The mechanisms involved in the long-lasting neuroprotective effect of fluoxetine against MDMA ('ecstasy')-induced degeneration of 5-HT nerve endings in rat brain

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1 It has been reported that co-administration of fluoxetine with 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') prevents MDMA-induced degeneration of 5-HT nerve endings in rat brain. The mechanisms involved have now been investigated.

2 MDMA (15 mg kg^{-1}, i.p.) administration produced a neurotoxic loss of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in cortex, hippocampus and striatum and a reduction in cortical [^{3}H]-paroxetine binding 7 days later. Fluoxetine (10 mg kg^{-1}, i.p., $\times 2$, 60 min apart) administered concurrently with MDMA or given 2 and 4 days earlier provided complete protection, and significant protection when given 7 days earlier. Fluvoxamine (15 mg kg^{-1}, i.p., $\times 2$, 60 min apart) only produced neuroprotection when administered concurrently.

3 Fluoxetine (10 mg kg^{-1}, $\times 2$) markedly increased the K_D and reduced the B_{max} of cortical [^{3}H]-paroxetine binding 2 and 4 days later. The B_{max} was still decreased 7 days later, but the K_D was unchanged. [^{3}H]-Paroxetine binding characteristics were unchanged 24 h after fluvoxamine (15 mg kg^{-1}, $\times 2$).

4 A significant cerebral concentration of fluoxetine plus norfluoxetine was detected over the 7 days following fluoxetine administration. The fluvoxamine concentration had decreased markedly by 24 h.

5 Pretreatment with fluoxetine (10 mg kg^{-1}, $\times 2$) failed to alter cerebral MDMA accumulation compared to saline pretreated controls.

6 Neither fluoxetine or fluvoxamine altered MDMA-induced acute hyperthermia.

7 These data demonstrate that fluoxetine produces long-lasting protection against MDMA-induced neurodegeneration, an effect apparently related to the presence of the drug and its active metabolite inhibiting the 5-HT transporter. Fluoxetine does not alter the metabolism of MDMA or its rate of cerebral accumulation.

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Abbreviations: ANOVA, analysis of variance; B_{max}, maximal number of binding sites; 2,3-DHBA, dihydroxybenzoic acid; 3,4-DHMA, dihydroxymethamphetamine; EDTA, ethylenediaminetetraacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; h.p.l.c., high performance liquid chromatography; 5-HT, 5-hydroxytryptamine; K_D, dissociation constant; MAO, monoamine oxidase; MDMA, ($\pm$) 3,4-methylenedioxymethamphetamine

Introduction

The recreationally used drug 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') has two distinct neurochemical effects on the concentration of 5-hydroxytryptamine (5-HT) in the brain of rats. The first effect is a rapid and dramatic increase in the extracellular concentration of 5-HT. This results from the ability of MDMA to stimulate 5-HT release (Gough *et al.*, 1991; Brodkin *et al.*, 1993), inhibit uptake (Steele *et al.*, 1987; Johnson *et al.*, 1991) and block metabolism of 5-HT by MAO-A (Leonardi & Azmitia, 1994). MDMA binds with high affinity to the 5-HT transporter protein (Poblete *et al.*, 1989), reverses the direction of neurotransmitter flow (Rudnick & Wall, 1992)

and exerts a pronounced stimulatory effect on 5-HT release in synaptosomes (Nichols *et al.*, 1982; McKenna *et al.*, 1991; Berger *et al.*, 1992), brain slices (Johnson *et al.*, 1986; Sprouse *et al.*, 1989; Fitzgerald & Reid, 1993) and cultured neurones (Azmitia *et al.*, 1990; Gu & Azmitia, 1993). 5-HT release has also been demonstrated *in vivo* by the use of intracerebral microdialysis (Schmidt *et al.*, 1987; Gough *et al.*, 1991; Gudelsky & Nash, 1996). The acute 5-HT releasing effect of MDMA involves both a calcium-dependent mechanism (Crespi *et al.*, 1997) and a calcium independent process mediated by the transporter (Johnson *et al.*, 1986). In fact, the release of 5-HT induced by MDMA *in vivo* is mostly attenuated by drugs that bind to the 5-HT transporter (fluoxetine, citalopram) thereby inhibiting the 5-HT uptake into presynaptic terminals (Schmidt, 1987; Schmidt *et al.*,

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1987; Azmitia et al., 1990; Hekmatpanah & Peroutka, 1990; Gudelsky & Nash, 1996; Mechan et al., 2000). The effect of MDMA on 5-HT release is reversible, the 5-HT extracellular concentration returning to control values 3–4 h after drug administration (Gudelsky & Nash, 1996; Mechan et al., 2000). Simultaneously with the 5-HT release, rats display both the 5-HT behavioural syndrome and hyperthermia (Slikker et al., 1989; Colado et al., 1993).

The second action of MDMA is that of producing, in several areas of the brain, a long-term loss of fine 5-HT axon terminals arising primarily from the dorsal raphe nucleus (O'Hearn et al., 1988). The degeneration has been demonstrated both histologically (O'Hearn et al., 1988; Molliver et al., 1990) and biochemically and is reflected in a substantial decrease in the concentration of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), and a reduction in the density of 5-HT uptake sites labelled with [³H]-paroxetine (Sharkey et al., 1991; Hewitt & Green, 1994; Colado et al., 1997b).

The mechanisms involved in producing this neurodegeneration are not fully understood at present, but recent data indicate that a major mechanism by which MDMA induces damage to 5-HT containing neurones in rat brain is by increasing the formation of hydroxyl free radicals (Colado & Green, 1995; Colado et al., 1997b; Yeh, 1999) which probably result from MDMA metabolism (Colado et al., 1995). The use of intracerebral microdialysis has demonstrated that systemic administration of MDMA increases the formation of 2,3-dihydroxybenzoic acid (2,3-DHBA) from salicylate in hippocampal and striatal dialysates (Colado et al., 1997b; Shankaran et al., 1999), a conversion that only occurs in the presence of a high concentration of free radicals. The fact that this effect was absent in rats with a lesion of the 5-HT nerve terminals indicates that these reactive species are probably formed in 5-HT nerve endings (Colado et al., 1997b). Additional evidence supporting the existence of an oxidative stress includes the report that the hydroxyl radical scavenger alpha-phenyl-N-tert-butyl nitrone (PBN) is neuroprotective (Colado & Green, 1995; Colado et al., 1997b; Yeh, 1999) and also abolishes the MDMA-induced rise in 2,3-DHBA (Colado et al., 1997b), the observation that MDMA increases lipid peroxidation in the brain (Sprague & Nichols, 1995; Colado et al., 1997a) and the fact that transgenic mice overexpressing CuZn superoxide dismutase are resistant to the neurotoxic actions of the drug (Cadet et al., 1995).

Initial studies based on both the pharmacology of MDMA and putative neuroprotective compounds, suggested a key role for dopamine in the expression of MDMA-induced neurotoxicity (Stone et al., 1988; Schmidt et al., 1990; Hewitt & Green, 1994). Most of these studies were performed in the absence of measures of body temperatures and consequently, a re-evaluation of the earlier reports was necessary. There is now evidence that α-methy-p-tyrosine and haloperidol only prevent MDMA-induced damage because they either induce hypothermia (Malberg et al., 1996) or prevent acute hyperthermia (Colado et al., 1999) and that the enhancing effect of L-DOPA on MDMA-induced neurodegeneration (Schmidt et al., 1991) might result from its ability to increase the MDMA-induced hyperthermia (Colado et al., 1999). In addition, the MAO-B inhibitor deprenyl has been shown to possess free radical scavenger activity (Thomas et al., 1997)

which could explain its neuroprotective effect against MDMA toxicity (Sprague & Nichols, 1995).

There is a large amount of evidence indicating the involvement of the 5-HT transporter in both the acute and in the long-term effects induced by MDMA. Fluoxetine, a selective 5-HT uptake inhibitor (Wong et al., 1983), when given with MDMA, prevents both the acute increase in hydroxyl radical formation which follows administration of the amphetamine derivative (Shankaran et al., 1999) and the long-lasting neurotoxic effects (Schmidt, 1987; Malberg et al., 1996). The neuroprotective effect of fluoxetine does not depend on an effect on body temperature since animals treated with fluoxetine and MDMA displayed a hyperthermic response similar to that observed when MDMA is given alone (Malberg et al., 1996; Mechan et al., 2000). Since MDMA interacts with the 5-HT transporter protein (Poblete et al., 1989) it seems reasonable to propose that fluoxetine may be preventing the uptake of MDMA or most likely, of a neurotoxic metabolite of MDMA into the 5-HT nerve endings (Malberg et al., 1996; Esteban et al., 2001).

Fluoxetine is extensively biotransformed (N-demethylation) by the hepatic cytochrome P450 2D6 enzyme system to norfluoxetine (Bergstrom et al., 1992; Hamelin et al., 1996), an active metabolite which is eliminated much more slowly than fluoxetine by both rats and humans (Gardier et al., 1994; Hamelin et al., 1996; Holladay et al., 1998). The plasma elimination half-lives of fluoxetine and norfluoxetine are respectively in the range 1–3 days and 7–15 days (Lemberger et al., 1985; Benfield et al., 1986; Stanford, 1996; Preskorn, 1997; Sanchez & Hyttel, 1999). Both compounds are highly lipophilic and accumulate in the brain where their concentrations are higher than those found in plasma (Gardier et al., 1994; Lefebvre et al., 1999). Norfluoxetine is as potent as fluoxetine in inhibiting 5-HT uptake and it is also more selective (Bolden-Watson & Richelson, 1993; Sanchez & Hyttel, 1999). Its presence in vivo therefore probably contributes substantially to the pharmacological and therapeutic effects of the parent compound (Horng & Wong, 1976; Fuller et al., 1978; Schmidt, 1987).

Fluvoxamine is also a selective 5-HT uptake inhibitor but with a different pharmacokinetic profile. Fluvoxamine undergoes oxidative metabolism but, in contrast to fluoxetine, it generates several metabolites, none of which appear to be pharmacologically active. In addition, CYP2D6 isoenzymes do not play a significant role in its metabolism (Van Harten, 1995; Preskorn, 1997).

The aim of this current study was to examine the mechanisms involved in the neuroprotective effect of fluoxetine against MDMA-induced neurotoxic loss of 5-HT in rat brain. To do this, MDMA was either co-administered with fluoxetine or given several days (2, 4 and 7 days) later. In addition, the study also investigated whether the long-term neuroprotective effect of fluoxetine was due to changes in the 5-HT transporter produced by the presence of either fluoxetine or norfluoxetine or, alternatively, it resulted from a pharmacokinetic interaction between fluoxetine and MDMA since, in rats, MDMA is metabolized to 3,4-dihydroxymethamphetamine (DHMA) by CYP2D1 and fluoxetine and norfluoxetine are potent inhibitors of CYP2D1. This investigation has also examined whether the neuroprotection induced by fluoxetine can be

extended to other 5-HT selective uptake inhibitors such as fluvoxamine.

Methods

Animals, drug administration and experimental protocol

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Male Dark Agouti rats (175 – 200 g, Harlan Iberica, Barcelona) were used. They were housed in groups of five, in conditions of constant temperature ($21 \pm 2^\circ C$) and a 12 h light/dark cycle (lights on: 0700 h) and given free access to food and water. ($\pm$) MDMA (obtained from the Ministry of Health, Spain), fluoxetine (Lilly) and fluvoxamine (Tocris) were dissolved in saline (0.9% NaCl) and given i.p. in a volume of 1 ml kg^{-1} for MDMA and fluvoxamine and 2 ml kg^{-1} for fluoxetine. Doses are reported in terms of the base.

Fluoxetine (10 mg kg^{-1}, i.p., twice, 60 min apart) was given 2, 4 and 7 days before MDMA (15 mg kg^{-1}, i.p.). In order to compare the neuroprotective effect of fluoxetine after these pretreatment periods with that when the drug was co-administered with MDMA, a further group of animals received fluoxetine 5 min before and 55 min after MDMA.

Fluvoxamine (15 mg kg^{-1}, i.p., twice, 60 min apart) was given either 24 h before MDMA (15 mg kg^{-1}, i.p) or 5 min before and 55 min after MDMA. Animals were always sacrificed 7 days after MDMA administration.

Rats were given a single administration of MDMA at the dose of 15 mg kg^{-1} since previous studies had demonstrated that this regimen produced an approximately 50% loss of several 5-HT parameters (O'Shea *et al.*, 1998). Fluoxetine and fluvoxamine were administered at doses altering the extra-cellular concentration of 5-HT (Bosker *et al.*, 1995; Mechan *et al.*, 2000).

Measurement of rectal temperature

Temperature was measured during the 6 h following MDMA administration by means of a digital readout thermocouple (Type K thermometer, Portec, U.K.) with a resolution of $\pm 0.1^\circ C$ and accuracy of $\pm 0.2^\circ C$ attached to a CAC-005 Rodent Sensor which was inserted 2.5 cm into the rectum of the rat, the animal being lightly restrained by holding in the hand. A steady readout was obtained within 10 s of probe insertion.

Measurement of 5-HT and its metabolite, 5-HIAA, in cerebral tissue

Rats were killed by cervical dislocation and decapitation, the brains rapidly removed and cortex, hippocampus and striatum dissected out on ice. Tissue was homogenized and 5-HT and 5-HIAA measured by high performance liquid chromatography (h.p.l.c.). Briefly, the mobile phase consisted of KH$_2$PO$_4$ (0.05 M), octanesulphonic acid (0.16 mM), ethylenediaminetetraacetic acid (EDTA, 0.1 mM) and methanol (16%), and was adjusted to pH 3 with phosphoric acid,

filtered and degassed. The flow rate was 1.3 ml min^{-1} and the working electrode potential was set at $+0.8$ V.

The h.p.l.c. system consisted of a pump (Waters 510) linked to an automatic sample injector (Loop 200 μl, Waters 712 WISP), a stainless steel reversed-phase column (Spher-isorb ODS2, 5 μm, 150×4.6 mm) with a precolumn and an amperometric detector (Waters M460). The current produced was monitored by using an integrator (Waters M745).

Measurement of fluoxetine, norfluoxetine, fluvoxamine and MDMA in cerebral tissue

Cortical tissue was homogenized in ice-cold sodium carbonate-sodium bicarbonate buffer (pH: 11.5) using an ultrasonicator. The homogenate was centrifuged at 18,000 r.p.m. for 20 min at $4^\circ C$. The supernatant was applied to a 145 mg C8 endcapped SPE Light column (International Sorbent Technology, Waters). The column was washed with methanol (2 ml) followed by distilled H$_2$O (2 ml) before sample (400 μl supernatant + 250 μl distilled H$_2$O + 100 μl protriptiline (5 μg ml^{-1}) or 100 μl fluoxetine (10 μg ml^{-1}) or 100 μl 3,4-methylenedioxyethylamphetamine (10 μg ml^{-1}) as internal standards for fluoxetine, fluvox-amine and MDMA, respectively) was applied. The column was washed with H$_2$O (2 ml) before selective elution of fluoxetine, norfluoxetine, fluvoxamine or MDMA with methanol (1 ml).

An aliquot (20 μl) of the resulting eluate was injected into a Waters h.p.l.c. system which consisted of a pump (Waters 510) linked to a manual sample injector (Loop 20 μl, Rheodyne), a stainless steel column (RP 18, 5 μm, 150×4.6 mm, XTerra) with a precolumn (RP 18, 5 μm, 20×3.9 mm, XTerra) and an ultraviolet/visible detector (Waters 2487). The current produced was monitored by using an integrator (Waters M745). For fluoxetine and norfluoxetine analysis the mobile phase consisted of 20 mM potassium dihydrogen phosphate (65%) and acetonitrile (35%, pH 2.5); the flow rate was set to 1.5 ml min^{-1} and u.v. absorption was measured at 210 nm. The retention times were 6.1 min for protriptiline (internal standard), 8.4 min for norfluoxetine and 9.6 min for fluoxetine. For fluvoxamine analysis, the experimental conditions were the same as above and the flow rate was set to 1 ml min^{-1}. The retention times were 10.6 min for fluvoxamine and 17.8 min for fluoxetine (internal standard). For MDMA analysis the mobile phase consisted of 20 mM potassium dihydrogen phosphate (75%) and acetonitrile (25%, pH 2.5); the flow rate was set to 0.8 ml min^{-1} and u.v. absorption measured at 235 nm. The retention times were 4.3 min for MDMA and 5.2 min for 3,4-methylenedioxyethylamphetamine (inter-nal standard). In each run all the peaks were completely resolved without any interference from endogenous com-pounds. Recoveries were determined by comparing peak height ratios of the extracted standards with those obtained by direct injection of the same amount of compounds ranged from 5 – 40 μg ml^{-1} for fluoxetine and norfluoxetine, from 2.5 – 40 μg ml^{-1} for fluvoxamine and from 1.25 – 80 μg ml^{-1} for MDMA. The limit of detection under the described conditions was 10 pg μl^{-1} for all the compounds with a signal to noise ratio of 3. Intra-assay variation was less than 5% and recovery greater than 90% except for MDMA (75%).

[³H]-Paroxetine binding in tissue homogenates

[³H]-Paroxetine binding was measured by the method described in detail by Hewitt & Green (1994). The animals were killed, the brain rapidly removed and dissected on ice within 2 min. Cortex from individual animals was homogenized in ice-cold Tris-HCl (50 mM; pH 7.4) containing (in mM): NaCl 120 and KCl 5, using an Ultra-Turrax. The homogenate was centrifuged at $30,000 \times g$ for 10 min at $4°C$. The supernatant was discarded and the wash procedure repeated twice more. The pellet was finally resuspended in the Tris buffer at a concentration of 5 mg tissue ml^{-1}. Aliquots of this membrane suspension were incubated for 90 min at room temperature with [³H]-paroxetine (specific activity 19.7 Ci $mmol^{-1}$; Du Pont) in the absence or presence of 5-HT for determination of non specific binding. The assay solution (1 ml) contained 100 μl of [³H]-paroxetine (0.02–4 nM) and 800 μl tissue preparation with the addition of either 100 μl 5-HT (100 μM) or 100 μl of buffer. After incubation, assay was terminated by rapid filtration through Whatman GF/B filters pretreated with 0.05% polyethylenimine using the Skatron Cell Harvester. The radioactivity was counted by scintillation spectrometry. Protein concentration was measured by the method of Lowry *et al.* (1951).

Statistics

Data from the monoamine and [³H]-paroxetine binding experiments were analysed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test when a significant F value was obtained (GraphPad Prism, San Diego, CA, U.S.A.).

The affinity constant (K_D) and maximal number of binding sites (B_{max}) for [³H]-paroxetine were calculated from saturation binding data with non-linear regression analysis fitted to a one-site binding model using the Prism software package. The analyses of K_D and B_{max} were performed using an unpaired *t*-test.

Statistical analysis of the temperature measurements were performed using the statistical computer package BMDP/386 Dynamic (BMDP Statistical Solutions, Cork, Eire). Data were analysed by means of two-way ANOVA with repeated measures (program 2 V) or, where missing values occurred, an unbalanced repeated measure model (program 5 V) was used. Both used treatment as the between subjects factor and time as the repeated measure. ANOVA was performed on both pre-treatment and post-treatment data.

Results

Effect of fluoxetine and fluvoxamine on the changes induced by MDMA in brain indole content and cortical [³H]-paroxetine binding

A single injection of MDMA (15 mg kg^{-1}, i.p.) produced a marked decrease in the concentration of 5-HT and 5-HIAA in the cortex, hippocampus and striatum (Figure 1) and a similar reduction in the cortical [³H]-paroxetine binding 7 days later (Figure 1). Two injections of fluoxetine

(10 mg kg^{-1}, i.p., 5 min prior to and 55 min after the MDMA), prevented the loss of indole content and attenuated the decrease in 5-HT uptake sites (Figure 1). Complete neuroprotection was observed when fluoxetine (2 injections, 60 min apart) was administered 2 and 4 days before MDMA (Figure 1). When fluoxetine was given 7 days before MDMA it still afforded a modest but significant protection against the MDMA-induced damage to 5-HT nerve endings (Figure 1).

While fluvoxamine (15 mg kg^{-1}, i.p., 5 min prior to and 55 min after the MDMA) also completely prevented the subsequent MDMA-induced reduction in brain indole content and [³H]-paroxetine binding density, it had no neuroprotective effect when given 24 h prior to the MDMA (Figure 2).

Effect of fluoxetine and fluvoxamine on saturation binding isotherms of [³H]-paroxetine in the cortex

Figure 3 shows saturation binding isotherms of [³H]-paroxetine in the cortex of saline and fluoxetine treated rats. In the saline groups, a non linear regression analysis of specific binding revealed a single saturable site (r^2 0.99) with a K_D of 0.203–0.256 nM and a B_{max} of 72–87 fmol mg^{-1} protein.

Administration of two doses of fluoxetine (10 mg kg^{-1}, 60 min apart) produced a pronounced increase in the dissociation constant (K_D) and a reduction in the maximal number of binding sites (B_{max}) for [³H]-paroxetine in the cerebral cortex both 2 and 4 days later (Figure 3). The B_{max} was still decreased 7 days later, although there was no change in the K_D value (Figure 3).

Twenty-four hours after fluvoxamine (15 mg kg^{-1}, i.p., two doses, 60 min apart) no significant change in the [³H]-paroxetine binding properties was observed in the drug-treated animals (K_D: 0.215 ± 0.05 nM, $n=4$; B_{max}: 82 ± 4.8 fmol mg^{-1} protein, $n=4$) compared with saline injected control rats (K_D: 0.237 ± 0.06 nM, $n=4$; B_{max}: 87 ± 5.4 fmol mg^{-1} protein, $n=4$).

Brain concentrations of fluoxetine, norfluoxetine and fluvoxamine

Following two doses of fluoxetine (10 mg kg^{-1}, i.p., 60 min apart) the levels of both fluoxetine and its main metabolite, norfluoxetine, were determined in cortex over the next 7 days. At the intervals examined, the peak concentration of fluoxetine occurred within the first 30 min interval declining progressively thereafter, reaching a low and constant concentration between 4–7 days after dosing (Figure 4). Norfluoxetine concentrations rose over the first 24–48 h, the concentration then diminishing slowly over the next 5 days (Figure 4).

The concentration of fluvoxamine 30 min after the second of two doses of the drug (15 mg kg^{-1}, i.p. 60 min apart) was 35 ± 6 nmol g^{-1} ($n=4$). The concentration then decreased rapidly, being 6 ± 0.03 nmol g^{-1} ($n=4$) at 6 h and 3 ± 0.2 nmol g^{-1} ($n=4$) at 24 h.

Effect of fluoxetine on indole concentration in the brain

Fluoxetine (10 mg kg^{-1}, two injections, 60 min apart) had no effect on 5-HT concentration at any time examined (2, 4, 7
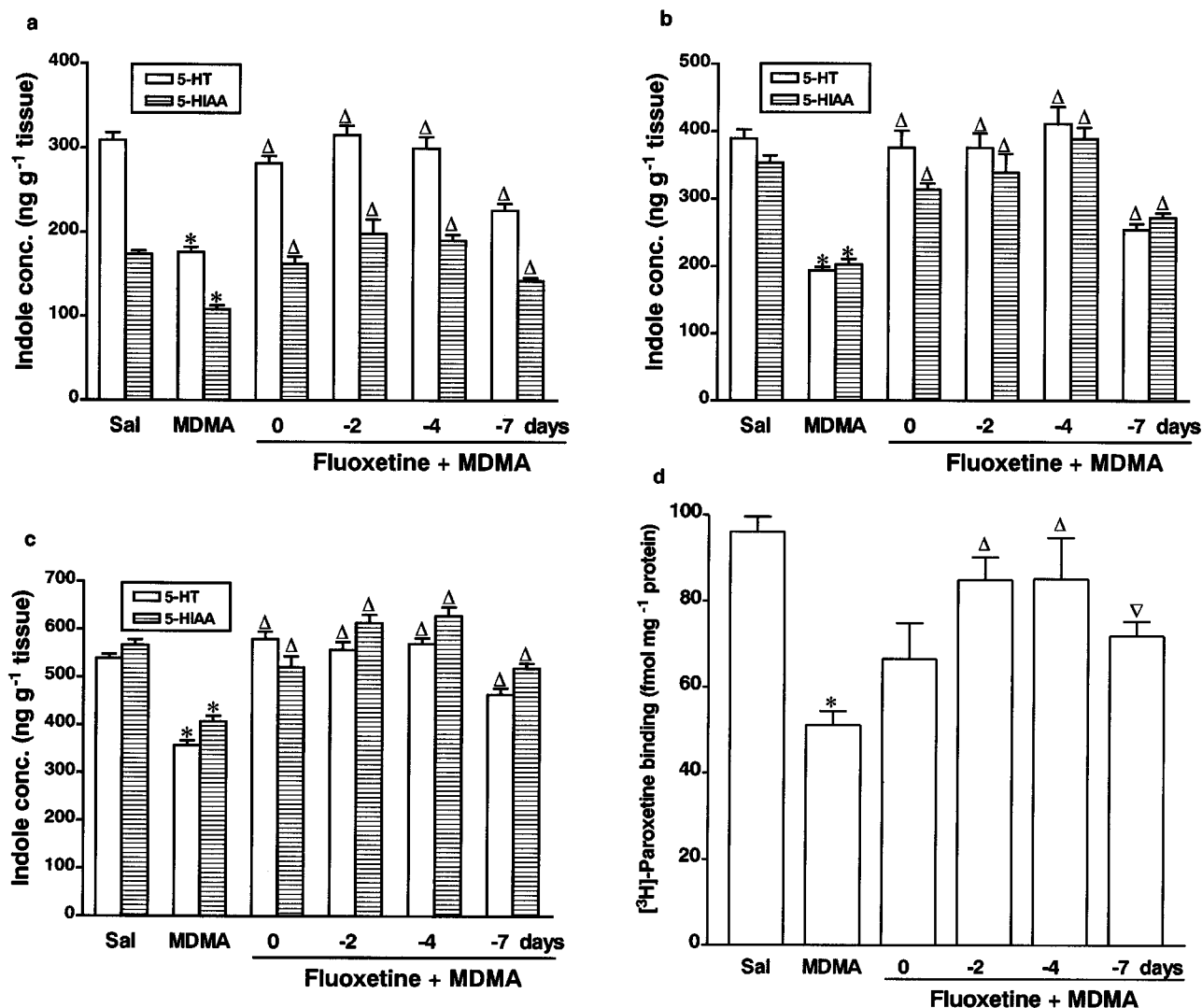
Figure 1 Effect of fluoxetine on the 3,4-methylenedioxymethamphetamine (MDMA)-induced decrease in indole concentration in (a) cortex, (b) hippocampus, (c) striatum and (d) density of [^{3}H]-paroxetine labelled 5-HT uptake sites in cortex. Two injections of fluoxetine (10 mg kg^{-1}, i.p) with an interval of 60 min were given 2, 4 or 7 days before MDMA (15 mg kg^{-1}, i.p.), rats being sacrificed 7 days later. A group of animals received fluoxetine 5 min before and 55 min after MDMA. Results shown as mean±s.e.mean ($n = 6-16$). Different from saline: *$P < 0.001$. Different from MDMA: $\triangledown P < 0.05$, $\triangle P < 0.001$.

and 14 days) following administration (Figure 5). However, there was a significant decrease in 5-HIAA concentration in cortex, hippocampus and striatum at both 2 and 4 days. No change in the brain 5-HIAA concentration was observed after that time (Figure 5).

Effect of fluoxetine on brain levels of MDMA

In order to investigate the possible effect of fluoxetine on the concentration of MDMA in the brain, rats were given fluoxetine (10 mg kg^{-1}, i.p., two doses, 60 min apart). Two days later the animals received a single dose of MDMA (15 mg kg^{-1}, i.p.) and the cortical concentration of MDMA was measured 60 min later. This time was chosen because the cerebral concentration of MDMA normally peaks 60 min after this dose of MDMA (Esteban *et al.*, 2001). There was no difference between MDMA levels

found in rats pretreated with fluoxetine (66±9 nmol g^{-1} tissue, $n = 4$) and those injected with saline (59±4 nmol g^{-1} tissue, $n = 4$).

Effect of fluoxetine and fluvoxamine on rectal temperature

Administration of MDMA (15 mg kg^{-1}, i.p.) produced a clear and sustained hyperthermia (Figure 6a,b). Fluoxetine (10 mg kg^{-1}, i.p.) either co-administered with MDMA (Figure 6a) or given 2, 4 (Figure 6a) or 7 days earlier failed to prevent the rise in temperature. Similarly, fluvoxamine (15 mg kg^{-1}, i.p.) given 5 min before and 55 min after MDMA did not modify the hyperthermic response (Figure 6b). Administration of fluoxetine or fluvoxamine alone (two doses 60 min apart) had no effect on rectal temperature (data not shown).
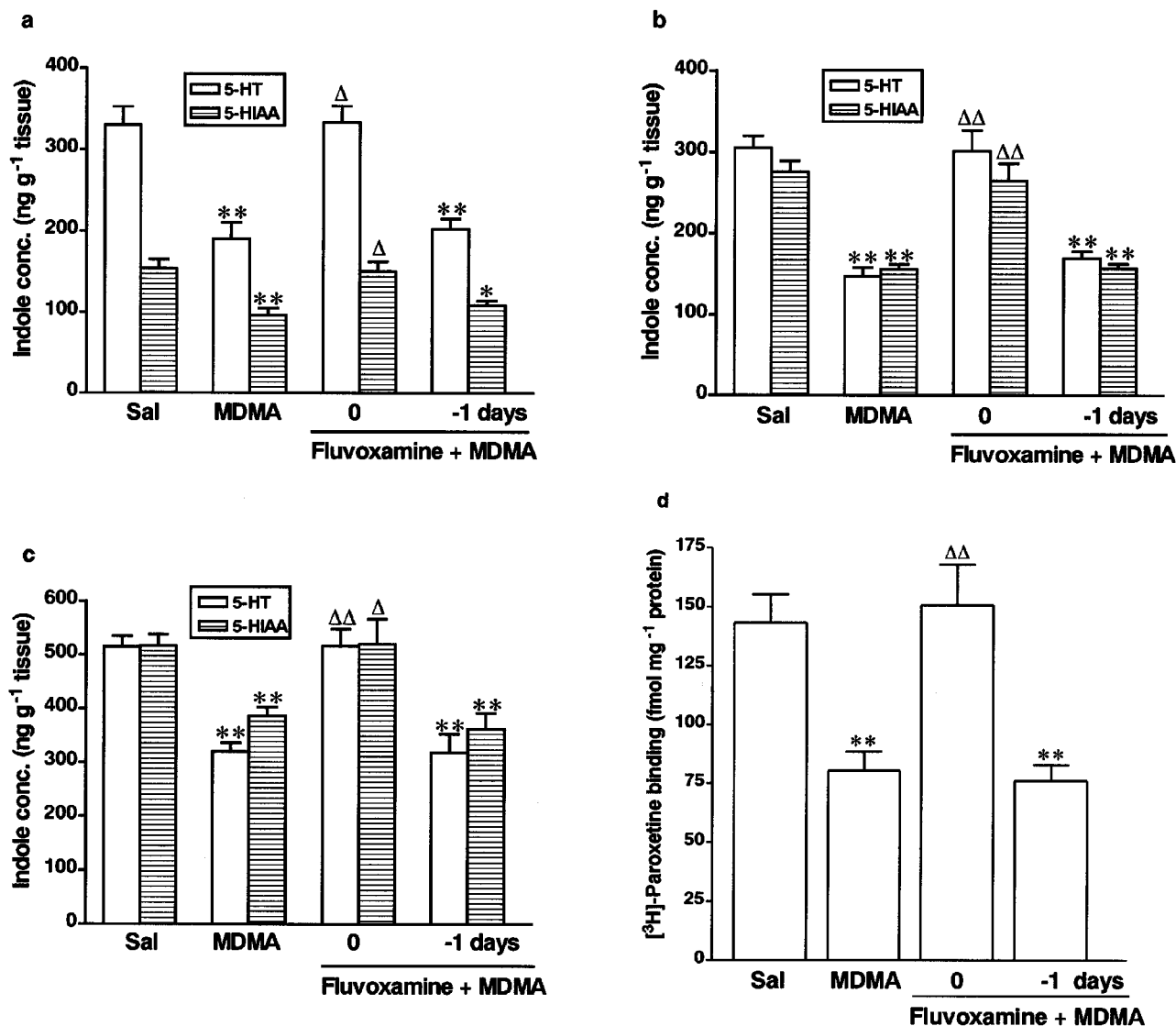
Figure 2 Effect of fluvoxamine on the 3,4-methylenedioxymethamphetamine (MDMA)-induced decrease in indole concentration in (a) cortex, (b) hippocampus, (c) striatum and (d) density of [³H]-paroxetine labelled 5-HT uptake sites in cortex. Two injections of fluvoxamine (15 mg kg⁻¹, i.p) with an interval of 60 min were given 24 h before MDMA (15 mg kg⁻¹, i.p.), rats being sacrificed 7 days later. A group of animals received fluvoxamine 5 min before and 55 min after MDMA. Results shown as mean ± s.e.mean ($n = 6-16$). Different from saline: $*P < 0.05$; $**P < 0.001$. Different from MDMA: $\triangle P < 0.01$, $\triangle\triangle P < 0.001$.

Discussion

This study shows for the first time that fluoxetine, when given 4 days before MDMA, provides complete protection against the delayed neurotoxic loss of 5-HT nerve terminals induced by this amphetamine derivative and still provides partial protection when given 7 days earlier. Other authors have previously reported that fluoxetine protected against MDMA-induced neurodegeneration, but in their studies the 5-HT uptake inhibitor was co-administered with the MDMA (Schmidt, 1987; Malberg et al., 1996; Shankaran et al., 1999) or given up to 6 h later (Schmidt, 1987; Shankaran et al., 1999). In the current study, protection was observed not only in the attenuation of the MDMA-induced decrease in 5-HT and 5-HIAA concentration in hippocampus, cortex and striatum, but also in the attenuation of the reduction in cortical 5-HT uptake sites

labelled with [³H]-paroxetine. The binding of [³H]-paroxetine decreases following administration of compounds shown histologically to produce neuronal damage, including, for example, selective 5-HT neurotoxins such as 5,7-dihydroxytryptamine (Habert et al., 1985) and MDMA (Hewitt & Green, 1994). Therefore, the ability of fluoxetine to attenuate the loss of 5-HT transporter gives confidence in the conclusion that fluoxetine is producing true neuroprotection.

When [³H]-paroxetine binding was used as a marker rather than the 5-HT content, the degree of protection seen when fluoxetine was given concurrently with MDMA was smaller than that observed when fluoxetine had been administered 2–4 days earlier (Figure 1). This effect is probably due to the existence of residual drug (fluoxetine or norfluoxetine) in the tissue interfering with the binding parameters 7 days after fluoxetine administration (Figure 3).
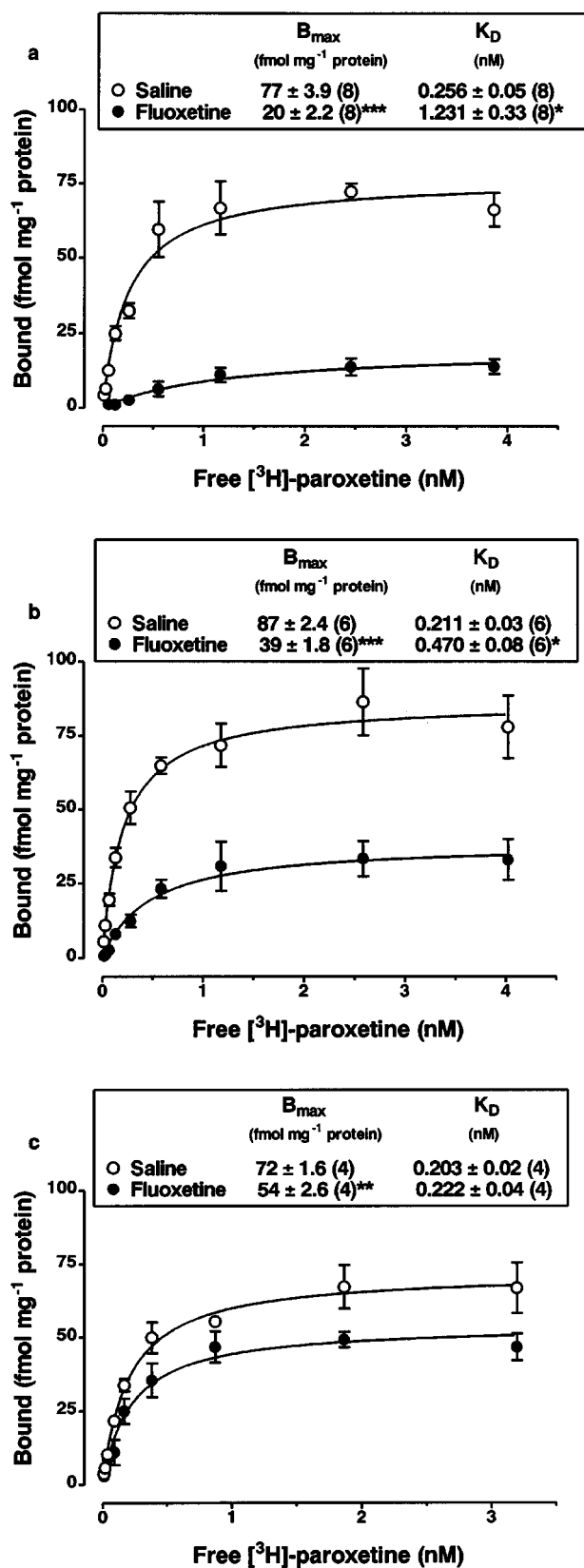
Figure 3 Saturation-binding analysis of [^{3}H]-paroxetine to the cortex of saline and fluoxetine injected rats 2 (a), 4 (b) and 7 (c) days after treatment. Rats received two doses of fluoxetine (10 mg kg^{-1}, i.p.) with an interval of 60 min. A saturation isotherm obtained from 4–8 experiments per group in triplicate is shown. B$_{max}$ and K_D of cortical [^{3}H]-paroxetine binding in saline- and fluoxetine-treated rats is shown as mean±s.e.mean. The data were best fitted to a single site model ($r^2 = 0.99$). Different from saline: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

The long-lasting neuroprotective effect of fluoxetine appears to be closely related to the presence in the brain of fluoxetine and its active and major metabolite norfluoxetine, whose presence was still detectable for up to 7 days after administration of fluoxetine. Both fluoxetine and norfluoxetine act primarily as 5-HT uptake inhibitors (Bolden-Watson & Richelson, 1993; Wong *et al.*, 1993; Sanchez & Hyttel, 1999) and both have similar efficacy as 5-HT uptake inhibitors (Wong *et al.*, 1993). Since the compounds have similar potency, it was possible to plot the concentration of [fluoxetine + norfluoxetine] in nmol g^{-1} versus the degree of protection seen over time (using the loss in 5-HT content in cortex). This revealed a simple hyperbolic plot indicating that protection probably relates to tissue drug concentration (Figure 7).

A plot of the changes in K_D value versus concentration of [fluoxetine + norfluoxetine] revealed, not unexpectedly, that there is a linear relationship between the combined concentration of these two highly liposoluble drugs and the K_D value ($r = 0.99$). There was also a reasonably linear relationship between the K_D and B$_{max}$ value at every time point studied ($r = 0.91$). It is probable that the B$_{max}$ change seen also relates to the presence of the drug interfering with the binding analysis rather than any down regulation of the receptor. This interpretation would be consistent with the failure of others to find down regulation of the uptake site even on chronic administration of 5-HT uptake inhibitor compounds (Cheetham *et al.*, 1993; Dean *et al.*, 1997). However we cannot rule out totally that a down regulation of the uptake site has occurred.

What appears clear is that a defined concentration of the drug must be present in the tissue to inhibit uptake and thereby provide neuroprotection. This view is supported by
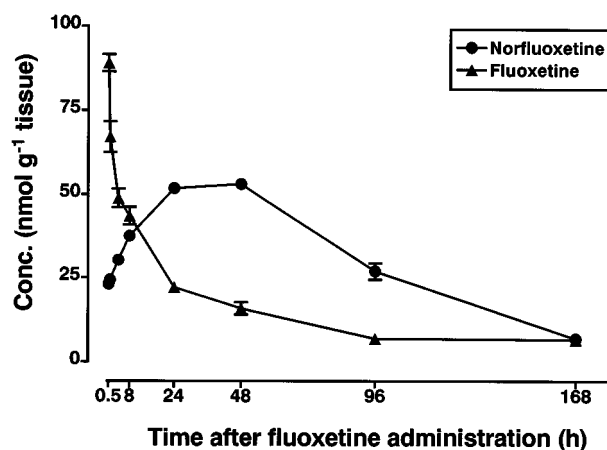

Figure 4 Time course of fluoxetine and norfluoxetine concentrations in cortex after administration of two doses of fluoxetine (10 mg kg^{-1}, i.p) with an interval of 60 min. Levels of the parent compound and its metabolite were measured at 0.5, 1, 4, 8, 24, 48, 96 and 168 h after last dose of fluoxetine. Results shown as mean±s.e.mean ($n = 5$ at each time point).
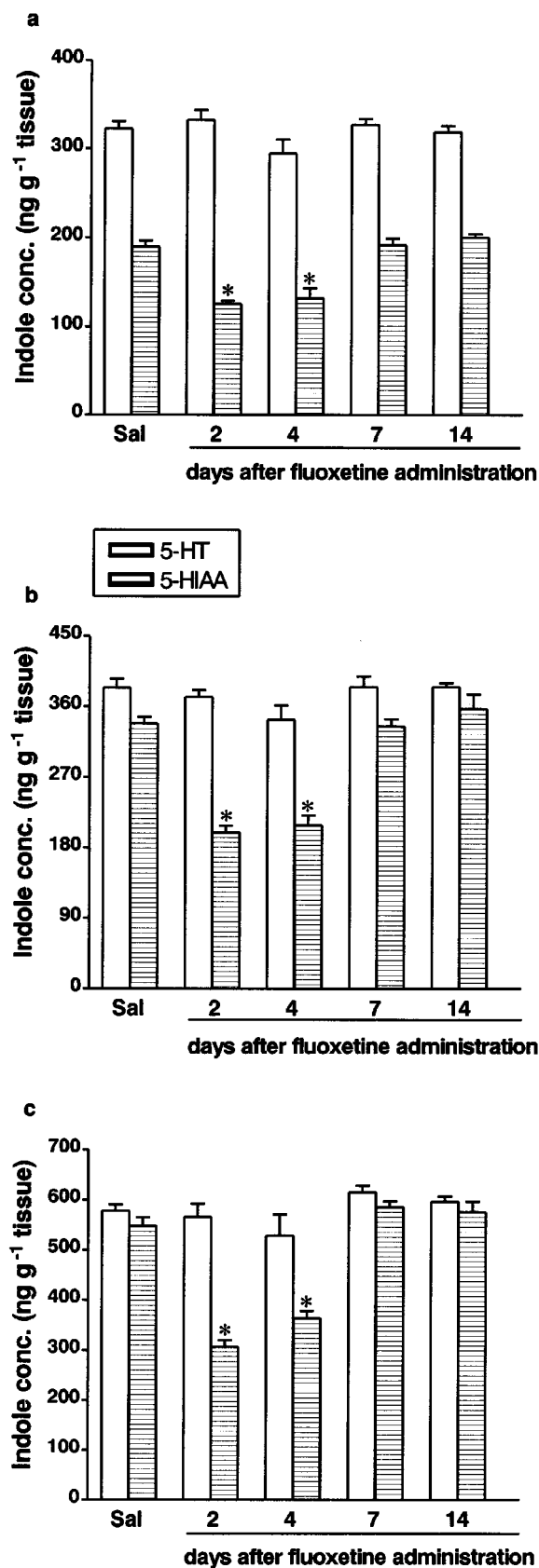
Figure 5 Effect of fluoxetine on indole concentration in (a) cortex, (b) hippocampus and (c) striatum 2, 4, 7 and 14 days after administration. Rats received two injections of fluoxetine (10 mg kg^{-1}, i.p) with an interval of 60 min. Results shown as mean$\pm$s.e.mean ($n = 6-12$). Different from saline: *$P < 0.001$.
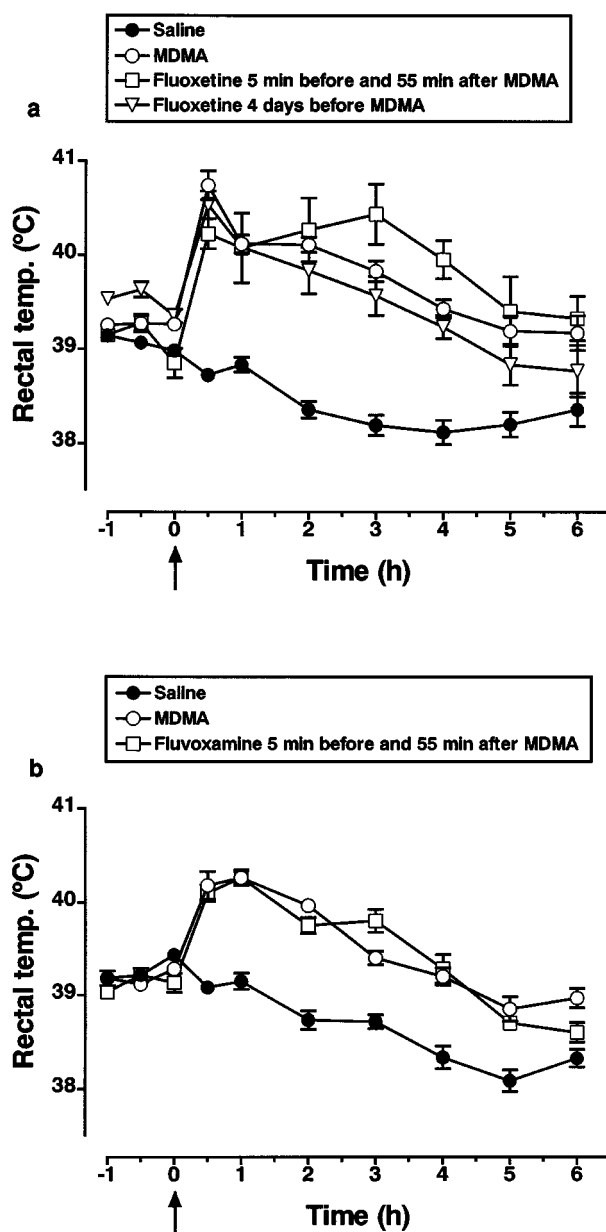

Figure 6 Effect of fluoxetine or fluvoxamine on the 3,4-methylene-dioxymethamphetamine (MDMA)-induced hyperthermia. (a) Rectal temperature of rats injected with fluoxetine (10 mg kg^{-1}, i.p., two doses, 60 min apart) or saline 4 days before MDMA (15 mg kg^{-1}, i.p.) or 5 min before and 55 min after MDMA. Arrow indicates MDMA injection. MDMA significantly increased temperature compared to saline treatment ($F_{(1,21)} = 116.4$; $P < 0.001$). Fluoxetine either co-administered with MDMA or injected 4 days before did not modify the hyperthermic response ($F_{(1,13)} = 0.283$ and $F_{(1,15)} = 2.61$, respectively). (b) Rectal temperature of rats injected with fluvoxamine (15 mg kg^{-1}, i.p.) or saline 5 min before and 55 min after MDMA. MDMA significantly increased temperature compared to saline treatment ($F_{(1,10)} = 133.1$; $P < 0.001$). Co-administration of fluvoxamine with MDMA did not modify the hyperthermic response ($F_{(1,10)} = 0.409$). Results shown as mean and vertical lines indicate s.e.mean ($n = 6-12$).

the study with fluvoxamine, since the rapid clearance of this compound was accompanied by loss of neuroprotection.

Fluoxetine not only provided long-lasting protection against the long-term neurotoxicity induced by MDMA but
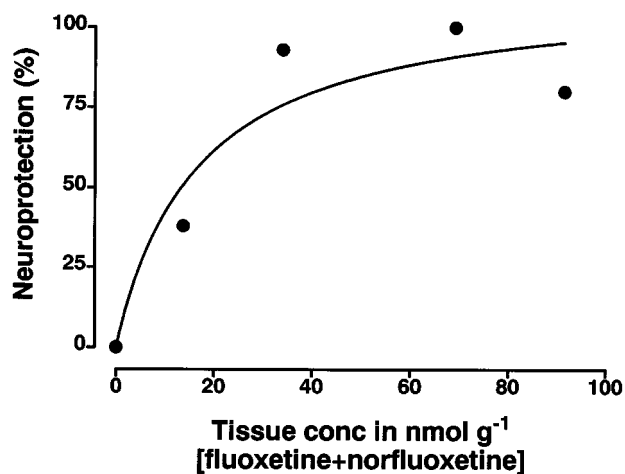
Figure 7 Plot of the mean concentration of fluoxetine plus norfluoxetine in cortical tissue at various times post fluoxetine injection (taken from data presented in Figure 4) and the degree of neuroprotection measured as the per cent of loss of cortical tissue 5-HT seen following fluoxetine administration to MDMA-treated rats versus the loss seen following MDMA alone.

also produced long-lasting prevention of the acute 5-HT depletion by p-chloroamphetamine. Administration to rats of one dose of fluoxetine (10 mg kg^{-1}, i.p.) between 4 and 24 h before p-chloroamphetamine completely prevented the reduction of the cortical 5-HT concentration induced by p-chloroamphetamine 4 h later. This effect was smaller when the pretreatment interval was 48 h (Fuller *et al.*, 1975).

There are several lines of evidence indicating that the continuous presence of selective 5-HT uptake inhibitors tends to produce changes in the function of the transporter within minutes (Blakely *et al.*, 1998; Benmansour *et al.*, 1999; Ramamoorthy *et al.*, 1998) and that the changes are due to protein kinase C (PKC) activation in a similar way to that occurring for the 5-HT receptors (Qian *et al.*, 1997). PKC activation in turns leads to transporter phosphorylation, transporter redistribution from the cell surface (sequestration) and loss of functional uptake activity. In contrast to 5-HT and amphetamines, antidepressants facilitate the 5-HT transporter phosphorylation and block 5-HT uptake (Ramamoorthy & Blakely, 1999). Since MDMA is a substrate for the 5-HT transporter, an internalization of cell-surface transporter protein and therefore, a decrease in the density of transporter proteins in the cell membrane induced by the presence of fluoxetine or norfluoxetine, could involve a significant and lasting blockade in the uptake of MDMA or most likely of a metabolic product to the presynaptic 5-HT nerve terminals (Esteban *et al.*, 2001).

Two and four days after fluoxetine administration, there is a decrease in brain 5-HIAA concentration whereas 5-HT content remains unaltered. This effect, previously described by others (Baldessarini *et al.*, 1992), is probably due to decreased 5-HT synthesis resulting from compensatory changes initiated by the increase in the synaptic 5-HT concentration (Bymaster & Wong, 1974). However, a reduction in 5-HT synthesis following fluoxetine administration is not the cause of fluoxetine-induced neuroprotection since administration of the tryptophan hydroxylase inhibitor

p-chlorophenylalanine (PCPA) does not influence the MDMA-induced decrease in cortical 5-HT uptake sites (Brodkin *et al.*, 1993).

All current evidence suggests that the neurotoxicity of MDMA is not due to the parent compound but to a toxic metabolite probably formed peripherally. Intracerebral administration of MDMA at a dose that produced a concentration similar or higher than that reached following a neurotoxic dose of MDMA (15 mg kg^{-1}) given peripherally, does not produce neurotoxic damage (Esteban *et al.*, 2001). There is evidence that intrastriatal or intracortical administration of glutathione and N-acetylcysteine conjugates of α-methyldopamine (a major metabolite of MDMA) causes significant decreases in striatal and cortical 5-HT concentrations 7 days later (Bai *et al.*, 1999). Nevertheless, the role for these compounds on MDMA neurotoxicity remains to be determined.

It is well known that in Dark Agouti rats MDMA is metabolized by CYP2D1, the rat equivalent to the human CYP2D6, and that fluoxetine and its metabolite, norfluoxetine, are potent inhibitors of CYP2D6 (Hamelin *et al.*, 1996). In addition, fluoxetine and norfluoxetine are detected in the brain several days after fluoxetine injection. Therefore, it could be argued that the protective effect of fluoxetine results from its ability to inhibit MDMA metabolism. This does not appear to be the case since fluoxetine did not alter the concentration of MDMA in the brain. When MDMA was administered to rats pretreated with fluoxetine 2 days earlier (a time when the concentration of both fluoxetine and its metabolite are still high), the cortical MDMA levels observed 60 min later were similar to that found in rats not given fluoxetine. We can state with some degree of confidence that fluoxetine does not prevent MDMA toxicity by inhibiting the metabolism of the drug. It thus seems likely that fluoxetine and its major metabolite are preventing the entry of a neurotoxic metabolite of MDMA into the nerve ending.

What can also be stated unequivocally is that the neuroprotective effect of fluoxetine or fluvoxamine against MDMA toxicity is not related to an effect on body temperature. The hyperthermic response immediately following MDMA was similar in rats receiving saline or either fluoxetine or fluvoxamine. In addition, these data support the notion that the rise in rectal temperature induced by MDMA is not obviously related to its ability to increase 5-HT release (Mechan *et al.*, 2000).

In summary this study shows that the neuroprotective effect of fluoxetine is due to the long term presence of either the parent compound or to its active metabolite, norfluoxetine in the brain. Both compounds block 5-HT transporter selectively, inhibit 5-HT uptake and presumably interfere with the entry of MDMA or a neurotoxic metabolite into the presynaptic nerve terminal.

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