Addition of a 5-HT Receptor Agonist to Methylphenidate Potentiates the Reduction of $^{123}$I]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 serotonergic 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 serotonergic 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 ($^{123}$I]β-CIT and $^{123}$I]FP-CIT). Groups of rats were treated with methylphenidate or saline for 4 days. Additional groups were treated with the selective 5-HT$_2$ 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, along 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 $^{123}$I]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-HT$_2$ receptors in patients using methylphenidate.


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INTRODUCTION

Several amphetamine-related compounds are neurotoxic to rodents, as shown by a profound and long-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 and Gibb, 1973) as well as serotonin (5-HT) (Hotchkiss and 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.

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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 (Ricarthe et al., 1993; Battaglia et al., 1988; Stone et al., 1986; Schmidt et al., 1987; Schmidt and Taylor, 1987; 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., 1998; Scheffel et al., 1998; 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 and 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 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-aminooindan (MMAI) causes 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 increasing DA synthesis and release through activation of 5-HT$_2$ receptors (Huang and Nichols, 1993; Schmidt et al., 1991). Thus, brain 5-HT$_2$ 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 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-HT$_2$ receptors by 5-HT reuptake inhibitors on DA neurons may be.

Based on 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 on 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 (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-HT$_2$ 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. 5-HT transporter densities were not measured in rats treated with fluoxetine alone, or in combination with methylphenidate. In Experiment 2, a combination of 10 mg/kg methylphenidate and 5 mg/kg quipazine, as in Experiment 1, was retested, along with a saline-treated control group. In this experiment, DA transporter density was measured 2 weeks (rather than 5 days) after drug treatment.

### Measuring DA and 5-HT transporter densities

Five days or 2 weeks after treatment, rats were injected i.v. with either ~1.85 MBq [123I]FP-CIT or [123I]β-CIT. [123I] labeling of FP-CIT and β-CIT was performed by oxidative radiiodideostannylation (Amerham Cygne, Technical University, Eindhoven, and Radionuclide Center, Vrije University, Amsterdam, The Netherlands, respectively) of their trimethylstan- nyl precursors obtained from RBI (Natick, MA). Both [123I]FP-CIT and [123I]β-CIT had a specific activity >185 MBq/nmol and a radiochemical purity of >97%.

Both [123I]FP-CIT and [123I]β-CIT bind to 5-HT as well as DA transporters. We have previously shown that [123I]FP-CIT binds in vivo predominantly to DA transporters (Booij et al., 1997). [123I]β-CIT has higher in vivo binding ratios for the 5-HT transporter than [123I]FP-CIT. Therefore, the combination of FP-CIT and these two radioligands was chosen to assess both the 5-HT and DA system. Two hours after injection of [123I]FP-CIT (Booij et al., 1997) and 1 h after injection of [123I]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. [123I] radioactivity of [123I]FP-CIT or [123I]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 × 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 nonspecifically bound radioligand. The specific binding at each time point 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 [123I]FP-CIT and [123I]β-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 ver. 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 [123I]FP-CIT or specific [123I]β-CIT binding when compared to saline-treated rats. No significant group effect was observed, either for [123I]FP-CIT binding or for [123I]β-CIT-binding (P = 0.184 and P = 0.254; Figs. 1, 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 [123I]FP-CIT or specific [123I]β-CIT binding when compared to saline-treated rats. A significant group effect was observed for specific [123I]FP-CIT, but not specific [123I]β-CIT binding (P = 0.016 and P = 0.540, respectively). ANOVA analysis revealed that specific [123I]FP-CIT binding was significantly higher in the hypothalamus of groups of rats treated with quipazine compared to controls (Fig. 1D).

**Is fluoxetine neurotoxic to DA neurons?**

When we compared specific [123I]FP-CIT binding of fluoxetine treated rats with saline-treated rats, no significant group effect was observed (P = 0.122; Fig. 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 [123I]FP-CIT or [123I]β-CIT-binding when compared to saline-treated rats. In specific [123I]FP-CIT, but not [123I]β-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 $[^{123}\text{I}]$FP-CIT binding was significantly lower in rats treated with the combination of methylphenidate with quipazine when compared to controls ($P = 0.016$ and $P = 0.049$; Fig. 1A,B, respectively). No difference in binding was observed in the striatum ($P = 0.439$; Fig. 1C) or hypothalamus ($P = 0.912$; Fig. 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 $[^{123}\text{I}]$FP-CIT or $[^{123}\text{I}]$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 $[^{123}\text{I}]$FP-CIT binding ($P = 0.044$), but not for $[^{123}\text{I}]$β-CIT binding ($P = 0.106$; Fig. 2). Using Dunnett’s test for post-hoc analysis of specific $[^{123}\text{I}]$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 $[^{123}\text{I}]$FP-CIT binding when compared to quipazine-treated rats ($P = 0.001$ and $P = 0.005$; Fig. 1A,B, respectively). In addition, in the frontal cortex $[^{123}\text{I}]$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 ($P = 0.023$; Fig. 1A).

Furthermore, in the hypothalamus a significantly higher $[^{123}\text{I}]$FP-CIT binding was observed in combined treated rats than in rats treated with methylphenidate alone ($P = 0.049$; Fig. 1D).

**Does $[^{123}\text{I}]$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 $[^{123}\text{I}]$FP-CIT binding in a group of rats treated with either a combination of methylphenidate and fluoxetine, or fluoxetine and methylphenidate alone (Fig. 1).

**Long-term effects**

Since the 5-day survival period was relatively short, Experiment 2 retested the effects of 10 mg/kg of methylphenidate with quipazine when compared to controls ($P = 0.016$ and $P = 0.049$; Fig. 1A,B, respectively). No difference in binding was observed in the striatum ($P = 0.439$; Fig. 1C) or hypothalamus ($P = 0.912$; Fig. 1D).
ylphenidate in addition of quipazine. Rats were allowed a 2-week postdrug survival period to ensure that methylphenidate's effects on presynaptic 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 $^{[123]}$I$\text{FP-CIT}$ binding in the frontal cortex or hippocampus were observed compared to controls ($P = 0.988$) (Fig. 1A,B, respectively).

**DISCUSSION**

Coadministration of quipazine and methylphenidate produced a significant reduction in specific $^{[123]}$I$\text{FP-CIT}$ 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 $^{[123]}$I$\text{FP-CIT}$ binding in the frontal cortex and hippocampus was observed, even though animals had been treated with identical dose regimes.

In rats, $^{[123]}$I$\text{FP-CIT}$ labels both DA and 5-HT transporters in vivo (Booij et al., 1997). However, $^{[123]}$I$\text{FP-CIT}$ binds in vivo predominantly to DA transporters. Therefore, reduced specific $^{[123]}$I$\text{FP-CIT}$ 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 $^{[123]}$I$\beta$-CIT has higher binding ratios for the 5-HT transporter than $^{[123]}$I$\text{FP-CIT}$ (Reneman et al., 1999), and no reductions were observed in $^{[123]}$I$\beta$-CIT binding after administration of methylphenidate in addition to quipazine, it is likely that DA and not 5-HT terminal markers were affected.

It could be argued that the decrease in $^{[123]}$I$\text{FP-CIT}$ 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 $^{[123]}$I$\text{FP-CIT}$ binding is that activation of 5-HT$_2$ receptors facilitated DA release from axonal terminals, leading to a reactive downregulation of presynaptic DA transporters. Several studies suggest that 5-HT may act via 5-HT$_2$ and/or other receptor subtypes to facilitate DA release, since it has been shown that stimulation of 5-HT$_2$ receptors facilitated MDMA-induced DA release (Gudelsky et al., 1994). A previous study demonstrated that treatment

**Fig. 2.** Specific binding of $^{[123]}$I$\beta$-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 (nonspecific 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: 10 mg/kg methylphenidate + 5 mg/kg quipazine 5 mg/kg; VI: quipazine.
with the selective 5-HT$_2$ receptor agonist 1-(2,5-dimet- 
thoxy-4-iodophenyl)-2-aminopropane alone had no ef- 
fect on DA synthesis, but it potentiated the increase in 
DA synthesis produced by amphetamine (Huang and 
Nichols, 1993). A recent study by Parsons et al. (1999) 
reported potentiation of cocaine-induced increases in 
nucleus accumbens DA after 5-HT$_{1B/1A}$ receptor ago- 
ist pretreatment. It was suggested by Schmidt et al. 
(1992) that the regulation of DA synthesis mediated by 
5-HT$_2$ receptors is likely to be a phasic effect which 
becomes significant only during stages of high seroto- 
nergic and dopaminergic transmission. Thus, the re-

sults of the present study could be viewed as a poten-
tiation of methylphenidate-induced release of DA 
through activation of 5-HT$_2$ receptors by quipazine in 
rat frontal cortex and hippocampus. This demonstrates 

that while activation of 5-HT$_2$ receptors alone has little 
effect on dopamine transporters, it may induce neuro-
toxic 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 hippocam-
pus 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 fron-
tal cortex and hippocampus (Elsworth et al., 1990). 

Interestingly, Schmidt et al. (1991) showed that coad-
ministration of L-dopa potentiated regional deficits pro-
duced by MDMA in which the striatum was the least 
affected of the three brain regions examined. It has 
been suggested that such considerations become rele-
vant 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 re-
duction of DA terminal markers. Possibly, synaptic 
5-HT levels were not high enough to stimulate 5-HT$_2$ 
receptors sufficiently. Only sufficient stimulation of 
5-HT$_2$ 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 stri- 
atal DA axonal markers has been evaluated in 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 $^{[123]}$I-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 neu-
rons, the effect of methylphenidate on 5-HT neuro-
transmission remained poorly defined. Only one inves-
tigation has, to our knowledge, studied the 5-HT 
neurotoxic potential of methylphenidate (Zaczek et al., 
1989). In line with this study, we observed no reduc-
tions in $^{[123]}$I-$\beta$-CIT-labeled 5-HT transporters follow-
ing administration of methylphenidate or in combina-
tion with quipazine. Since we did not observe any 
changes in 5-HT and DA terminal markers after meth-
lyphenidate administration, methylphenidate seems to 
lack both DA as well as 5-HT neurotoxic potential. 
However, the present study suggests that the DA neu-
rotoxic potential of methylphenidate on brain regions 
other than the striatum need to be further clarified.

The present findings, coupled with the previous find-
ings of Huang and Nichols (1993) and Gudelsky et al. 
(1994), may have important practical as well as clinical 
implications. From a clinical point of view, the concom-
itant use of psychostimulants, such as methylpheni-
date, and drugs activating 5-HT$_2$ receptors (e.g., 
lysergic acid diethylamide, which is a 5-HT$_2$ receptor 
agonist) should be avoided until a better understand-
ing 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-HT$_2$ re-
ceptors. 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 serotonin-
nerve system: stimulation of 5-HT$_2$ receptors in the presence 
of methylphenidate potentiates methylphenidate-in-
duced 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 do-
paminergic neurons. As normal function of the DA 
transporter is to regulate the action of released DA, 
disruption of DA transporter function can lead to del-
eterious effects such as changes in dopaminergic trans-
mision and behavior (Giros et al., 1996).

In summary, our results indicate that the combina-
tion of a selective 5-HT$_2$ receptor agonist and methyl-
phenidate 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 on brain regions which do not have a massive DA input, such as the frontal cortex and hippocampus.

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