion. The data of these experiments are summarized in Table 4.

Table 4 shows, that EtOH and MDMA, or their combination, significantly increased the basal [^{3}H] outflow from striatal slices (i.e. they led to a drug-induced [^{3}H] outflow) also in the absence of fluvoxamine throughout superfusion. Moreover, statistical comparison of the data of Table 4 with those of Figure 5 revealed that in the absence of fluvoxamine the MDMA- (or EtOH-) induced [^{3}H] outflow was

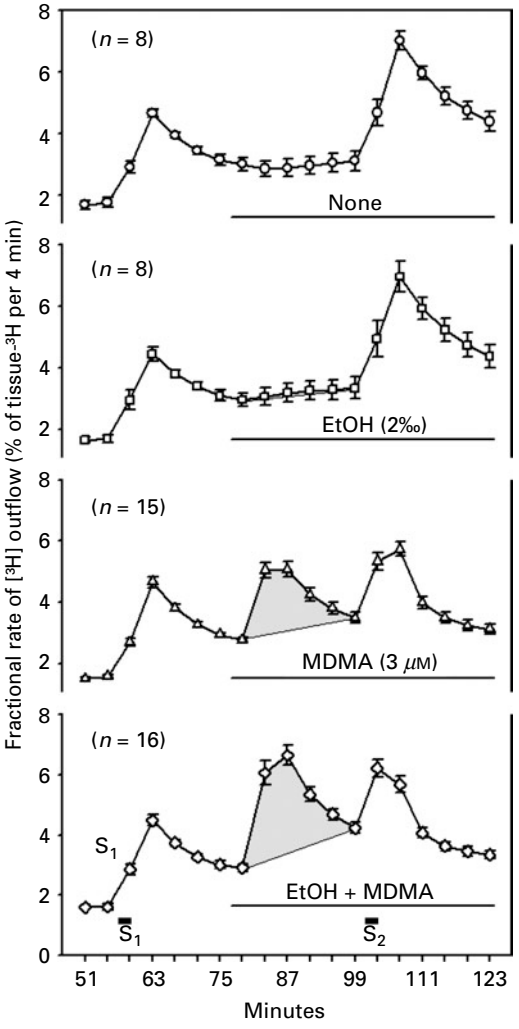

Figure 4. Rat striatum [dopamine (DA) release)], superfusion in the presence of fluvoxamine. Time-course of [^{3}H] outflow from rat striatal slices preincubated with [^{3}H]DA. Following preincubation with [^{3}H]DA (0.1 μmol/l, in the presence of 1 μM fluvoxamine), the striatal slices were continuously superfused in the presence of 1 μM fluvoxamine and stimulated twice electrically, as indicated by the short horizontal bars: S_1, S_2 (360 rectangular pulses at 3 Hz, 2 ms, 4 V/chamber, 26–28 mA). The outflow of [^{3}H] from the slices is shown as the fractional rate of [^{3}H] outflow (in percent of tissue-^{3}H per 4-min fraction). Ethanol (EtOH, 2‰), MDMA (3 μM), or the combination of both drugs were added to the medium from 24 min before S_2 onwards as indicated by the longer horizontal bars; the outflow of [^{3}H] induced by the latter drugs is represented by the shaded areas under the curves (see also Figure 5). For further details see text (means $\pm$ S.E.M., n = number of slices).

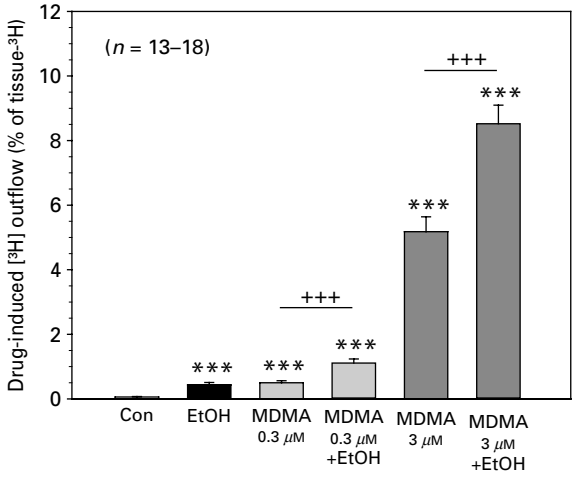

Figure 5. Rat striatum [dopamine (DA) release)], superfusion in the presence of fluvoxamine. Effects of EtOH and MDMA (alone, or in combination) on the spontaneous outflow of [^{3}H] in rat striatal slices preincubated with [^{3}H]DA. Following preincubation with [^{3}H]DA (0.1 μmol/l, in the presence of 1 μM fluvoxamine), the striatal slices were continuously superfused in the presence of 1 μM fluvoxamine and stimulated twice electrically as shown in Figure 4. Ethanol (EtOH, 2‰), MDMA (3 μM), or the combination of both drugs were added to the medium from 24 min before S_2 onwards. Their effects on the spontaneous outflow of [^{3}H] ('drug-induced [^{3}H] outflow') were calculated as the shaded areas under the curves (see Figure 4) and are shown as a percentage of the tissue-^{3}H content before drug addition. Statistics: means $\pm$ S.E.M., n = number of slices; *** $p < 0.001$ vs. controls (Con, no drug addition); $^{+++}$ $p < 0.001$ vs. corresponding MDMA concentration alone.

significantly more pronounced ($p < 0.001$, except for 3 μM MDMA + EtOH; n.s.). On the other hand, both the electrically evoked [^{3}H] overflow at S_1 and the basal [^{3}H] outflow (b_1) were significantly ($p < 0.001$) smaller in the absence of fluvoxamine throughout superfusion and the inhibitory effects of 3 μM MDMA $\pm$ EtOH on the electrically evoked [^{3}H] overflow (S_2/S_1 in percent of controls; see above) almost disappeared in the absence of fluvoxamine.

[^{3}H]ACh release in striatal slices

Preincubation of striatal slices in the presence of [^{3}H]choline (0.1 μM) and fluvoxamine (1 μM), led to the accumulation of 1.63 ± 0.05 pmol/slice [^{3}H] ($n = 76$). Subsequently the slices were superfused in the presence of the selective 5-HT reuptake blocker fluvoxamine (1 μM). The basal outflow of [^{3}H] (b_1) in the fraction before the first electrical field stimulation period (S_1) corresponded to 1.17 ± 0.02% of tissue-^{3}H ($n = 76$), whereas the electrically evoked [^{3}H] overflow at S_1 amounted to 3.18 ± 0.06% of tissue-^{3}H ($n = 76$). Figure 6 shows the time-course of tritium outflow

Table 4. Rat striatum (DA release); superfusion in the absence of fluvoxamine: effects of EtOH and MDMA (alone, or in combination) on spontaneous and electrically evoked [^{3}H] outflow in rat striatal slices preincubated with [^{3}H]DA

Drug added 24 min before S_2	Conc.	Drug-induced [^{3}H] outflow (% of tissue-^{3}H)	Electrically evoked [^{3}H] overflow (S_2/S_1; % of controls)	n
None (controls)	–	0.133 ± 0.03	100.00 ± 2.26	17
EtOH	2‰	0.74 ± 0.07***	106.80 ± 2.95	17
MDMA	0.3 μM	1.37 ± 0.15***	92.23 ± 4.03	15
EtOH + MDMA	2‰ + 0.3 μM	2.28 ± 0.23*** ++	85.49 ± 4.32*	14
MDMA	3 μM	8.94 ± 0.57***	97.03 ± 3.54	14
EtOH + MDMA	2‰ + 3 μM	9.70 ± 0.55***	92.42 ± 3.96	18

Following preincubation with [^{3}H]DA (0.1 μmol/l, in the presence of 1 μM fluvoxamine), the slices were continuously superfused in the absence of fluvoxamine and stimulated twice electrically as shown in Figure 4. Tissue accumulation of [^{3}H]DA amounted to 2.72 ± 0.06 pmol/slice (n = 102), the basal [^{3}H] outflow b_1 to 2.21 ± 0.04 % of tissue-^{3}H (n = 102) and the electrically evoked overflow at S_1 to 3.70 ± 0.12 % of tissue-^{3}H (n = 102). EtOH and MDMA, alone or in combination were added to the medium from 24 min before S_2 onwards (see Figure 4). Their effects on the spontaneous outflow of [^{3}H] ('drug-induced [^{3}H] outflow') are given as a percentage of the tissue-^{3}H content before drug addition. Their effects on the electrically evoked overflow at S_2 are shown as S_2/S_1 ratios in percent of the corresponding controls (no drug addition before S_2).
Values are means ± S.E.M., n = number of slices.
* $p < 0.05$, *** $p < 0.001$ vs. controls; ++ $p < 0.01$ vs. MDMA (0.3 μM).

from striatal slices pre-incubated with [^{3}H]choline [given as fractional rates of [^{3}H] outflow (mean ± S.E.M., n = 6)].

In contrast to the experiments on the evoked 5-HT or DA release (see above), MDMA and EtOH or their combination were added to the superfusion medium not 24 min but only 4 min before S_2. Pilot experiments had shown that using this time schedule the peak amount of MDMA-induced outflow of [^{3}H] from striatal slices preincubated with [^{3}H]DA coincided with the electrically evoked overflow of [^{3}H] from striatal slices preincubated with [^{3}H]choline. Moreover, only experiments with the higher MDMA concentration (3 μM) were performed and the electrical field stimulation parameters differed from those used for [^{3}H]5-HT or [^{3}H]DA release, respectively (see Figure legends).

Although from Figure 6 it is less obvious, Figure 7 clearly shows that the electrically evoked overflow of [^{3}H] from rat striatal slices preincubated with [^{3}H]choline was significantly diminished by 2‰ EtOH or 3 μM MDMA, and also by their combination.

Figure 7 also shows that the inhibitory effects of MDMA and its combination with EtOH were completely antagonized by the DA D_2 receptor antagonist L-sulpiride. Nevertheless, the inhibitory effects of 3 μM MDMA were surprisingly small compared to the tremendous drug-induced effects on striatal slices preincubated with [^{3}H]DA (see Figure 5). Moreover, in pilot experiments on striatal slices from Wistar rats both direct and indirect effects of dopaminergic drugs were much more pronounced (S_2/S_1 ratios as a percentage of untreated controls): 1 μM apomorphine (29.61 ± 2.00 %; n = 54, $p < 0.001$ vs. controls); 1 μM bromocriptine (78.49 ± 2.51 %; n = 12, $p < 0.01$ vs. controls); 1 μM nomifensin (57.17 ± 4.74 %; n = 17, $p < 0.001$ vs. controls); 1 μM L-sulpiride (164.73 ± 4.88 %; n = 51, $p < 0.001$ vs. controls). Therefore the question arose, whether inhibition of the metabolism of DA could increase the inhibitory effects of 3 μM MDMA in striatal slices preincubated with [^{3}H]choline. Figure 7 shows, however, that the presence of the monoamine oxidase B (MAO-B) inhibitor selegeline (1 μM) and the carboxy-*O*-methyl transferase (COMT) inhibitor entacapone (0.1 μM) throughout the entire experiment (i.e. during preincubation and superfusion of the slices) did not increase the inhibitory effect of MDMA (3 μM), but rather led to a small but significant ($p < 0.05$ vs. MDMA alone) decrease of the effect.

The experiments shown in Figure 8 were undertaken to shed some light on the receptors involved in the inhibitory effects of 3 μM MDMA alone and in combination with 2‰ EtOH on the electrically evoked [^{3}H] overflow in rat striatal slices preincubated with [^{3}H]choline. In all of these experiments the antagonists were present throughout superfusion in addition to 1 μM fluvoxamine.

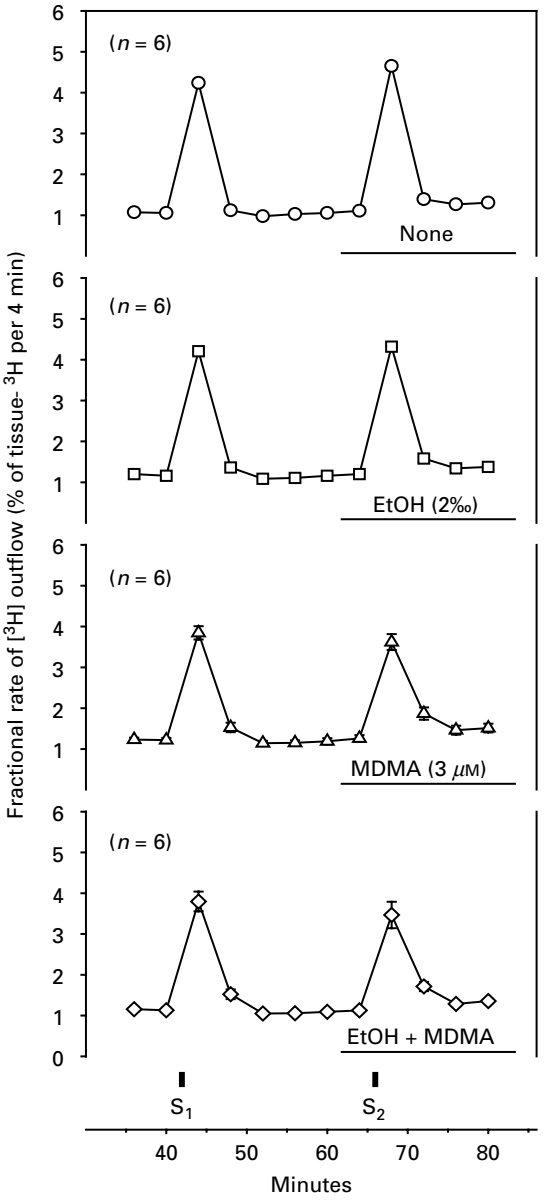

Figure 6. Rat striatum (acetylcholine release), superfusion in the presence of fluvoxamine. Time-course of [^{3}H] outflow from rat striatal slices preincubated with [^{3}H]choline. Following preincubation with [^{3}H]choline (0.1 μmol/l, in the presence of 1 μM fluvoxamine), the striatal slices were continuously superfused in the presence of 1 μM fluvoxamine and stimulated twice electrically, as indicated by the short horizontal bars: S_1, S_2 (36 rectangular pulses at 3 Hz, 2 ms, 4 V/chamber, 26–28 mA). The outflow of [^{3}H] from the slices is shown as the fractional rate of [^{3}H] outflow (in percent of tissue-^{3}H per 4-min fraction). Ethanol (EtOH, 2‰), MDMA (3 μM), or the combination of both drugs were added to the medium from 4 min before S_2 onwards as indicated by the longer horizontal bars. Drug effects on the electrically evoked [^{3}H] overflow are shown in Figure 7. For further details see text (means ± S.E.M., n = number of slices).

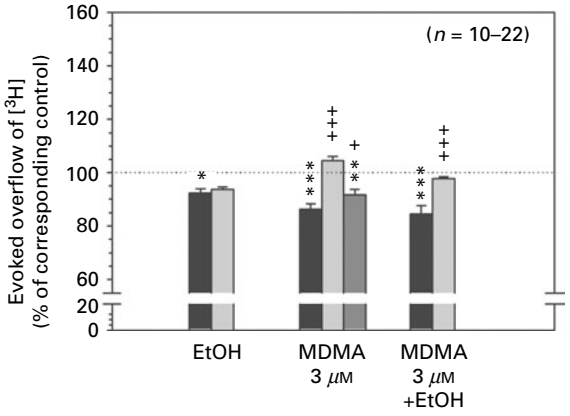

Figure 7. Rat striatum (acetylcholine release), superfusion in the presence of fluvoxamine. Inhibitory effects of ethanol (EtOH, 2‰), MDMA (3 μM), or their combination on the electrically evoked [^{3}H] overflow of rat striatal slices preincubated in the presence of [^{3}H]choline: interaction with 1 μM L-sulpiride or the metabolic inhibitors selegeline (1 μM) and entacapone (0.1 μM). Black bars: following preincubation with [^{3}H]choline (0.1 μM, in the presence of 1 μM fluvoxamine), the striatal slices were continuously superfused in the presence of 1 μM fluvoxamine and stimulated twice electrically, as shown in Figure 6. Ethanol (EtOH, 2‰), MDMA (3 μM), or the combination of both drugs were added to the medium from 4 min before S_2 onwards. Light grey bars: as above, but the superfusion medium contained in addition 1 μM L-sulpiride. Dark grey bar: as above, but both incubation and superfusion medium contained in addition 1 μM selegeline and 0.1 μM entacapone. Statistics: means ± S.E.M., n = number of slices; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. corresponding controls (no drugs before S_2); $^{+}$ $p < 0.05$, $^{+++}$ $p < 0.001$ vs. MDMA (± EtOH) alone.

The significance of differences shown in Figure 8 was always calculated vs. the effects of MDMA ± EtOH in the presence of fluvoxamine alone throughout superfusion. Thus the inhibitory effects of MDMA ± EtOH were, either completely or only in part, antagonized by the 5-HT_2 receptor antagonists olanzapine (Ol) and ritanserin (Rit), and by the NMDA antagonists D,L-AP-5 (AP5) and MK-801 (MK). The 5-HT_3 antagonist tropanyl-3,5-dimethylbenzoate (Tr) had no significant influence on the effect of MDMA ± EtOH. Interestingly, the nicotinic antagonist mecamylamine (Mec) and the NK_1-receptor antagonist L-732,138 (L732) appeared to reduce the effects of MDMA only in the absence of EtOH. It should be noted that most of the antagonists present throughout superfusion also affected the basal outflow of [^{3}H] and the evoked overflow of [^{3}H] at S_1 (see Table 5 for details).

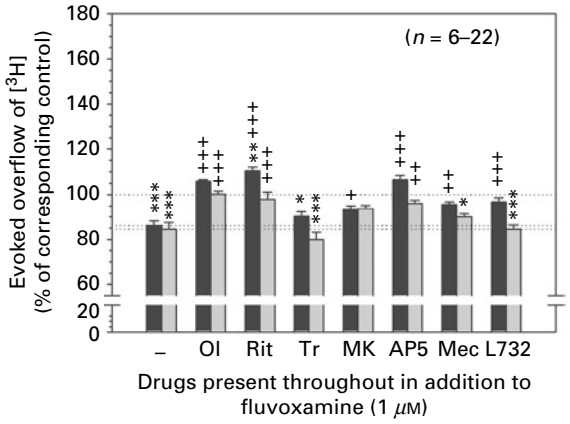

Figure 8. Rat striatum (acetylcholine release), superfusion in the presence of fluvoxamine. Inhibitory effects of MDMA (3 μM, ■), or of its combination with ethanol (EtOH, 2‰, □), on the electrically evoked [^{3}H] overflow of rat striatal slices preincubated in the presence of [^{3}H]choline: interaction with various drugs present throughout superfusion in addition to 1 μM fluvoxamine. Following preincubation with [^{3}H]choline (0.1 μmol/l, in the presence of 1 μM fluvoxamine), the striatal slices were continuously superfused either in the presence of 1 μM fluvoxamine alone (–), or in the additional presence of either olanzapine (Ol, 1 μM), ritanserin (Rit, 3 μM), tropanyl-3,5-dimethylbenzoate (Tr, 10 μM), MK-801 (MK, 1 μM), D,L-AP-5 (AP5, 100 μM), mecamylamine (Mec, 10 μM), or L-732,138 (L732, 1 μM), as indicated below the columns. During superfusion they were stimulated twice electrically, as shown in Figure 6. MDMA, or its combination with EtOH were added to the medium from 4 min before S_2 onwards (see Figure 6). Statistics: means ± S.E.M., n = number of slices; * $p < 0.05$, *** $p < 0.001$ vs. corresponding controls (no drugs before S_2); $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. MDMA, or MDMA + EtOH, respectively, alone.

Discussion

Using a dose–response approach, we first confirmed that EtOH co-administered with MDMA potentiated the locomotor effects of MDMA (for a detailed discussion of this issue, see e.g. Hamida et al., 2007). Using rats that were naive to MDMA and EtOH, we then investigated the effects of MDMA and EtOH, alone or in combination, on the *spontaneous outflow* and the *electrically evoked overflow* of [^{3}H] in slices of the rat striatum pre-incubated with [^{3}H]5-HT, [^{3}H]DA or [^{3}H]choline (vs. the hippocampus and neocortex for 5-HT release). In this context it should be emphasized that the doses of MDMA administered to rats in vivo (Figure 1) as well as the concentrations of MDMA used in the in-vitro experiments ($\leqslant 3\,\mu$M) are of relevance for studies in humans (de la Torre et al., 2000; Irvine et al., 2006). The effects of a combined application of MDMA + EtOH are of particular interest because both drugs are often taken together by abusers (Barrett et al., 2006). Moreover, recent studies in rats provided behavioural and physiological evidence for synergistic interactions between them (Cassel et al., 2005; Hamida et al., 2007). Such interactions could involve pharmacokinetic and/or pharmacodynamic components. Concerning pharmacodynamic interactions, EtOH and MDMA might alter the functional properties within complex multisynaptic circuitries and/or interact locally on presynaptic neuropharmacological substrates participating in the regulation of neurotransmitter release.

In the present study, we focused on this second possibility by using a slice superfusion technique. Electrically evoked overflow of [^{3}H] from brain slices preloaded with the appropriate marker is a valuable model for the study of the presynaptic modulation of action potential-induced, exocytotic neurotransmitter release (Birthelmer et al., 2003a,b; Hertting et al., 1980; Jackisch et al., 1980). Conversely, the spontaneous [^{3}H] outflow from such slices (Ehret et al., 2001; Starke et al., 1981; Steppeler et al., 1982; Zumstein et al., 1981) and most probably also that induced by direct drug application mainly consists of a mixture of the efflux of both the [^{3}H] transmitter itself and its tritiated metabolites.

5-HT release in rat striatum, hippocampus and cortex

MDMA (0.3 and 3 μM) and – less effectively – EtOH enhanced the spontaneous outflow of [^{3}H] in slices of the rat striatum (Figure 2, Table 1), hippocampus (Table 2) and neocortex (Table 3) preincubated with [^{3}H]5-HT. Moreover, the electrically evoked 5-HT release was significantly increased, but only by the highest concentration of MDMA and only in striatal slices (Tables 1–3). EtOH significantly potentiated the MDMA-induced release of [^{3}H], but only in striatal slices, at the low MDMA concentration (0.3 μM) and in the presence of nomifensine. In this context it is noteworthy that the concentration of 3 μM is close to the peak plasma concentration of MDMA found in humans after the consumption of a MDMA dose of 100–150 mg (i.e. 1.3–2.2 μM in de la Torre et al., 2000; see also Irvine et al., 2006).

Given systemically or applied directly to the VTA, EtOH stimulates 5-HT release (Yan, 1999; Yan et al., 1996), and this effect might involve a local action (Thielen et al., 2001), as confirmed herein. Such local mechanisms might also include effects on the SERT, which is supported by the finding of Daws et al.

Table 5. Rat striatum (ACh release): effects of various antagonists on basal and electrically evoked [^{3}H] outflow in rat striatal slices preincubated with [^{3}H]choline (0.1 μM, in the presence of 1 μM fluvoxamine)

Drugs present in addition to 1 μM fluvoxamine	Conc. (μM)	Basal [^{3}H] outflow (b_1) (% of tissue-^{3}H)	Evoked [^{3}H] overflow (S_1) (% of tissue-^{3}H)	n
None	1	1.17 ± 0.02	3.18 ± 0.06	76
L-sulpiride	1	1.31 ± 0.04***	4.55 ± 0.11***	46
Entacapone + selegeline[a]	0.1 + 1	1.35 ± 0.04***	3.77 ± 0.12***	21
Ritanserin	3	1.49 ± 0.04***	3.48 ± 0.15*	23
Olanzapine	1	1.52 ± 0.05***	4.47 ± 0.16***	24
Tropanyl-3,5-dimethyl-benzoate	10	1.41 ± 0.05***	1.63 ± 0.09***	20
Mecamylamine	10	1.43 ± 0.04***	3.92 ± 0.13***	36
MK-801	1	1.77 ± 0.05***	3.20 ± 0.16	21
DL-AP-5	100	1.59 ± 0.05***	3.18 ± 0.19	20
L-732,138	1	1.98 ± 0.09***	2.94 ± 0.09*	33

Following preincubation the slices were superfused continuously in the presence of 1 μM fluvoxamine either alone or in the presence of additional drugs, as indicated and stimulated twice electrically as shown in Figure 6. The basal outflow (b_1 value) and the electrically evoked overflow at the first stimulation period (S_1) are shown in percent of the tissue-^{3}H concentration (see Methods section).

Values are means ± S.E.M.; n = number of slices.

[a] These drugs were also present during preincubation with [^{3}H]choline.

* $p < 0.05$, *** $p < 0.001$ vs. 1 μM fluvoxamine alone throughout.

(2006) that the selective SERT inhibitor fluvoxamine and EtOH increased extracellular 5-HT levels of 5-HT in an additive manner. These results, however, do not mean that this mechanism is the only one to operate in vivo. Moreover, this effect was not due to alterations of neuronal electrical properties, as EtOH did not modify the electrically evoked overflow of [^{3}H]5-HT. In the presence of the DAT inhibitor nomifensine, the application of 0.3 or 3 μM MDMA significantly increased the release of 5-HT from striatal slices, as shown earlier (Gudelsky and Nash, 1996; Mechan et al., 2002; Sabol and Seiden, 1998). This enhancement involves a carrier-mediated mechanism and depends on vesicular stores of 5-HT (Wichems et al., 1995).

Using EtOH in addition to 0.3 μM MDMA, the MDMA-induced 5-HT release from striatal slices in the presence of nomifensine was larger than that elicited by MDMA alone (Figure 3). This is the first main finding of the present work. It points towards a synergistic effect of both drugs in the striatum, which appears to undergo saturation as it was not found with 3 μM MDMA. One explanation for this EtOH potentiation of the MDMA effect on 5-HT release could be related to an EtOH-induced increase of the availability of MDMA within the slices. Indeed, in the mouse, a combined MDMA + EtOH treatment increased striatal d-MDMA levels by 4- to 7-fold (Johnson et al., 2004). However, in the present experiment, MDMA and EtOH were directly applied on the slices, whereas in mice the drugs were given systemically (four times, every 2 h, 15.0 mg/kg MDMA s.c.), raising the possibility that EtOH simply facilitated the distribution of MDMA to the brain. Thus, the mechanism accounting for the increase in 5-HT release after local application of both MDMA and EtOH on striatal slices remains to be identified, although the observation that both MDMA (Mlinar and Corradetti, 2003) and EtOH (Daws et al., 2006) appear to interact with the SERT suggest the possibility of additive effects on 5-HT reuptake. Nevertheless it should be emphasized that according to earlier observations (Crespi et al., 1997), the effect of MDMA on DA release should be markedly weakened, if not abolished in the presence of the DAT inhibitor nomifensine during superfusion. Therefore the present data suggest that the potentiation by EtOH of MDMA-induced 5-HT release may appear even under the condition of a lower dopaminergic tonus, or when the 5-HT/DA balance is largely in favour of 5-HT.

In order to check for a possible participation of endogenously released DA, similar experiments were performed in which nomifensine was present during the pre-incubation but not the superfusion period (Table 1). We observed that the electrically evoked overflow of [^{3}H] elicited by the first stimulation

(S_1, i.e. before addition of drugs) was decreased when nomifensine was omitted during the superfusion, an effect most probably due to non-specific blocking actions of nomifensine on the SERT. Particularly interesting under this condition is the finding that both the drug-induced spontaneous [^{3}H] outflow (treatment of the slices with MDMA, or MDMA + EtOH) and the drug effects on electrically evoked 5-HT release (S_2/S_1 ratios) did not significantly differ from the values in the presence of nomifensine. Again, these observations do not support a participation of DA in the effects of combined MDMA + EtOH applications on the spontaneous and electrically evoked overflow of [^{3}H]5-HT from striatal slices.

Regarding the effects of MDMA, EtOH or their combination in the hippocampus or neocortex, our results show that both drugs and their combination increased 5-HT release in these brain regions. Regardng EtOH, these observations are in line with previous results (Thielen et al., 2002). It is also well known from the literature that MDMA stimulates this release (Esteban et al., 2001) by an action on both the SERT and on intraneuronal vesicular stores of 5-HT (Mlinar and Corradetti, 2003). MDMA is also able to induce the release of 5-HT from cortical synaptosomes (Kramer et al., 1994). Finally, intravenous injections of MDMA trigger the release of 5-HT in the hippocampus and frontal cortex (Gartside et al., 1997). As such, our results with MDMA alone are in agreement with the literature. However, in contrast to our observations in the striatum, in neither of these two structures did the combination of MDMA + EtOH lead to a further increase of 5-HT release, suggesting a region-specific effect of combined MDMA + EtOH applications in the striatum.

DA release in the rat striatum

The DA-releasing effect of MDMA is also well documented in the literature, and an enhanced 5-HT release seems to participate in this effect (Goni-Allo et al., 2006; Gudelsky and Nash, 1996; Koch and Galloway, 1997; Yamamoto et al., 1995). Since it has been shown that the SERT is one of the main targets of MDMA (Rudnick and Wall, 1992) and that selective SERT inhibitors prevent at least part of the 5-HT releasing effect of MDMA (Berger et al., 1992; Wichems et al., 1995), some of our experiments were performed in the presence of the SERT blocker fluvoxamine in order to reduce the influence of 5-HT on DA release, which – by acting on various 5-HT receptor types – may be both inhibitory or facilitatory (Alex and Pehek, 2007; Fink and Göthert, 2007). In support of the findings mentioned above, we observed that the MDMA-induced release of DA was significantly higher in the absence (i.e. when the action of MDMA on the SERT was normal; Table 4) than in the presence of fluvoxamine (i.e. when MDMA-induced 5-HT release was diminished by blockade of SERT; Figure 5).

Interestingly, in the presence of fluvoxamine, i.e. under conditions in which the effect of MDMA on serotonergic terminals was most probably attenuated (Gudelsky and Nash, 1996; Mechan et al., 2002), the electrically evoked release of DA (S_2/S_1 ratio) was decreased by MDMA (see text), effects which disappeared in the absence of the SERT blocker (Table 4). Moreover, in the presence of fluvoxamine the MDMA-induced [^{3}H] outflow was facilitated by EtOH at both MDMA concentrations (Figure 5: 0.3 μM, +100%; 3 μM, +65%), whereas in its absence, EtOH facilitated the MDMA-induced release of DA only at the lower concentration of MDMA (Table 4: 0.3 μM, +66%). It should be borne in mind, however, that in such experiments fluvoxamine was present throughout superfusion and thus may have contributed to a substantial increase of the serotonergic tonus before MDMA was applied. Since it has been shown that 5-HT inhibits the evoked DA release in the striatum (Ennis et al., 1981) via 5-HT_{1B} (Sarhan and Fillion, 1999; Sarhan et al., 2000) or 5-HT_{2C} receptors (Alex et al., 2005), the finding that the MDMA-induced release of DA at both MDMA concentrations was less pronounced in the presence (Figure 5) than in the absence of fluvoxamine (Table 4) might be caused by this inhibitory effect of 5-HT. Taken together these observations also indicate that one of the possible targets for EtOH in MDMA-treated rats could be the level of inhibition which 5-HT exerts on DA terminals. In fact, our data suggest that EtOH may have counterbalanced the effects of fluvoxamine on MDMA-induced DA release. Indeed, using the combination of EtOH + MDMA, the spontaneous [^{3}H] outflow was quite comparable, whether the SERT was blocked or not.

How can these in-vitro data help to understand the effects of MDMA and EtOH observed in vivo? In-vivo microdialysis experiments have shown that MDMA increases extracellular levels of 5-HT much more potently than those of DA, suggesting that the inhibition exerted by 5-HT on DA terminals is rather high in vivo (Baumann et al., 2007; Green et al., 2003). Therefore, it seems possible to speculate that the simultaneous use of EtOH and MDMA in vivo could contribute to attenuate the 5-HT mediated inhibition of DA release, resulting in a larger efflux of DA and

to an increased locomotor activity (Cassel et al., 2005; Hamida et al., 2007). Although this suggestion seems to contradict our findings on 5-HT release (see above) we speculate that EtOH potentiates the MDMA-induced locomotor effects of MDMA when the *5-HT/DA balance* in the striatum is largely in favour of 5-HT. Future experiments using intrastriatal in-vivo microdialysis in the presence of MDMA, EtOH and their combination should help to test this possibility.

ACh release in the rat striatum

Cholinergic interneurons provide a dense intrinsic innervation in the striatum (Phelps et al., 1985) and play a key role in modulating its activity. Therefore, studying the effects of MDMA on striatal cholinergic neurotransmission is relevant, especially regarding the effects of MDMA on DA release and the D_2 receptor-mediated inhibitory effects of DA on ACh release (Alcantara et al., 2003; Drukarch et al., 1989; Hertting et al., 1980; Ikarashi et al., 1997; Stoof et al., 1982, 1992).

In the present study, 3 μM MDMA, 2‰ EtOH or their combination were added to the superfusion medium in the fraction just preceding the electrical field stimulation of the striatal slices for two reasons: first, because MDMA had no effect on baseline [^{3}H] outflow under these conditions (Figure 6) and second, because we wanted to elicit a maximal MDMA-induced increase in endogenous DA at the time of electrically evoked release of ACh (this point was tested in pilot experiments). Both MDMA and EtOH produced a small but significant reduction in the electrically evoked release of [^{3}H]ACh in these slices. This inhibitory effect was, however, not altered by the addition of EtOH (Figure 7).

These findings appear to be in contrast to previous in-vivo and in-vitro studies (Acquas et al., 2001; Fischer et al., 2000), which both reported on a stimulatory action of MDMA on ACh release. There were differences, however, in the experimental conditions used [in-vivo microdialysis (Acquas et al., 2001) vs. in-vitro superfusion studies], in the respective superfusion buffers [10 μM physostigmine (Fischer et al., 2000) vs. no physostigmine (present study)], in the concentrations of MDMA applied [in vivo (3.2 mg/kg i.v.; Acquas et al., 2001); in vitro (10–300 μM MDMA; Fischer et al., 2000), vs. 3 μM (present study)], and in the respective stimulation conditions. For instance, in-vivo low doses of MDMA failed to alter the basal ACh release (Acquas et al., 2001) and a similar explanation could apply to our current ex-vivo observations, in which the highest concentration of MDMA was 10 times lower than the EC_{50} described by Fischer et al. (2000).

On the other hand, the inhibitory effect of MDMA on the release of [^{3}H]ACh was surprisingly weak, especially in view of the very powerful stimulatory effect of this drug on the release of striatal dopamine, and the well-known potent inhibitory effect of drugs acting directly on presynaptic D_2 receptors or increasing the endogenous dopaminergic tone in the striatum (see Results, and Agid et al., 1975; Herman et al., 1988; Hertting et al., 1980; Stoof et al., 1982). Therefore, by blocking the DA-metabolizing enzymes MAO-B and COMT, we tested the possibility that MDMA had in fact induced mainly the release of non-active DA metabolites instead of DA itself. However, the inhibitory effect of 3 μM MDMA was not increased in the presence of a combination of the MAO-B blocker selegeline (Knoll, 1987) and the COMT inhibitor entacapone (Holm and Spencer, 1999), suggesting that the weak effect of MDMA on ACh release was not related to a larger metabolic degradation of endogenously released DA. Nevertheless, it should be noted that the metabolic inhibitors significantly increased the accumulation of [^{3}H]choline in striatal slices (data not shown), as well as the basal and the evoked overflow of [^{3}H]ACh (Table 5). These observations may be tentatively interpreted as reflecting an acute down-regulation of both D_2 autoreceptors and heteroreceptors due to increased tissue levels of unmetabolized DA, which in turn affected both dopaminergic and cholinergic transmissions.

As regards the involvement of presynaptic receptors in the small but significant inhibitory effects of 3 μM MDMA, 2‰ EtOH, and their combination, the following observations were made using various receptor antagonists (in addition to fluvoxamine) throughout superfusion: (i) D_2/D_3 receptor blockade (with L-sulpiride) abolished the inhibitory effects of MDMA, but not that of EtOH (Figure 7); (ii) blockade of 5-HT_2 receptors (with olanzapine or ritanserin) antagonized the inhibitory effects of MDMA, and also that of its combination with EtOH, whereas 5-HT_3 receptor blockade (with tropanyl-3,5-dimethylbenzoate; Fozard and Gittos, 1983) left the inhibitory effects of MDMA and EtOH unchanged (Figure 8); (iii) NMDA receptor blockade at the glutamate-binding site (with D,L-AP-5) antagonized the inhibitory effects of MDMA and of its combination with EtOH, whereas the uncompetitive NMDA receptor channel blocker MK-801 (dizozilpine) had small antagonistic effects (Figure 8); (iv) nicotinic ACh receptor (nAChR) blockade (with mecamylamine), or that of NK_1 receptors (with L-732,138), antagonized the inhibitory

effects of MDMA, but not that of its combination with EtOH (Figure 8).

Thus, MDMA appears to affect the electrically evoked ACh release via the activation of dopaminergic D_2 receptors. These results are in line with the inhibitory effect of D_2 receptor activation on the striatal cholinergic neurotransmission (Alcantara et al., 2003; Drukarch et al., 1989; Hertting et al., 1980; Ikarashi et al., 1997; Stoof et al., 1982, 1992), as well as with the ability of MDMA to stimulate DA release (Green et al., 2003). Moreover, L-sulpiride increased both the basal and the evoked ACh release, suggesting a tonic activation of D_2 receptors by endogenous DA, which is consistent with in-vivo findings (Bertorelli and Consolo, 1990; Ikarashi et al., 1997).

The present data also show that 5-HT_2 receptors play a role in these effects, whereas 5-HT_3 receptors are not involved. Thus, the observation that olanzapine and ritanserin, two 5-HT_2 receptor antagonists (Bymaster et al., 1999; Leysen et al., 1985) completely antagonized the MDMA-mediated inhibition of ACh release indicates a participation of the 5-HT_2 receptor in the effect of MDMA.

The striatum also possesses a high density of NMDA receptors (Greenamyre and Young, 1989) and striatal cholinergic interneurons receive excitatory glutamatergic inputs from the motor cortex or the thalamus (Lapper and Bolam, 1992). The non-competitive NMDA receptor antagonist MK-801 (Ramoa et al., 1990) slightly diminished the inhibitory effects of MDMA on the evoked ACh release, suggesting that MDMA might in part interfere with NMDA receptor ion-channel function. Moreover, DL-AP-5, a potent and competitive antagonist at the glutamate-binding site of the NMDA receptor (Davies and Watkins, 1982), completely antagonized the inhibitory effects of MDMA, suggesting that glutamatergic transmission is involved in the inhibitory effects of MDMA on electrically evoked ACh release.

Blockade of neuronal nAChRs with 10 μM mecamylamine (Panagis et al., 2000) only slightly diminished the inhibitory effect of MDMA on evoked ACh release indicating that MDMA also acts, in part, via this cholinergic receptor. Interestingly, studies on striatal synaptosomes and slices showed that the release of DA can be induced by activation of nAChRs located on dopaminergic nerve terminals (Grady et al., 1992b; Kaiser and Wonnacott, 2000; Sakurai et al., 1982; Wonnacott, 1997). Moreover, there are also nAChRs on glutamatergic axon terminals in the striatum, leading to glutamate release following their activation, which in turn may either stimulate dopaminergic terminals to release DA (Kaiser and Wonnacott, 2000) or cholinergic interneurons to release ACh (Lupp et al., 1992). Thus, part of the effect of MDMA on the evoked ACh release may be mediated by indirect neuronal loops involving both glutamatergic and dopaminergic transmission following stimulation of nAChRs.

Finally, tachykinin receptors of the NK_1 type, which are activated by substance P, also seem to participate in the action of MDMA on the evoked release of striatal ACh in rats. Indeed, blockade of this receptor with the potent and highly selective competitive antagonist L-732,128 (MacLeod et al., 1994) fully antagonized the inhibitory effects of MDMA.

As regards the effects of EtOH on striatal release of ACh, it has been shown previously by in-vivo microdialysis that EtOH (1 and 2 g/kg), after a short increase, considerably decreased the basal levels of ACh in the medial prefrontal cortex (Jamal et al., 2005), an effect possibly due to a decrease in the activity of choline acetyltransferase (Jamal et al., 2007). Another group also showed an inhibition of basal and stimulated ACh release in the hippocampus in vivo following systemic application of 2.4 g/kg EtOH, whereas local infusion of $\leqslant$10 mM EtOH into the hippocampus led to a stimulation of ACh release (Henn et al., 1998). In this context it is important to note that the concentration of 2‰ EtOH used in the present experiments represents a concentration of 34.3 mM.

The findings mentioned above are extended by the present work as we found EtOH to inhibit the evoked release of ACh in vitro in the striatum, an effect which may also imply local mechanisms considering that EtOH was directly applied on the slices. Regarding the interaction of EtOH with specific receptors and neurotransmitter release (see Harris, 1999; Mascia et al., 2001; Yoshimoto et al., 1992), EtOH is known to potentiate the function of neuronal $GABA_A$ receptors. Furthermore, 10–20 mM EtOH appears to inhibit the NMDA receptor-gated ion channel, although the sensitivity of NMDA receptors to EtOH seems to be dependent on the subunit composition of the receptor and thus may vary from region to region (Tabakoff and Hoffman, 1996). Finally, NMDA receptor-evoked ACh release is significantly reduced in the presence of EtOH (at 2–6‰) (Darstein et al., 1998). Thus the inhibitory effect of EtOH alone on ACh release observed in the present study might be explained in part by an inhibitory effect on NMDA-evoked ACh release.

Two of the findings mentioned above reveal some clues on the possible mechanism of interaction between the effects of EtOH and MDMA on cholinergic

transmission in the striatum. Thus, when nAChRs or NK_1 receptors were blocked with selective antagonists, the inhibitory effects of MDMA on the evoked ACh release were attenuated or prevented, respectively, only in the absence of EtOH. Regarding nAChRs it should be remembered (see above) that nicotine also indirectly affects DA release via nAChRs located on glutamatergic neurons (Kaiser and Wonnacott, 2000). Since EtOH itself interacts with NMDA receptors, this indirect loop may be affected in the presence of the nAChR antagonist mecamylamine. Moreover, it has been shown that EtOH also directly modulates nAChR functions, effects which seem to be important for several behavioural effects of the drug, including self-administration (Borghese et al., 2003; Soderpalm et al., 2000). On the other hand, interactions of EtOH with substance P-containing neurons or with NK_1 receptors are so far unknown. Nevertheless our data point to a possible involvement of those neurons or receptors in the interaction of EtOH with MDMA on striatal ACh release.

Taken together our present findings on striatal ACh release, similar to those on striatal DA and 5-HT release, suggest that the effects of MDMA and EtOH involve several local neurotransmitter systems, which interact more or less indirectly in an extremely complicated way.

Conclusions

Regarding the complex mechanisms behind the effects of MDMA and/or EtOH on the modulation of striatal neurotransmission several new observations emerged from the present study which support the hypothesis that EtOH may affect the abuse liability of MDMA. For example, using the combination of MDMA with EtOH there is a striking parallelism both in locomotor activity (Figure 1) and drug-induced DA release (Figure 5), supporting the role of striatal DA levels in the control of locomotion (Martin-Iverson and Burger, 1995; Mehler-Wex et al., 2006). Moreover, even if the effects mediating the action of these drugs of abuse on striatal ACh release need further investigation, the use of specific antagonists allowed us to suggest that several receptors and local neuronal interactions may be implicated. Finally, this work demonstrates for the first time, a local synergistic interaction of EtOH and MDMA on the spontaneous release of DA and 5-HT in the striatum. In no case, however, should the current findings be considered as demonstrating an exclusive mechanism accounting for this synergism. Thus, it also seems likely that the effects of the combined application of EtOH + MDMA on behavioural parameters (such as hyperactivity or hyperthermia) only partly rely upon local mechanisms. Indeed, if our results show that one (or more) local mechanism(s) might participate in these interactions, they neither exclude an additional in-vivo action via more complex polysynaptic loops that could also involve extra-striatal neurons, nor do they discard the possibility of an additional involvement of pharmacokinetic factors, also observed, e.g. during cocaine abuse following EtOH consumption (Cami et al., 1998; Farre et al., 1997; Foltin et al., 1995). Obviously, the two latter issues need further investigation.

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Statement of Interest

None.

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