Investigating the potential neurotoxicity of ecstasy (MDMA). An imaging approach
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Chapter 5.1

Use of amphetamine by recreational users of ecstasy (MDMA) is associated with reduced striatal dopamine transporter densities: a $[^{[123]}\beta$-CIT SPECT study

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Abstract

Rationale - Tablets sold as ecstasy do often not only contain 3,4-methylenedioxymethamphetamine (MDMA) but other compounds well known to cause dopaminergic neurotoxicity, such as (meth)amphetamine. Furthermore, the use of ecstasy in the Netherlands is often combined with the use of amphetamine. However, little is known about the effects of ecstasy use or the combination of ecstasy and amphetamine use on dopamine (DA) neurons in the human brain. Objectives - To investigate the effects of ecstasy as well as the combined use of ecstasy and amphetamine on the density of nigrostriatal DA neurons.

Methods - [123]I-β-CIT SPECT was used to quantify striatal DA transporters. Striatal [123]I-β-CIT binding ratios of control subjects ($n=15$) were compared with binding ratios of ecstasy users ($n=29$) and individuals with a history of combined ecstasy and amphetamine use ($n=9$) after adjustment for age. Results - Striatal [123]I-β-CIT binding ratios were significantly lower in combined ecstasy and amphetamine users compared to sole ecstasy users (6.75 vs. 8.46, respectively: -20.2%, $p=0.007$). Binding ratios were significantly higher in ecstasy users when compared to controls (8.46 vs. 7.47, respectively: +13.2%, $p=0.045$).

Conclusions - These initial observations suggest that the sole use of ecstasy is not related to dopaminergic neurotoxicity in humans. In contrast, the reported use of amphetamine by regular users of ecstasy seems to be associated with a reduction in nigrostriatal DA neurons.

Introduction

Amphetamine and some of its analogues have been shown to be neurotoxic to dopamine (DA) and/or serotonin (5-HT) neurons in animals. For instance, after administration of methamphetamine, animals develop long-lasting decreases in brain DA and 5-HT axonal markers, including the neurotransmitters themselves (i.e., DA and 5-HT), and their transporter sites (Seiden et al., 1976; Villemagne et al., 1998; Wagner et al., 1980). Administration of amphetamine to animals, including non-human primates, results in decreases in DA levels and DA transporter densities (Melega et al., 1996; Steranka 1983; 1980). Furthermore, the popular recreational drug 3,4-methylenedioxyamphetamine (MDMA, "Ecstasy") has been shown to be neurotoxic to brain 5-HT neurons in animals and possibly humans (Renenan et al., 2001a; 2001b; McCann et al., 1998a; Ricautre et al., 2000; Semple et al., 1999). Brain levels of DA and its metabolite homovanillic acid (HVA) are not reduced by low doses of MDMA but after higher doses, suggesting that while MDMA is more toxic to 5-HT than DA systems, it can also damage DA neurons (Taffe et al., 2001; Commins et al., 1987)

While the potential neurotoxic effects of MDMA on DA neurons have been extensively studied in animals, little is known about the dopaminergic effects of MDMA in the human brain. Only two studies have investigated the effects of MDMA on DA neurons by evaluating cerebrospinal fluid HVA (McCann et al., 1994) and DA transporter densities using SPECT (Semple et al., 2000). HVA and DA transporter densities in MDMA users were comparable with control subjects. Furthermore, since the composition of tablets sold as ecstasy do often not only contain MDMA, but also other compounds well known to cause DA neurotoxicity, such as amphetamine and methamphetamine, it is important to study its effects in the human brain directly. The Drugs Information and Monitoring System (DIMS), a unique project in the Netherlands to chemically monitor the ecstasy market, reported that in 1997 a substantial proportion (32%) of the street substances being marketed as ecstasy contained amphetamine or methamphetamine (Spruit et al., 1999), ranging from a low of 7 to 23% (on average 32 mg) per tablet (Konijn et al., 1997).

Furthermore, a recent survey in the Netherlands investigated the prevalence of the combined use of ecstasy and amphetamine (the use of methamphetamine is uncommon in the Netherlands). It was found that in 26% of the 847 cases ecstasy was often or always combined with the use of amphetamine (Van de Wijngaart et al., 1997).

These observations press for a further investigation of the effects of ecstasy, as well as the combined use of ecstasy and amphetamine on brain DA neurons in human beings. The development of [123]I-iodine-2β-carbomethoxy-3β-(4-iodophenyl) tropane (β-CIT), has made it possible to image concomitantly DA and 5-HT transporters in the human brain using single photon emission computed tomography (SPECT) (Brücke et al., 1993). The DA transporter is a structural element of the DA neuron that is substantially decreased in animals given DA neurotoxins, such as methamphetamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Villemagne et al., 1998; Booij et al., 1997a).

This study compared the density of striatal [123]I-β-CIT labelled DA transporters in ecstasy users. Furthermore, the effects of combined ecstasy and amphetamine use on [123]I-β-CIT labelled DA transporters were analyzed. Previous studies in rodents have demonstrated that drugs that bind to the 5-HT trans-
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porter enhance binding of \(^{3}H\)β-CIT to the DA transporter (Fujita et al., 1997; Scheffel et al., 1994), although this was not observed with other DA transporter ligands (Lavalaye et al., 2000; Booji et al., 1998). Therefore, striatal \(^{3}H\)β-CIT binding ratios in a group of combined ecstasy and amphetamine users were compared both with ecstasy users and healthy controls. This study was part of a larger \(^{3}H\)β-CIT investigation, in which the effects of ecstasy on the 5-HT system were studied in more detail (Reneman et al., 2001a; 2000b).

Methods and materials

Participants

Ecstasy users were compared with ecstasy-naive but drug using controls. Subjects were recruited with flyers distributed at venues associated with the "rave scene" in Amsterdam with the help of UNITY, an agency which provides harm reduction information and advice. Experimental and control groups were thus recruited from the same community sources. Subjects selected were matched for gender and age, between 18 and 45 years, otherwise healthy, and with no psychiatric history. 38 ecstasy users and 15 ecstasy-naive subjects were recruited. The 15 ecstasy-naive subjects ("controls") were healthy subjects with no self-reported prior use of ecstasy. Of the 38 ecstasy users, 9 reported that they intentionally usedamphetamine in the 3 months prior to this study ("Ecstasy + amphetamine users"; Table 1). Participants agreed to abstain from use of all psychoactive drugs for at least 3 weeks before the study, and were asked to undergo urine drug screening (with an enzyme-multiplied immunoassay for amphetamines, barbiturates, benzodiazepine metabolites, cocaine metabolite, opiates, and marijuana) before enrolment. After testing urine samples, exclusion criteria were: a positive drug screen; pregnancy; a severe medical or neuropsychiatric illness that precluded informed consent, and a lifetime psychiatric disorder. Subjects were interviewed with a structured computer assisted diagnostic psychiatric interview (Composite International Diagnostic Interview: CIDI, version 2.1) to screen for current axis I psychiatric diagnoses. The institutional Medical Ethics Committee approved the study. After complete description of the study to the subjects, written informed consent was obtained from all participants.

Imaging

For SPECT scanning the Strichmann Medical Equipment 810X tomographic system was used. This 12-detector single-slice scanner has a full-width at half-maximum (FWHM) resolution of approximately 7.5 mm. Each acquisition consisted of at least 15 slices, (acquired in a 64 x 64 matrix), 3 min per slice, and with a slice distance of 5 mm. The energy window was set at 135±190 keV. Subjects lay in the supine position with the head aligned in a parallel to the obitomeatal line, and were positioned such that the scanning volume initially included the cerebellum. Acquisition was commenced 20 h after i.v. injection of approximately 140 MBq \(^{123}I\)β-CIT (radio-labeling as described by Neumeyer et al., 1991), a time when specific binding to the striatum is maximal and stable for up to 24 h following injection (Brücke et al., 1993; Laruelle et al., 1994). For analysis of \(^{3}H\)β-CIT binding, a standard template with regions of interest (ROIs) was constructed manually from MR images. For positioning we used these MR images as a guide. The template with a ROI for the left and right striatum, a brain region rich in DA transporters, was placed on three consecutive SPECT slices, demonstrating best visualization of the striatum (Booji et al., 1997b). An additional template was constructed with a ROI for the cerebellum. An investigator unaware of the participant's history performed ROI analysis. The uptake in the cerebellum, presumed free from DA transporters, was used as a reference for background radioactivity (non-specific binding + free ligand). Striatal \(^{3}H\)β-CIT binding was calculated by dividing total binding in the striatum by binding in the cerebellum.

Statistics

Differences between the three groups with regard to demographic variables and other drug exposure were analyzed using ANOVA (log transformed if necessary).

The difference in mean \(^{3}H\)β-CIT labelled DA transporters was analyzed using a general linear regression model. The starting model included group (3 levels), gender (2 levels), age (linear) and extent of previous cannabis use (linear, log transformed).

Pearson correlation analyses was performed between striatal \(^{3}H\)β-CIT binding ratios, and extent of previous MDMA and amphetamine use. Results were considered significant at p < 0.05. Data were analyzed using SPSS (SPSS Software Inc, Chicago, IL, USA version 9.0).

Results

Participants

Ecstasy + amphetamine users were younger than controls and sole ecstasy users, though this did not reach statistical significance (p = 0.10). The groups were similar for gender distribution (Table 1).
The two ecstasy using groups were similar with regard to use of ecstasy, alcohol and other drugs, except for the use of amphetamine (Table 1). Ecstasy + amphetamine users indicated using more cannabis than controls (Table 1). Similar and only occasional use of LSD (lysergic acid diethylamide), 'magic mushrooms' and cocaine was reported in both groups (data not shown).

**SPECT imaging**

Gender and extent of previous cannabis use were dropped from the starting model as they had no significant contribution (p = 0.75 and 0.62, respectively). Age had a highly significant effect (p = 0.000) on mean \([^{[3]}\)fB-CIT binding ratios and was therefore kept in the model. Comparisons of groups revealed that binding ratios were significantly higher in ecstasy users compared to controls (p = 0.045). \([^{[3]}\)fB-CIT binding ratios were significantly lower in ecstasy + amphetamine users compared to sole ecstasy users (p = 0.007), but not when compared to controls (p = 0.275) (Table 1 and Figure 1).

There was no correlation between the extent of previous ecstasy use and striatal \([^{[3]}\)fB-CIT binding

**Table 1. Demographics and comparison of striatal \([^{[3]}\)fB-CIT binding ratios between controls, ecstasy and ecstasy + amphetamine users**

<table>
<thead>
<tr>
<th></th>
<th>Controls n = 15</th>
<th>Ecstasy users n = 29</th>
<th>Ecstasy + amphetamine users n = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>26.1 (5.5)</td>
<td>26.1 (5.6)</td>
<td>22.1 (8.8)</td>
</tr>
<tr>
<td>Men/women</td>
<td>7/8</td>
<td>15/14</td>
<td>6/3</td>
</tr>
<tr>
<td>Ecstasy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of use (y)</td>
<td></td>
<td>5.1 (2.9)</td>
<td>4.4 (1.9)</td>
</tr>
<tr>
<td>Usual dose (tablets)</td>
<td></td>
<td>1.7 (0.7)</td>
<td>2.3 (0.7)</td>
</tr>
<tr>
<td>Lifetime dose (tablets)</td>
<td></td>
<td>32.4 (52)</td>
<td>358 (610)</td>
</tr>
<tr>
<td>Time since last tablet (months)</td>
<td></td>
<td>3.4 (4.6)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>Amphetamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphetamine (no. times used past year)</td>
<td></td>
<td></td>
<td>47.5 (45.0)</td>
</tr>
<tr>
<td>Mean amphetamine dose (g)</td>
<td></td>
<td></td>
<td>0.41 (0.31)</td>
</tr>
<tr>
<td>Alcohol and other drugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol (units/week)</td>
<td>10.6 (9.8)</td>
<td>10.3 (8.6)</td>
<td>12.3 (13.9)</td>
</tr>
<tr>
<td>Tobacco (cig./day)</td>
<td>1.0 (0.5)</td>
<td>8.5 (8.0)</td>
<td>12.7 (13.2)</td>
</tr>
<tr>
<td>Cannabis (no. joints last 3 months)</td>
<td>1.7 (3.1)</td>
<td>54.1 (107.0)</td>
<td>87.0 (102.8)</td>
</tr>
<tr>
<td><strong>SPECT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated striatal ([^{[3]})fB-CIT binding ratios</td>
<td>7.47 (SE 0.39)</td>
<td>8.46 (SE 0.28)*</td>
<td>6.75 (SE 0.52)*</td>
</tr>
</tbody>
</table>

1 Data are expressed in mean ± SD values except for estimated \([^{[3]}\)fB-CIT binding ratios
2 Significantly different from controls (Log transformed: ANOVA F=5.7, df=3, p<0.01; Post hoc comparison p<0.01)
3 Estimated marginal means after correction for the difference in age distribution (evaluated at 25.6 y of age)
4 Significantly different from controls (Overall ANOVA F=4.9 df=2, p<0.01; Post hoc comparison p<0.01)
5 Significantly different from ecstasy users (Overall ANOVA F=4.9 df=2, p<0.01; Post hoc comparison p<0.01)
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Discussion
The results of this preliminary study suggest that while sole ecstasy use does not decrease striatal DA transporter densities in human beings, the combined use of amphetamine and ecstasy may be associated with reduced striatal DA transporter densities.

To our knowledge, this is the first study reporting on the effects of amphetamine on striatal dopamine transporter densities in human brain. PET studies in amphetamine treated monkeys have shown reductions in striatal [123I]fluoro-L-DOPA uptake in vervet monkeys (Melega et al., 1996; 1997). Furthermore, studies on rat striatal DA system have established that chronic amphetamine exposure results in neurotoxicity characterized by decreases in dopamine levels and DA transporter densities, swollen nerve terminals and degenerated axons (Ridley et al., 1982; Ryan et al., 1990). Given the large body of evidence directly documenting the DA neurotoxic potential of amphetamine in rodents and non-human primates, our data provide preliminary evidence that recreational use of combined amphetamine and ecstasy use might be neurotoxic to DA neurons.

While few studies have investigated the effects of amphetamine in humans, recently a number of studies have reported on the effects of methamphetamine in the human brain. Like amphetamine, methamphetamine is an amphetamine derivative known to cause damage to DA neurons in animals treated with this drug (Seiden et al., 1976; Wagner et al., 1980). The present findings are in good agreement with these studies in methamphetamine users. For instance, using PET, reductions in DA terminal markers have been demonstrated in methamphetamine-treated monkeys and human methamphetamine users using the DA transporter ligands [3H][C]WIN-35,248 and [3H]d-threo-methylphenidate (Villemaigne et al., 1998; McCann et al., 1998b). Reductions in DA transporter densities in methamphetamine users have been associated with motor and cognitive impairments (Volkow et al., 2001) and the severity of persistent psychiatric symptoms (Sekine et al., 2001).

The presently observed absence of neurotoxic effects of ecstasy on human DA neurons is in good agreement with previous animal studies which failed to find any damage to DA neurons following MDMA treatment (Green et al., 1995; Steele et al., 1994). It is also in agreement with a recent [123I]r-CIT SPECT study (Semple et al., 2000), in which no reductions in striatal binding ratios were observed between ecstasy users and control subjects. McCann and colleagues (1994) also found no evidence of neurotoxic effects of MDMA on DA neurons in the human brain, since HVA levels in the cerebral spinal fluid of ecstasy users did not differ from controls.

The present finding of absence of neurotoxic effects of ecstasy on human DA is of interest for a number of reasons. First, our findings may indicate that the effects of drugs with known DA neurotoxic effects (such as amphetamine) in tablets sold as 'ecstasy' in the Netherlands, may be too small to be neurotoxic. It can not be excluded that the ecstasy tablets taken by our subjects did not contain amphetamine or methamphetamine. However, there is substantial support that a considerable proportion of the ecstasy users in this study must have, unintentionally, been exposed to amphetamine or methamphetamine, since 83% of the ecstasy users indicated having used ecstasy in 1997. In that particular year, 32% of all ecstasy drug samples (n = 7009) tested by DIMS contained amphetamine or methamphetamine (on average 32 mg per tablet; Spruite et al., 1999). Second, it has been suggested that a recent case of parkinsonism in a chronic human ecstasy user (Baggott et al., 1999) may have resulted from a neurotoxic effect of MDMA on the nigrostriatal dopaminergic neurons. It was suggested by Mintzer and co-workers (1999) that the parkinsonism may have resulted from amphetamine or methamphetamine present in ecstasy tablets taken by the patient. However, in the present study we did not observe evidence indicating loss of DA neurons in sole ecstasy users, whereas in the group of combined ecstasy and methamphetamine users [3H]r-CIT binding ratios were only approximately 12% lower when compared to ecstasy users. It is well known that parkinsonian signs do not occur before more than 50% of DA terminals are degenerated (Fearnley & Lees 1991). Therefore, we can now say that the parkinsonian signs were most probably not caused by use of ecstasy or the combined use of ecstasy withamphetamine or methamphetamine. Third, the present findings stress the importance to perform studies like these in well matched groups: not only with respect to age and gender, but the use of other drugs as well. Ideally, two groups under study will differ only on one variable which is the focus of the study. In the present study, combined ecstasy and amphetamine users differed only in the use of ecstasy, suggesting that it is the use amphetamine, and not ecstasy, that may lead to loss of nigrostriatal neurons.

Previous studies in rodents have demonstrated increased binding of [123I]r-CIT to the DA transporter short after administration of 5-HT reuptake inhibitors (Fujita et al., 1997; Scheffel et al., 1994). In line with
this, we presently observed increased striatal binding of \([{14}^\text{C}]\)β-CIT in ecstasy users when compared to controls. One possible explanation for this enhancement of binding is regulation of the DA transporter through inhibition of 5-HT uptake. It has been suggested that (rapid) regulation, such as post-translational regulation, is evoked by the inhibition of 5-HT uptake, for instance by MDMA. However, since ecstasy users were scanned at least 3 weeks after the last MDMA tablet taken, future experimental studies will have to find out what the long-term effects of 5-HT reuptake inhibitors (or MDMA) are on DA transporter densities. Whatever the underlying mechanism, the present findings clearly demonstrate the need for careful matching of study groups. To investigate the effects of amphetamine use on DA neurons in ecstasy users it is of crucial importance to compare DA transporter densities between ecstasy using subjects that only differ on the intentional use of amphetamine, rather than to control subjects, such as performed in the present study.

Displacement studies in animals and humans have shown that striatal uptake of β-CIT is associated with DA transporters (Laruelle et al., 1993; 1994). Moreover, it has been shown that striatal \([{14}^\text{C}]\)β-CIT binding, measured 24 h p.i. adequately reflects the density of DA transporters (Laruelle et al., 1994).

When considered with results of previous SPECT studies directly documenting the validity of SPECT with \([{12}^\text{C}]\)β-CIT for detecting MPTP-induced DA neurotoxicity (Shaya et al., 1992), the present findings of reduced DA transporter densities in combined ecstasy and amphetamine users may be related to damage to striatal DA axons and axon terminals. It should be kept in mind, however, that it is an assumption that a decrease in DA transporter density directly reflects axonal loss. The presently observed decreases in DA transporter densities could also be related to a neuro-adaptive process, not associated with actual DA nerve terminal degeneration.

Several potential limitations of the current study should be mentioned. The absence of effects of ecstasy use on human DA neurons may be related to the fact that we had to rely upon participant's report of drug abuse. As with all retrospective studies, it is impossible to determine exactly what drug was taken, and to ensure abstention from MDMA. Urine screening was performed to detect concealed recent MDMA use. In future studies, hair-sample analysis would be a useful way to assess more appropriately what drug was taken at what time and to ascertain previous use of MDMA. However, of particular relevance to this study is that we were able to get a fairly good impression on what an ecstasy tablet is likely to have contained in the Netherlands, because of the DIMS project. Second, there is a possibility that pre-existing differences between amphetamine users and amphetamine non-users underlie differences in DA transporter densities. People with low DA transporter densities may be predisposed to use amphetamine and to have low DA transporter densities. Future studies taking the recently described functional polymorphism in the promoter for the DA transporter gene into account, could be of interest (Heinz et al., 2000a; 2000b). However, the two groups of amphetamine users were both regular users of ecstasy and differed only on the point that one subgroup had a history of amphetamine use. It is therefore unlikely that pre-existing differences or the use of other drugs than amphetamine should account for changes in striatal DA transporter densities. Considering the small sample size of the amphetamine group, the results of the present study should be interpreted as preliminary.

We conclude that the use of ecstasy seems not to be associated with dopaminergic neurotoxicity in humans. In contrast, the reported use of amphetamine in regular ecstasy users seems to be associated with toxic damage to dopaminergic neurons, in line with previous studies in animals. These initial observations suggest potential harmful effects of amphetamine on DA system.

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Chapter 5.2

Addition of a 5-HT receptor agonist to methyl-phenidate potentiates the reduction of $[^{123}]$FP-CIT binding to dopamine transporters in rat frontal cortex and hippocampus

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Abstract
The neurotoxic potential of amphetamine and related drugs is well documented. However, methylphenidate, an amphetamine derivative used in the treatment of attention deficit hyperactivity disorder, and known to increase synaptic dopamine (DA) levels, seems to lack neurotoxic potential. It is hypothesized that both dopaminergic and serotoninergic systems are involved in the neurotoxicity of amphetamine derivatives. The purpose of the present study was to evaluate the neurotoxic potential of methylphenidate and to test whether stimulation of the serotoninergic system may confer neurotoxic properties to methylphenidate for DA or serotonin (5-HT) neurons. In addition, the present study was undertaken to evaluate the necessity to perform future SPECT studies in individuals using both methylphenidate and 5-HT acting agents. We therefore measured monoaminergic transporters in rat brain using radioligands suitable for SPECT imaging ([123I]β-CIT and [123I]FP-CIT). Groups of rats were treated with methylphenidate or saline for 4 days. Additional groups were treated with the selective 5-HT2 receptor agonist quipazine or the selective 5-HT reuptake blocker fluoxetine, alone or in combination with methylphenidate. Binding studies were performed 5 days after the last treatment. In a second experiment, methylphenidate in combination with quipazine, alone with a control group, was retested. In this experiment, monoaminergic terminal density was estimated 2 weeks (rather than 5 days) after drug treatment. Five days, but not 2 weeks, after treatment a significant reduction in specific [123I]FP-CIT binding was observed in the frontal cortex and hippocampus of rats treated with methylphenidate in combination with quipazine. These changes probably do not reflect neurotoxic changes of frontal cortex and hippocampal DA terminal markers, but a compensatory downregulation of DA transporters. These findings suggest potential harmful effects of concomitant use of drugs directly activating 5-HT2 receptors in patients using methylphenidate.

Introduction
Several amphetamine-related compounds are neurotoxic to rodents, as shown by a profound and lasting decrease in the concentration of monoamines in the brain. For example, administration of high doses of methamphetamine results in loss of dopamine (DA) (Koda & Gibb, 1973) as well as serotonin (5-HT) (Hotchkiss & Gibb, 1980). In contrast, the amphetamine derivatives 3,4-methylenedioxymethamphetamine (MDMA or “Ecstasy”) and fenfluramine are relatively selective in producing neurotoxic damage to 5-HT neurons, while sparing dopaminergic neurons. Damage to 5-HT neurons was demonstrated by reductions in brain 5-HT content, 5-hydroxyindoleacetic acid, or the density of 5-HT uptake sites (Ricaurte et al., 1993; Bataglia et al., 1988; Stone et al., 1986; Schmidt et al., 1987a; 1987b; O’Hearn et al., 1988). Moreover, recent positron emission tomography (PET) and single photon emission computed tomography (SPECT) studies have shown decreases in the number of central 5-HT neurons in MDMA-treated primates and human MDMA-users, as well as loss of DA neurons in methamphetamine users (McCann et al., 1996; Scheffel et al., 1995; Semple et al., 1999).

The precise mechanisms underlying the neurotoxic effects of amphetamines are not yet known. Several lines of evidence now indicate that the neurotoxicity of MDMA and related drugs is closely linked to DA release (Abekawa et al., 1994; O’Dell et al., 1991; Schmidt et al., 1985; Wagner et al., 1983). For example, pretreatment with L-3,4 dihydroxyphenylalanine (L-DOPA) potentiates the long-term serotonergic deficits induced by MDMA (Schmidt et al., 1991), and there appears to exist a linear correlation between the acute increase of extracellular DA and the extent of serotonergic toxicity (Nash & Nichols, 1991).

In spite of this and other evidence favoring a role for DA in the neurotoxicity of these agents, there are at least two considerations which have been suggested to contradict this conclusion. First, fenfluramine, an agent with extreme weak DA-releasing properties, is known to be neurotoxic to 5-HT neurons (McCann et al., 1997). Second, methylphenidate, an amphetamine derivative clinically used in the treatment of attention deficit hyperactivity disorder (ADHD) and narcolepsy (Ellison et al., 1978; Faraj et al., 1974) and known to increase synaptic DA levels, lacked neurotoxic potential in three studies which have evaluated the neurotoxic potential of methylphenidate (Wagner et al., 1980; Zaczek et al., 1989; Yuan et al., 1997). Apparently, an increase in extracellular DA, although necessary, is not a sufficient condition for neurotoxicity of amphetamine-related drugs.

It has recently been suggested that both dopaminergic and serotonergic systems are involved in the neurotoxicity of amphetamine derivatives (Schmidt et al., 1990). For example, the potent 5-HT releaser, 5-methoxy-6-methyl-2-aminoindan (MMAI), will cause long-term central 5-HT deficits only when combined with amphetamine (Johnson et al., 1991). Interestingly, it was recently shown that 5-HT released by MDMA plays a role in 5-HT neuron toxicity by incre-
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...ing DA synthesis and release through activation of 5-HT2 receptors (Huang & Nichols, 1993; Schmidt et al., 1991). Thus, brain 5-HT2 receptors and DA content appear to play a major role in the neurotoxicity of amphetamine-related compounds.

Recently, a possible alarming tendency to add 5-HT acting agents to methylphenidate has been noted in the treatment of children with ADHD. The combination of methylphenidate with, for instance, fluoxetine, led to a significant therapeutic improvement (Badet et al., 1998; Masand et al., 1998; Myronuk et al., 1996; Findling, 1996). Theoretically, as previously discussed, this new combination of medication could be neurotoxic to DA and/or 5-HT neurons. To date, however, it is not known what the effects on DA and 5-HT neurons are when methylphenidate is used in combination with direct or indirect 5-HT acting agents. Several studies have shown that 5-HT reuptake inhibitors prevent 5-HT neurotoxicity induced by several amphetamine-derivatives (McCann et al., 1995). By occupying the 5-HT transporter, 5-HT reuptake inhibitors possibly prevent the parent amphetamine and/or a toxic metabolite from entering the 5-HT neuron. We therefore hypothesized that the combination of methylphenidate with a 5-HT reuptake inhibitor may not be neurotoxic to 5-HT neurons. However, it is not known what the effect of methylphenidate in combination with indirect stimulation of 5-HT2 receptors by 5-HT reuptake inhibitors on DA neurons may be.

Based upon these considerations, from a clinical as well as a theoretical point of view, it seems relevant to: 1) study whether the combination of methylphenidate and 5-HT acting agents is neurotoxic to DA and/or 5-HT neurons. 2) further evaluate the neurotoxic potential of methylphenidate, not only on DA but on 5-HT neurons as well, and; 3) evaluate the necessity to perform future SPECT studies in individuals treated with methylphenidate in combination with a 5-HT acting agent. Therefore, in the present studies neurotoxicity was assessed by the extent of regional loss of monoaminergic transporters in rat brain using radioligands suitable for SPECT imaging.

Materials and methods

Male Wistar rats (obtained from Broekman Institute B.V., Someren, the Netherlands), 200-250 g, were used in these experiments. All experiments involving animals were approved by the local Animal Care Committee.

Animal experiments

In experiment 1, studying short-term effects, methylphenidate was administered at a dose of 10 mg/kg or 40 mg/kg. Each dose was tested in a group of five rats, given orally every 12 h, for 4 consecutive days. A control group (n = 5) received saline, according to the same schedule and route of administration. Two groups of rats (n = 5 per group) received 5 mg/kg of the 5-HT2 receptor agonist quipazine (Sigma-Aldrich, Zwijndrecht, the Netherlands; Titeler et al., 1987; Glennon, 1987) or the 5-HT reuptake blocker, fluoxetine (Eli Lilly, Nieuwegein, the Netherlands) i.p. every 24 h for 4 consecutive days. Finally, in one group of rats (n = 5) 5 mg/kg quipazine, and in another group of rats 5 mg/kg fluoxetine, was co-administered 6 h (every 24 h) after 10 mg/kg methylphenidate was given, according to the above schedule and route of administration, for 4 consecutive days. Five days after treatment, DA and 5-HT transporter densities were measured, as described below.

Measuring DA and 5-HT transporter densities

Five days or 2 weeks after treatment, rats were injected i.v. with either approximately 1.85 MBq [3H]FP-CIT or [3H]β-CIT. [3H]Labeling of FP-CIT and β-CIT was performed by oxidative radioiododestannylation (Amersham Cygne, Technical University, Eindhoven, and Radionuclide Center, Vrije University, Amsterdam, the Netherlands, respectively) of their trimethylstannyl precursors obtained from RBI (Natick, MA). Both [3H]FP-CIT and [3H]β-CIT had a specific activity > 185 MBq/nmol and a radiochemical purity of > 95%.

Both [3H]FP-CIT and [3H]β-CIT bound to 5-HT as well as DA transporters. We have previously shown that [3H]FP-CIT binds in vivo predominantly to DA transporters (Booij et al., 1997). [3H]β-CIT has higher in vivo binding ratios for the 5-HT transporter than [3H]FP-CIT. Therefore, the combination of these two radioligands was chosen to assess both the 5-HT and DA system. Two hours after injection of [3H]FP-CIT (Booij et al., 1997) and 1 h after injection of [3H]β-CIT (Reneman et al., 1999), animals were killed by bleeding via heart puncture under ether anesthesia. The brains were quickly removed, dissected into frontal cortex, hippocampus, striatum, hypothalamus and cerebellum, and weighed. [3H]Radioactivity of [3H]FP-CIT or [3H]β-CIT in each region was assayed in a...
gamma counter. The data were corrected for radioactivity decay back to the time of preparation of the injection syringes in order to compare relative concentrations in the tissues taken and to relate the results to the injected dose. The amount of radioactivity was expressed as a percentage of the injected dose multiplied by the body weight per gram tissue weight (% ID x kg/g tissue), as described previously (Rijks et al., 1996). For both radioligands, the cerebellum was used as a reference region for the estimation of free and non-specifically bound radioligand. The specific binding was estimated by subtraction of radioactivity in cerebellum from total radioactivity in the region of interest.

Statistical analyses
We tested the main effect of drug administration on specific radioligand binding in the four brain regions studied by general linear model-based multivariate ANOVA (MANOVA), taking possible correlations between brain regions studied and multiple comparisons into account. When MANOVA revealed a significant effect, we investigated differences in regional \(^{\text{[\text{3}]}\text{FP-CIT}}\) and \(^{\text{[\text{1}]}\text{IT}}\)-CIT binding between groups by one-way ANOVA. When more than two different groups were compared, we used Dunnett’s test for post hoc analysis. Data are presented as means ± SEM. *p < 0.05 was taken to be significant with a two-tailed test. We analyzed all data with SPSS version 9.0 (Chicago, IL).

Results
SHORT-TERM EFFECTS
Is methylphenidate neurotoxic to 5-HT and/or DA neurons?
We investigated whether treatment with methylphenidate (10 or 40 mg/kg) led to significant reductions in specific \(^{\text{[\text{3}]}\text{FP-CIT}}\) or specific \(^{\text{[\text{1}]}\text{IT}}\)-CIT binding, when compared to saline-treated rats. No significant group effect was observed, either for \(^{\text{[\text{3}]}\text{FP-CIT}}\) binding, nor

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**Figure 1.** Specific binding of \(^{\text{[\text{3}]}\text{FP-CIT}}\) in frontal cortex (A), hippocampus (B), striatum (C), and hypothalamus (D). Specific binding (expressed as % ID x kg/g tissue) was calculated as total regional activity minus activity in the cerebellum (non-specific binding) and represent the mean ± SE of 5 rats. I: saline; II: 10 mg/kg methylphenidate; III: 40 mg/kg methylphenidate; IV: 10 mg/kg methylphenidate + 5 mg/kg quipazine (short-term); V: 5 mg/kg quipazine; VI: 10 mg/kg methylphenidate + 5 mg/kg fluoxetine; VII: 5 mg/kg fluoxetine; VIII: saline; IX: 10 mg/kg methylphenidate + 5 mg/kg quipazine (long-term). *p < 0.05 **p < 0.02.
Investigating the potential neurotoxicity of Ecstacy (MDMA): An imaging approach

Figure 2. Specific binding of $[^{3}H]j$CIT in frontal cortex (A), hippocampus (B), striatum (C), and hypothalamus (D). Specific binding (expressed as % ID x kg/g tissue) was calculated as total regional activity minus activity in the cerebellum (non-specific binding), and represent the mean ± SE of five rats. I: saline; II: 10 mg/kg methylphenidate; III: 40 mg/kg methylphenidate; IV: 10 mg/kg methylphenidate + 5 mg/kg quipazine; V: quipazine.

for $[^{3}H]j$CIT-binding ($p = 0.184$ and $p = 0.254$; Figures 1 and 2, respectively).

Is quipazine neurotoxic to 5-HT and/or DA neurons?
In this analysis we investigated whether treatment with quipazine led to significant reductions in specific $[^{3}H]j$FP-CIT or specific $[^{3}H]j$CIT binding when compared to saline-treated rats. A significant group effect was observed for specific $[^{3}H]j$FP-CIT, but not specific $[^{3}H]j$CIT-binding ($p = 0.016$ and $p = 0.540$, respectively). ANOVA analysis revealed that specific $[^{3}H]j$FP-CIT binding was significantly higher in the hypothalamus of groups of rats treated with quipazine compared to controls (Figure 1D).

Is fluoxetine neurotoxic to DA neurons?
When we compared specific $[^{3}H]j$FP-CIT binding of fluoxetine treated rats with saline-treated rats, no significant group effect was observed ($p = 0.122$; Figure 1).

Is the combination of methylphenidate and quipazine neurotoxic to DA and/or 5-HT neurons?
We investigated whether treatment with the combination of methylphenidate (10 mg/kg) with quipazine (5 mg/kg) led to significant reductions in $[^{3}H]j$FP-CIT or $[^{3}H]j$CIT-binding when compared to saline-treated rats. In specific $[^{3}H]j$FP-CIT, but not $[^{3}H]j$CIT binding, a significant group effect was observed ($p = 0.029$ and $p = 0.817$, respectively). ANOVA analysis revealed that in the frontal cortex and hippocampus, specific $[^{3}H]j$FP-CIT binding was significantly lower in rats treated with the combination of methylphenidate with quipazine when compared to controls ($p = 0.016$ and $p = 0.049$; Figures 1A and 1B, respectively). No difference in binding was observed in the striatum ($p = 0.439$; Figure 1C) or hypothalamus ($p = 0.912$; Figure 1D).

Is the combination of methylphenidate and quipazine significantly different from either methylphenidate (10 mg/kg) or quipazine alone?
We investigated whether treatment with the combination of methylphenidate (10 mg/kg) with quipazine (5 mg/kg) led to significant reductions in $[^{3}H]j$FP-CIT
or [3H]β-CIT binding when compared to groups of rats treated with methylphenidate (10 mg/kg) or quipazine alone. MANOVA revealed a significant group effect for specific [3H]FP-CIT binding (p = 0.044), but not for [3H]β-CIT binding (p = 0.106; Figure 2). Using Dunnett's test for post hoc analysis of specific [3H]FP-CIT binding, it was shown that in the frontal cortex and hippocampus of rats treated with the combination of methylphenidate and quipazine there were significant reductions in [3H]FP-CIT binding when compared to quipazine-treated rats (p = 0.001 and p = 0.005; Figure 1A, B, respectively). In addition, in the frontal cortex, [3H]FP-CIT binding in rats treated with the combination of methylphenidate and quipazine, was significantly lower compared to rats treated with methylphenidate (10 mg/kg) alone (p = 0.023; Figure 1A). Furthermore, in the hypothalamus a significant higher [3H]FP-CIT binding was observed in combined treated rats, than in rats treated with methylphenidate alone (p = 0.049; Figure 1D).

Does [3H]FP-CIT binding differ significantly in rats treated with the combination of methylphenidate and fluoxetine from rats treated with either methylphenidate (10 mg/kg) or fluoxetine alone?

No significant group effect was observed (p = 0.531) when we compared specific [3H]FP-CIT binding in a group of rats treated with either a combination of methylphenidate and fluoxetine, or fluoxetine and methylphenidate alone (Figure 1).

LONG-TERM EFFECTS

Since the 5-day survival period was relatively short, Experiment 2 retested the effects of 10 mg/kg of methylphenidate in addition of quipazine. Rats were allowed a 2-week postdrug survival period to ensure that methylphenidate's effects on pre-synaptic monoaminergic terminals were indeed long-lasting. Animals were treated with methylphenidate in combination with quipazine, together with a saline-treated control group. Two weeks after treatment no reductions in specific [3H]FP-CIT binding in the frontal cortex or hippocampus were observed when compared to controls (p = 0.988) (Figures 1A and 1B, respectively).

Discussion

Co-administration of quipazine and methylphenidate produced a significant reduction in specific [3H]FP-CIT binding in the frontal cortex and hippocampus when rats were examined 5 days after treatment. However, when animals were examined 2 weeks after combined quipazine and methylphenidate administration no significant reduction in [3H]FP-CIT binding in the frontal cortex and hippocampus was observed, even though animals had been treated with identical dose regimes.

In rats, [3H]FP-CIT labels both DA and 5-HT transporters in vivo (Booij et al., 1997). However, [3H]FP-CIT binds in vivo predominantly to DA transporters. Therefore, reduced specific [3H]FP-CIT binding, as observed in the present study after administration of methylphenidate in combination with quipazine, probably reflects changes in DA terminal markers. In addition, since [3H]β-CIT has higher binding ratios for the 5-HT transporter than [3H]FP-CIT (Reneman et al., 1999), and no reductions were observed in [3H]β-CIT binding after administration of methylphenidate in addition of quipazine, it is likely that DA and not 5-HT terminal markers were affected.

It could be argued that the decrease in [3H]FP-CIT binding caused by coadministration of methylphenidate and quipazine is due to neurotoxic loss of DA transporters terminals. A more favorable explanation, however, for the observed short-term changes in frontal cortex and hippocampal [3H]FP-CIT binding is that activation of 5-HT2 receptors facilitated DA release from axonal terminals, leading to a reactive down-regulation of pre-synaptic DA transporters. Several studies suggest that 5-HT may act via 5-HT2 and/or other receptor subtypes to facilitate DA release, since it has been shown that stimulation of 5-HT2 receptors facilitated MDMA-induced DA release (Gudelsky et al., 1994). A previous study demonstrated that treatment with the selective 5-HT2 receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane alone had no effect on DA synthesis, but it potentiated the increase in DA synthesis produced by amphetamine (Huang & Nichols, 1993). A recent study by Parsons et al. (1999) reported potentiation of cocaine-induced increases in nucleus accumbens DA after 5-HT1B/1A receptor agonist pretreatment. It was suggested by Schmidt et al. (1992) that the regulation of DA synthesis mediated by 5-HT2 receptors is likely to be a phasic effect which becomes significant only during stages of high serotonergic and dopaminergic transmission. Thus, the results of the present study could be viewed as a potentiation of methylphenidate-induced release of DA through activation of 5-HT2 receptors by quipazine in rat frontal cortex and hippocampus. This demonstrates that while activation of 5-HT2 receptors alone has little effect on dopamine transporters, it may induce neurotoxic effects of methylphenidate.

Remarkably, in the present study changes in DA terminal markers were observed in rat frontal cortex.
and hippocampus but not in the striatum. However, it is worth noting that the frontal cortex and hippocampus do receive a dopaminergic input (Bischoff et al., 1979; Mennicken et al., 1992), although this input is certainly more massive in the striatum. Other studies have reported dopaminergic degeneration in the frontal cortex and hippocampus (Eisworth et al., 1990). Interestingly, Schmidt et al. (1991) showed that coadministration of L-DOPA potentiated regional deficits produced by MDMA in which the striatum was the least affected of the three brain regions examined. It has been suggested that such considerations become relevant only by assuming a terminal-terminal interaction between 5-HT and dopaminergic systems, a structural aspect still unresolved.

The addition of a 5-HT reuptake blocker, fluoxetine, to methylphenidate did not result in a significant reduction of DA terminal markers. Possibly, synaptic 5-HT levels were not high enough to stimulate 5-HT2 receptors sufficiently. Only sufficient stimulation of 5-HT2 receptors, in the presence of an amphetamine-derivative, will elicit a marked increase in DA release and a subsequent downregulation of DA transporters.

The neurotoxic potential of methylphenidate on striatal DA axonal markers has been evaluated by only three studies (Yuan et al., 1997; Wagner et al., 1980; Zaczek et al., 1989). In these studies, animals treated with methylphenidate did not develop long-lasting changes in regional brain catecholamine axon markers. The present finding that methylphenidate alone lacks short-, as well as long-term DA neurotoxicity is in agreement with these three previous reports. However, if we were not to correct for multiple comparisons, 5 days after treatment, specific \([123]F-P-FP-CIT\) binding in the frontal cortex of the group of rats treated with 40 mg/kg of methylphenidate would be significantly lower than saline-treated rats. A similar trend is observed in the hippocampus of rats treated with methylphenidate. This would suggest that high doses of methylphenidate produce short-term changes in DA terminal markers of rat frontal cortex, and possibly hippocampus. However, future studies with a 2-week drug free interval need to be conducted.

While the above-mentioned studies investigated the neurotoxic potential of methylphenidate on DA neurons, the effect of methylphenidate on 5-HT neurotransmission remained poorly defined. Only one investigation has, to our knowledge, studied the 5-HT neurotoxic potential of methylphenidate (Zaczek et al., 1989). In line with this study, we observed no reductions in \([123]IB-P-CIT\) labeled 5-HT transporters following administration of methylphenidate or in combination with quipazine. Since we did not observe any changes in 5-HT and DA terminal markers after methylphenidate administration, methylphenidate seems to lack both DA as well as 5-HT neurotoxic potential. However, the present study suggests that the DA neurotoxic potential of methylphenidate on brain regions other than the striatum need be further clarified.

The present findings, coupled with previous findings of Huang and Nichols (1993) and of Gudelsky et al. (1994), may have important practical as well as clinical implications. From a clinical point of view, the concomitant use of psychostimulants, such as methylphenidate, and drugs activating 5-HT2 receptors (e.g., lysergic acid diethylamide, which is a 5-HT2 receptor agonist) should be avoided until a better understanding of the interactions between these drugs is available and neurotoxic effects can be ruled out. Therefore, future SPECT/PET studies may be conducted in children/adolescents treated for ADHD with methylphenidate and concomitant (ab)use of drugs activating 5-HT2 receptors. On the other hand, our data do not support the hypothesis that the combination of methylphenidate with fluoxetine (or other SSRIs) is harmful to DA nerve terminals. From a theoretical point of view, the present findings are of interest since they strongly suggest an interaction between the dopaminergic and serotonergic system: stimulation of 5-HT2 receptors in the presence of methylphenidate potentiates methylphenidate-induced DA release, resulting in changes in DA terminal markers in the frontal cortex and hippocampus. In addition, the results from this study demonstrate a newly observed effect of amphetamine analogs on dopaminergic neurons. As normal function of the DA transporter is to regulate the action of released DA, disruption of DA transporter function can lead to deleterious effects such as changes in dopaminergic transmission and behavior (Giros et al., 1996).

In summary, our results indicate that the combination of a selective 5-HT2 receptor agonist and methylphenidate produces short-term changes in DA terminal markers in rat frontal cortex and hippocampus. These changes probably do not reflect neurotoxic changes in DA terminal markers, but a compensatory downregulation due to facilitation by 5-HT2 receptors of methylphenidate-induced DA release. These findings suggest potential harmful effects of concomitant use of drugs directly activating 5-HT2 receptors in children and adolescents treated for ADHD with methylphenidate. Finally, a trend was observed in the present study which suggests that methylphenidate...
may have short-term DA neurotoxic effects of on brain regions which do not have a massive DA input, such as the frontal cortex and hippocampus.

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