



## Study on the neuroprotective effect of fluoxetine against MDMA-induced neurotoxicity on the serotonin transporter in rat brain using micro-PET

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### ABSTRACT

3, 4-Methylenedioxymethamphetamine (MDMA, “ecstasy”) has toxic effects on serotonergic neurons in the brain. Our aim was to determine whether *N,N*-dimethyl-2-(2-amino-4-[<sup>18</sup>F]-fluorophenylthio) benzylamine (4-[<sup>18</sup>F]-ADAM; a serotonin transporter imaging agent) and micropositron emission tomography (micro-PET) can be used to examine *in vivo* the effect of fluoxetine on MDMA-induced loss of serotonin transporters in rat brain. Male Sprague–Dawley rats were injected with fluoxetine [1 dose, 5 mg/kg, subcutaneously (s.c.)] followed by MDMA (twice a day for 4 consecutive days, 10 mg/kg, s.c.). Micro-PET with 4-[<sup>18</sup>F]-ADAM was performed on days 4, 10, 17, 24, and 31. In addition, the time course of occupancy by fluoxetine at 4-[<sup>18</sup>F]-ADAM sites was measured. Specific 4-[<sup>18</sup>F]-ADAM uptake ratios (SURs) were calculated from the micro-PET imaging data for various brain regions. Immunohistochemistry was performed 7 days after the last micro-PET scan. From day 4 to day 31, SURs were markedly decreased (by approximately 55–75% compared to control values) in all brain regions in MDMA-treated rats. The effect of MDMA was markedly attenuated (approximately 30–50%) by fluoxetine. The fluoxetine-induced decrease in uptake in different brain regions was 40–75% at 90-min postinjection, and this decrease returned to baseline values in most brain regions by day 31. The distribution and intensity of serotonin transporter (SERT) immunostaining in the brain paralleled the PET imaging results, suggesting that a single dose of fluoxetine provides long-lasting protection against MDMA-induced loss of SERT and that such neuroprotection is detectable *in vivo* by 4-[<sup>18</sup>F]-ADAM micro-PET.

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### Introduction

3,4-Methylenedioxymethamphetamine (MDMA, “ecstasy”) is a ring-substituted analog of methamphetamine with amphetamine-like stimulant effects (Baumann et al., 2007) and is one of the most popular recreational drugs used by adolescents (Parrott, 2004). According to a national survey, 10% of 12th graders in the United States have taken MDMA at least once (Banken, 2004). The serious adverse side effects which have been reported for MDMA include tachycardia, hypertension, hyperthermia, serotonin (5-HT) syndrome, hyponatremia, and kidney and liver failure (Henry et al., 1992; Schifano, 2004). Abuse of MDMA carries a high risk of complications stemming from acute and long-term neurophysiologic and neurocognitive toxicity (Adinoff, 2004).

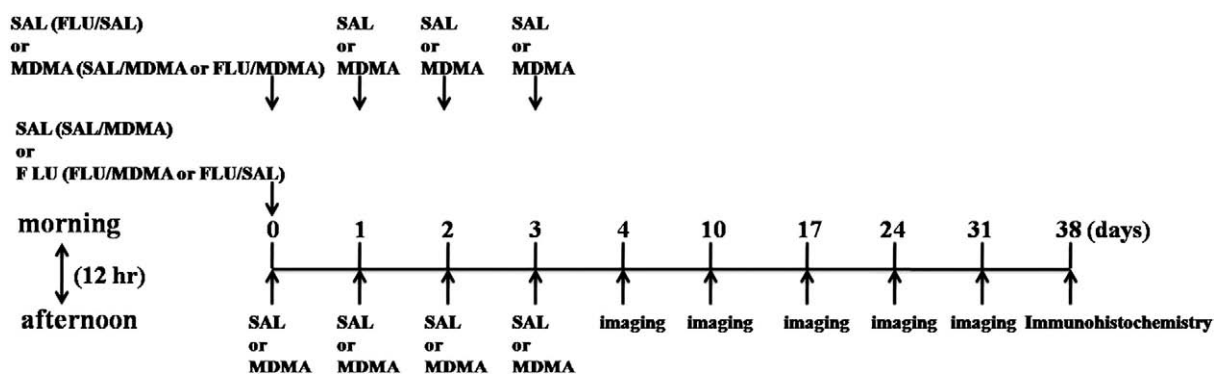
Numerous studies have demonstrated that MDMA and other illicit drugs produce long-lasting changes in the serotonergic system,

including decreases in the level of serotonin and in the density of the serotonin transporter (SERT; Green et al., 2003; Hatzidimitriou et al., 1999; Semple et al., 1999), a facilitated diffusion transporter in the plasma membrane of serotonergic nerve terminals and cell bodies that is considered to be a reliable marker of the integrity of serotonergic neurons (Nielsen et al., 2006; Zhou et al., 1998). The serotonin transporter has been implicated in the mechanism of MDMA-induced serotonin release and depletion (Rudnick and Wall, 1992). In SERT-knockout mice, MDMA-rewarding effects are abolished, suggesting that SERT may constitute a key target in the development of new treatment regimens for MDMA abuse (Trigo et al., 2007). Previously, MDMA-induced 5-HT neurotoxicity was studied exclusively in postmortem samples from MDMA abusers. However, immunohistochemically stained brain slices do not necessarily reflect conditions in the living brain. In addition, biochemical changes associated with different stages of MDMA abuse cannot be evaluated precisely in postmortem samples.

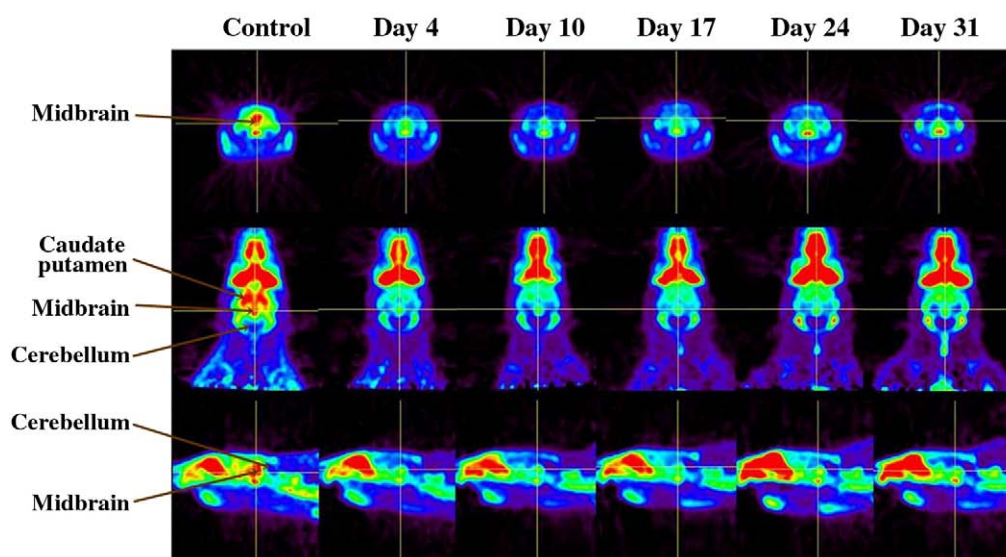
Noninvasive imaging modalities [e.g., positron emission tomography (PET) and single photon emission-computed tomography (SPECT)] in

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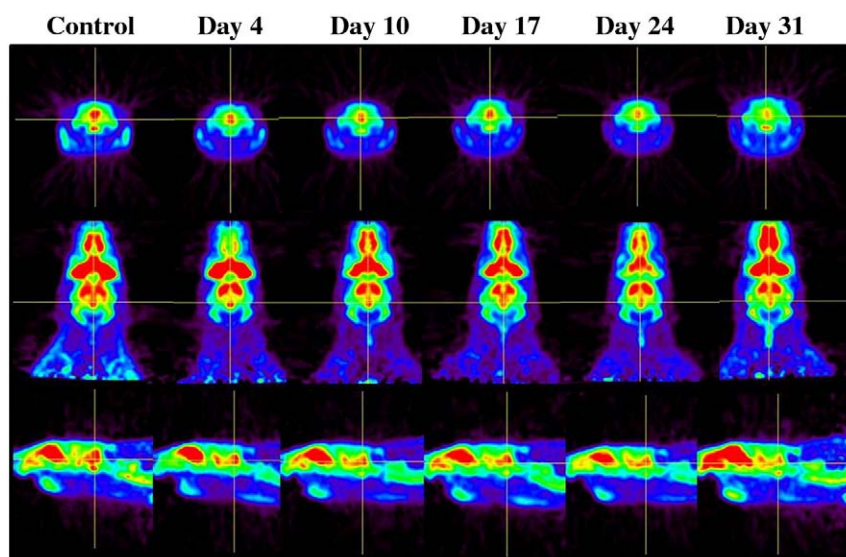
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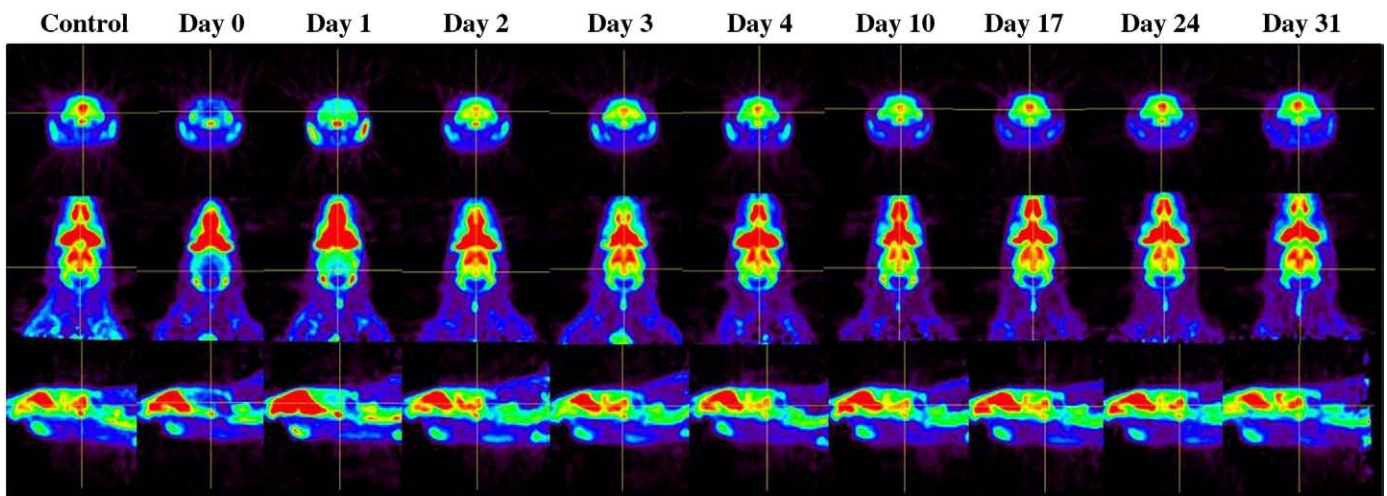
**Fig. 1.** Experimental schedule of drug treatments. The SAL/MDMA group was treated with a single dose of saline followed immediately by MDMA [10 mg/kg, subcutaneously (s.c.)] in the morning and afternoon on days 0, 1, 2, and 3. The FLU/MDMA and FLU/SAL groups were treated with a single dose of fluoxetine (FLU, 5 mg/kg, s.c.) followed immediately by MDMA (in the former group) or saline (in the latter group) in the morning and afternoon on days 0, 1, 2, and 3. 4- $^{18}\text{F}$ -ADAM micro-PET imaging was performed on days 4, 10, 17, 24, and 31, and immunohistochemistry was performed on day 38.



**Fig. 2.** 4- $^{18}\text{F}$ -ADAM/micro-PET images of coronal, transverse, and sagittal sections of rat brain on days 4–31 from the beginning of the 4-day treatment with SAL/MDMA. 4- $^{18}\text{F}$ -ADAM uptake in all brain regions was obviously lower in the SAL/MDMA group than in the control group.



**Fig. 3.** 4- $^{18}\text{F}$ -ADAM/micro-PET images of coronal, transverse, and sagittal sections of rat brain on days 4–31 from the beginning of the 4-day treatment with FLU/MDMA. 4- $^{18}\text{F}$ -ADAM uptake in all brain regions in the FLU/MDMA group was comparable to that in the control group.



**Fig. 4.** 4-[ $^{18}\text{F}$ ]-ADAM/micro-PET images of coronal, transverse, and sagittal sections of rat brain from 90 min (day 0) to day 31 after FLU/SAL treatment. 4-[ $^{18}\text{F}$ ]-ADAM uptake in all brain regions in the FLU/SAL group on days 4–31 was comparable to that in the control group.

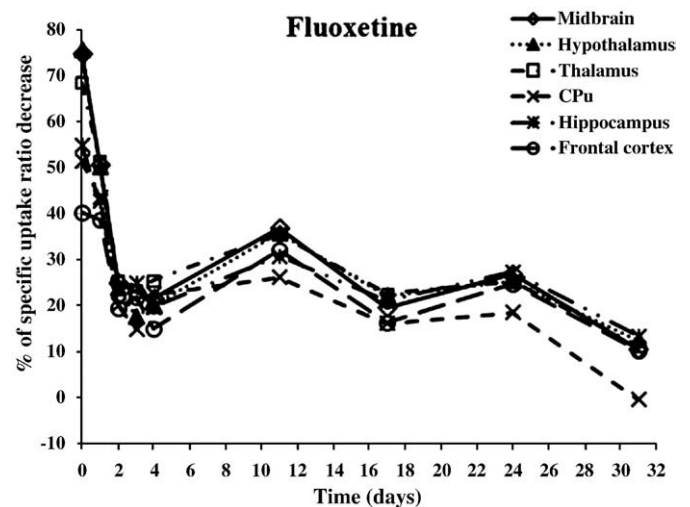
conjunction with targeted tracer compounds have been applied successfully in animals to visualize SERT in brain tissues (Brust et al., 2003; Ginovart et al., 2003; Hartvig et al., 2002; Jarkas et al., 2005). The use of radioligands and PET may provide the means to determine directly the effects of MDMA and how alterations of SERT are related to toxic changes in humans. Indeed, MDMA neurotoxicity has been studied in nonhuman primates and humans by [ $^{11}\text{C}$ ]-McN5652 and [ $^{11}\text{C}$ ]-DASB coupled with PET (Buchert et al., 2003; McCann et al., 1998; Scheffel et al., 1998; Szabo et al., 2002). Recently, we developed a new [ $^{18}\text{F}$ ]-labeled SERT radioligand, *N,N*-dimethyl-2-(2-amino-4-[ $^{18}\text{F}$ ]-fluorophenylthio) benzylamine (4-[ $^{18}\text{F}$ ]-ADAM) (Shiue et al., 2003b), with high affinity and high specificity for SERT (Shiue et al., 2003a). The use of this SERT imaging agent with PET imaging and autoradiography has been validated in a 5,7-dihydroxytryptamine (5,7-DHT)-lesioned rat model, a *p*-chloroamphetamine (PCA)-induced 5-HT depletion rat model, and in rats treated with paroxetine, a selective serotonin reuptake inhibitor (SSRI; Ma et al., 2009). Therefore, it is possible this method can be used reliably for directly evaluating the neurotoxicity of MDMA in the living brain and for monitoring the response to therapy in cases of MDMA-induced neurotoxicity.

Rats have been used widely in studies of MDMA-induced neurotoxicity (Bogen et al., 2003; Commins et al., 1987; Shankaran and Gudelsky, 1999), and the neuroprotective effect of fluoxetine (an SSRI) has been studied in a rat model of MDMA intoxication (Berger et al., 1992; Schmidt, 1987). However, the mechanism remains unclear, though it probably involves inhibition of the SERT interaction with MDMA and its neurotoxic metabolites (Sanchez et al., 2001) or a decrease in the formation of hydroxyl free radicals (Hrometz et al., 2004; Shankaran et al., 1999). Several reports suggest that SERT carries MDMA into serotonergic neurons. Colado et al. (1997) reported that fenfluramine (known to induce neurotoxic damage in serotonergic nerve terminals) suppressed the MDMA-induced formation of free radicals. This was supported by the administration of MDMA with SSRIs (e.g., fluoxetine and citalopram), which prevented subsequent extracellular oxidative stress (Shankaran et al., 1999), long-term serotonin depletion, and serotonin uptake site decreases, indicating that free radical production might occur following activation of SERT by MDMA (Battaglia et al., 1988; Green et al., 2003; Sanchez et al., 2001; Schmidt, 1987). However, the time course of fluoxetine alone and the fluoxetine-MDMA interaction with SERT and serotonergic changes resulting from such interactions are still unknown in rats. Therefore, we used 4-[ $^{18}\text{F}$ ]-ADAM and micro-PET to study the long-term effects (up to 31 days) of fluoxetine alone and in combination with MDMA on SERT. Part of this study has been presented as an abstract (Ma et al., 2008).

## Materials and methods

### Animals and drug treatments

Male Sprague–Dawley (SD) rats (250–300 g in weight) were housed at the National Defense Medical Center (Taipei, Taiwan) in animal facilities with a constant temperature of  $23 \pm 2^\circ\text{C}$  and a controlled light/dark cycle (light from 7:00 AM to 7:00 PM). 3,4-Methylenedioxymethamphetamine (purity, 98%) was obtained from the Investigation Bureau of Taiwan, and fluoxetine (FLU) was purchased from Sigma-Aldrich (St. Louis, MO). 3,4-Methylenedioxymethamphetamine and FLU were dissolved in saline (SAL; 0.9% NaCl) at a final concentration of 10 and 5 mg/ml, respectively. Rats were treated with SAL or FLU [5 mg/kg, subcutaneously (s.c.)] followed by SAL or MDMA (twice a day for 4 consecutive days, 10 mg/kg, s.c.). Rats were grouped as follows: SAL/MDMA (saline followed by MDMA injection,  $n=6$  for each time point), FLU/MDMA (fluoxetine followed by MDMA injection,  $n=6$  for each time point), FLU/SAL (fluoxetine followed by saline injection,  $n=6$  for each time



**Fig. 5.** Decrease in specific uptake ratio (SUR) of 4-[ $^{18}\text{F}$ ]-ADAM in brains of rats pretreated with fluoxetine (5 mg/kg, subcutaneously). Fluoxetine-induced inhibition of 4-[ $^{18}\text{F}$ ]-ADAM uptake was markedly higher 90 min postinjection in most brain regions. The SURs of 4-[ $^{18}\text{F}$ ]-ADAM in normal rats taken as baseline values on day 0 were 4.18 for the midbrain, 4.01 for the hypothalamus, 3.57 for the thalamus, 3.51 for the caudate putamen, 2.86 for the hippocampus, and 2.46 for the frontal cortex.



point), and normal controls ( $n=6$ ). The schedule of the experiment is shown in Fig. 1. In addition, in the FLU/SAL group, the brain biodistribution of 4-[ $^{18}\text{F}$ ]-ADAM was assessed in micro-PET scans beginning 90 min postfluoxetine injection on day 0 up to day 31 ( $n=6$  for each time point). Saline was injected instead of fluoxetine in the normal control group. The 4-[ $^{18}\text{F}$ ]-ADAM micro-PET scans were performed on day 0, and the calculated specific uptake ratios (SURs) were used as baseline values. All research protocols were approved by the institutional animal care and use committee.

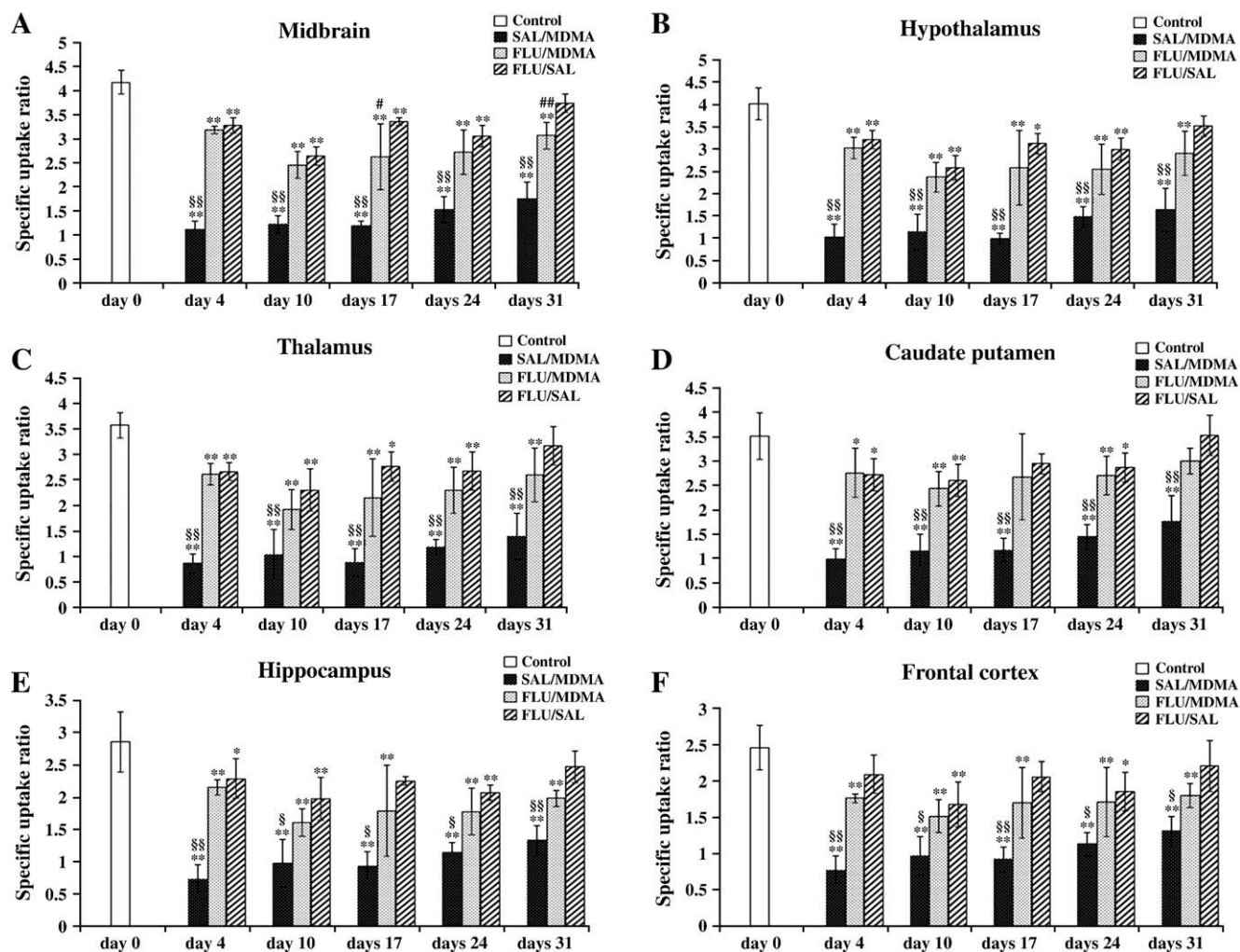
### Radiopharmaceuticals

The radioligand 4-[ $^{18}\text{F}$ ]-ADAM was synthesized in an automated synthesis module as described previously (Peng et al., 2008) but with slight modifications. In brief, the precursor, *N,N*-dimethyl-2-(2,4-dinitrophenylthio) benzylamine in 0.5 ml dimethyl sulfoxide was added to dried potassium [ $^{18}\text{F}$ ]-fluoride/Kryptofix 222 ( $\text{K}^{18}\text{F}/\text{K}_{2.2.2}$ ) residue, and the solution was heated and then cooled to room temperature. The intermediate was reduced with  $\text{NaBH}_4/\text{Cu}(\text{OAc})_2$  and then purified by high-performance liquid chromatography (HPLC). The 4-[ $^{18}\text{F}$ ]-ADAM ligand showed a radiochemical yield of

$1.5 \pm 0.3\%$ , a specific activity of  $1.75 \pm 0.77 \text{ Ci}/\mu\text{mol}$ , and a synthesis time of 120 min.

### Image data acquisition and analyses

Imaging was performed according to a previous report (Ma et al., 2009) with minor modifications. Rats were anesthetized by passive inhalation of a mixture of isoflurane/oxygen (5% isoflurane for induction and 2% for maintenance). Sixty minutes after 4-[ $^{18}\text{F}$ ]-ADAM ( $14.8\text{--}18.5 \text{ MBq}$ ;  $0.4\text{--}0.5 \text{ mCi}$ ) was injected in the tail vein, PET image acquisition was initiated [each fully three-dimensional acquisition was collected in list mode for 30 min using a micro-PET R4 scanner (Concorde MicroSystems, Knoxville, TN; energy window, 350–650 keV; timing window, 6 ns)]. Images were reconstructed using the Fourier rebinning algorithm and two-dimensional filtered backprojection (ramp filter with cutoff at Nyquist frequency). The reconstructed images were analyzed with ASIPro VM 6.3.3.1 software (Concorde MicroSystems). Volumes of interest for the midbrain, hypothalamus, thalamus, caudate putamen, hippocampus, frontal cortex, and cerebellum were drawn manually on the reconstructed PET images with the use of a rat brain atlas (Paxinos and Watson,



**Fig. 6.** Specific uptake ratios (SURs) of 4-[ $^{18}\text{F}$ ]-ADAM/micro-PET on days 4–31 from the beginning of the 4-day treatment in rat brain regions. (A) Midbrain. (B) Hypothalamus. (C) Thalamus. (D) Caudate putamen. (E) Hippocampus. (F) Frontal cortex. The SURs in all brain regions in the SAL/MDMA group were markedly decreased compared with that in the control and FLU/MDMA groups. Data are expressed as mean  $\pm$  SD,  $n=6$  for each time point. The SURs of 4-[ $^{18}\text{F}$ ]-ADAM in normal rats were taken as baseline values on day 0. \* $p<0.05$ , \*\* $p<0.01$  compared to the control and SAL/MDMA, FLU/MDMA, or FLU/SAL groups; § $p<0.05$ , §§ $p<0.01$  compared to the SAL/MDMA and FLU/MDMA groups; # $p<0.05$ , ## $p<0.01$  compared to the FLU/MDMA and FLU/SAL groups.

1998) and magnetic resonance images as reported previously (Ma et al., 2009; Fig. 1). The SURs were expressed as (target region-cerebellum)/cerebellum (Ma et al., 2009).

#### Immunohistochemistry

Immunohistochemistry was performed according to a previous report (Zhou et al., 1998) with minor modifications. Rats were anesthetized with 7.0% chloral hydrate [5 ml/kg, intraperitoneally (i. p.)] and perfused through the ascending aorta with 0.9% normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were removed, postfixed in the same fixative for 2 h, and cryoprotected overnight in a solution of 30% sucrose in 0.1 M PBS at 4 °C. Sagittal sections (40 µm) cut by cryostat (Leica CM 3050; Leica Microsystems Nussloch, GmbH, Nussloch, Germany) were rinsed in PBS, incubated in 1% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min, washed extensively, incubated in blocking solution (1% normal goat serum in PBS 0.1 M plus 1% Triton X-100) to reduce background, incubated overnight at 4 °C with rabbit anti-SERT antibody (1:2000; Chemicon International, Temecula, CA), rinsed, incubated with goat anti-rabbit biotinylated IgG (1:200; Vector, Burlingame, CA) for 1 h, incubated with avidin-biotin complex (1:200; Vectastain ABC kit, Vector) for 0.5 h, washed, exposed to 0.05% diaminobenzidine (dissolved in 0.1% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris buffer, pH 7.6) for 10 min, washed three times with distilled water, and then mounted on gelatin-coated glass slides.

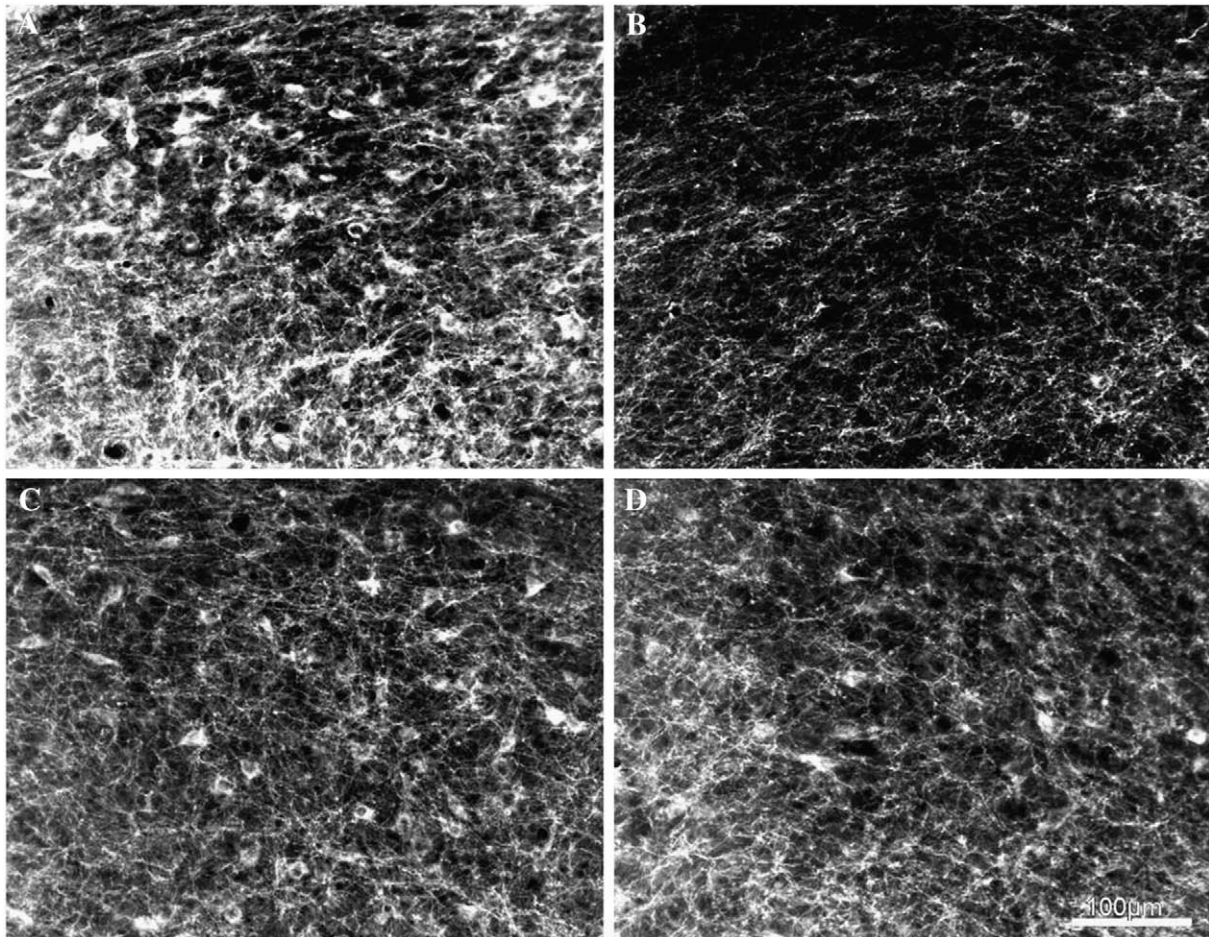
#### Statistical analysis

The SURs in different brain regions were compared by Student's *t*-test for two independent groups. One-way analysis of variance (ANOVA) with post-hoc Bonferroni adjustment was used for statistical evaluation. A *p* value of less than 0.05 (indicated by \*, §, #) or less than 0.01 (indicated by \*\*, §§, ##) was considered statistically significant. Data are presented as mean ± SD.

#### Results

The distribution of 4-[<sup>18</sup>F]-ADAM in rat brain is shown in Figs. 2–4 (control rats, column 1, Fig. 2; MDMA-treated rats on days 4, 10, 17, 24, and 31, columns 2–6, Fig. 2; fluoxetine + MDMA-treated rats on these days, Fig. 3; fluoxetine-treated rats on these days and days 0–3, Fig. 4). The SURs of 4-[<sup>18</sup>F]-ADAM in rat brain are shown in Figs. 5 and 6. Brain uptake of 4-[<sup>18</sup>F]-ADAM was significantly less in rats pretreated with MDMA than in control rats (*p* < 0.01; Fig. 6A–F) or FLU/MDMA rats (*p* < 0.05; Fig. 6A–F).

4-[<sup>18</sup>F]-ADAM uptake in the FLU/MDMA and FLU/SAL groups was quite similar and high on day 4 (*p* > 0.05; Fig. 6A–F). The SERT density decreased more frequently on day 10 and day 24 and increased on day 31. The uptake ratio of both groups fluctuated with time but showed the same pattern among brain regions. However, between-group differences in midbrain uptake ratio developed later (*p* < 0.05 for day 17, *p* < 0.01 for day 31; Fig. 6A). In the SAL/MDMA group, the SUR in



**Fig. 7.** Dark-field photomicrograph of SERT immunoreactivity in the dorsal raphe. (A) Control group, (B) SAL/MDMA group, (C) FLU/MDMA group, and (D) FLU/SAL group. The densities of serotonergic fibers and cell bodies were clearly decreased after MDMA treatment (SAL/MDMA vs. FLU/MDMA, FLU/SAL, or control group).



various regions decreased by ~75% compared to control (range from 0.72 to 1.12 vs. 2.86 to 4.18) on day 4. The decrement remained constant at about 70% on days 10–17 and was approximately 55–60% on days 24 and 31. The region of largest decrease occurred in the thalamus (0.85 on day 4 and 1.39 on day 31) and the region of smallest decrease occurred in the frontal cortex (0.77 on day 4 and 1.31 on day 31). In contrast, in the FLU/MDMA group, on day 4, the decrease in SUR (compared to control SUR) in various brain regions was ~25%, and days 24–31, it was ~30%. On the other hand, the SUR was markedly higher in the FLU/MDMA group (approximately 30–50% higher) than in the SAL/MDMA group at the same time points. The difference from baseline value on day 31 was significant in most brain regions, except for the caudate putamen. In the FLU/SAL group, the SUR in various brain regions decreased by ~20% on day 4 and returned to within ~90% of baseline in most brain regions on day 31 ( $p > 0.05$ ).

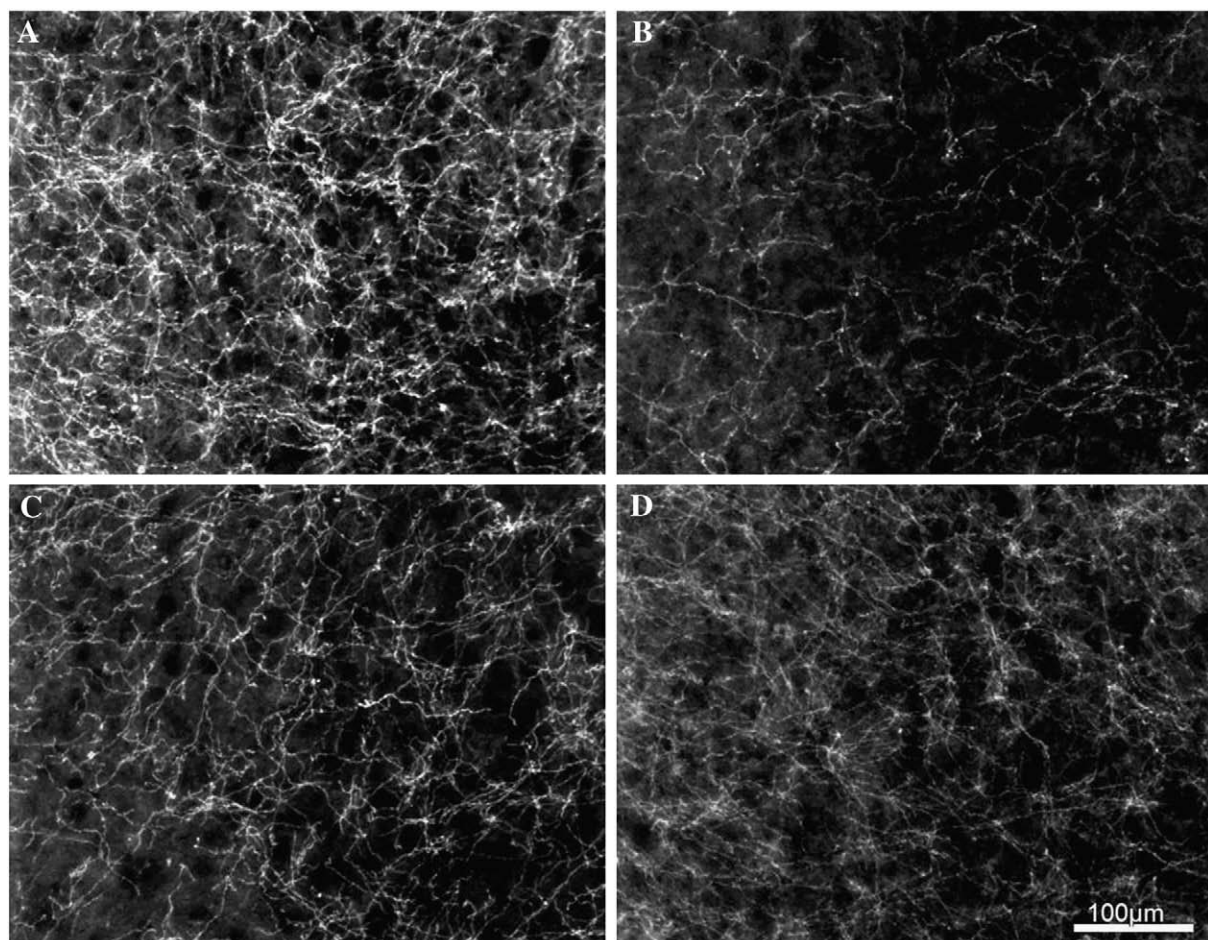
The time course of fluoxetine-induced reduction in 4-[ $^{18}\text{F}$ ]-ADAM uptake in various brain regions is shown in Fig. 5. In the FLU/SAL group, 4-[ $^{18}\text{F}$ ]-ADAM uptake was markedly inhibited as soon as 90 min after fluoxetine administration. Compared to the control group, the SUR (in percent) decreased in different brain regions by ~40–75% of control at 90 min postinjection. The SURs in midbrain and hypothalamus were 1.05 and 0.97, respectively, reduced by ~75% compared to control (4.18 in midbrain and 4.01 in hypothalamus), and frontal cortex was 1.47, decreased by ~40% compared to control (2.46) at 90 min after fluoxetine administration.

At the end of the study (day 38), immunohistochemical localization data were obtained and compared to the micro-PET

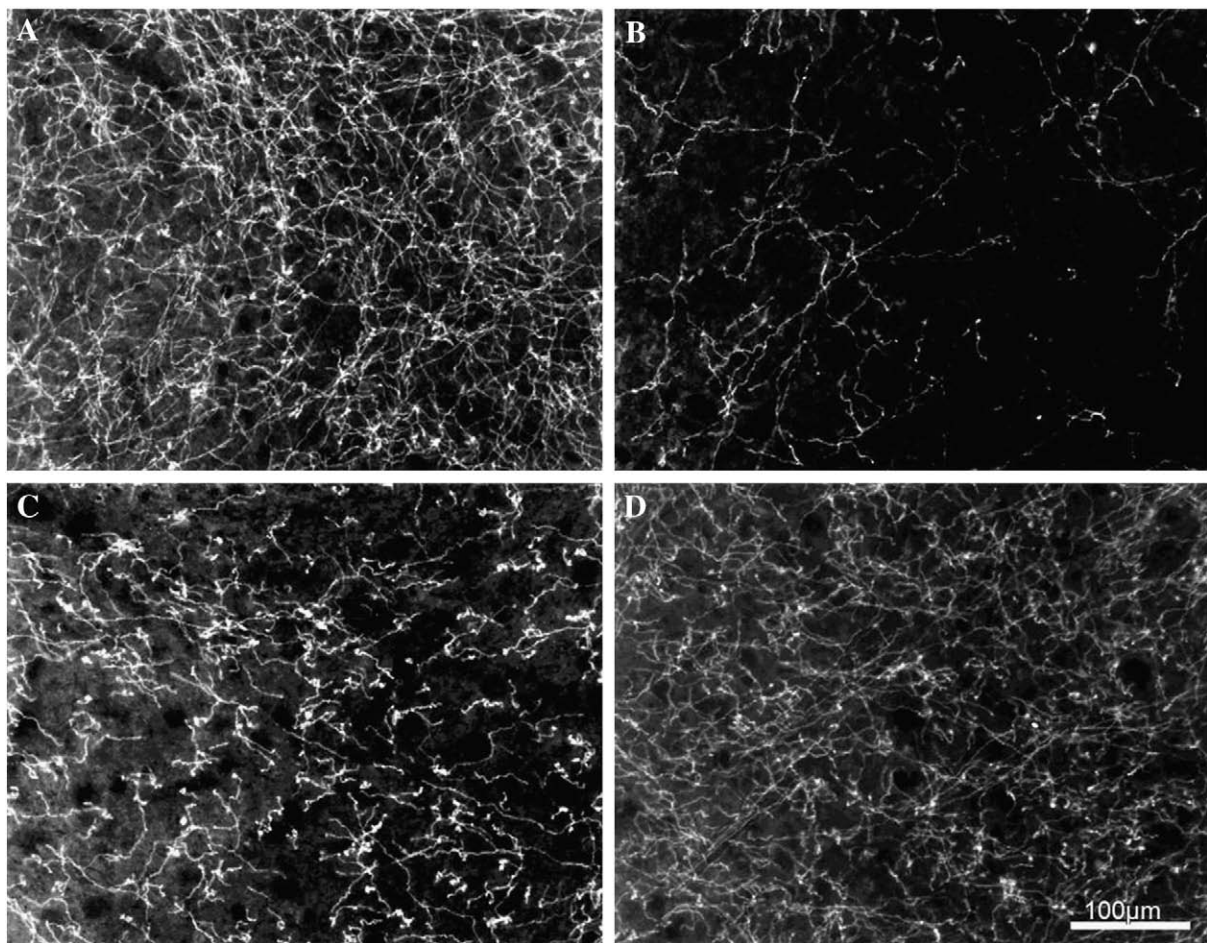
data. A dense meshwork of fibers and high densities of SERT labeling were found in all brain regions in control animals (Figs. 7A–12A). In the midbrain, labeled cell bodies were detected in the dorsal raphe (Fig. 7) and median raphe (data not shown). In addition to the raphe nuclei, including the substantia nigra and superior colliculus, a widespread and heterogeneous distribution of SERT immunoreactivity was observed. Lower densities of SERT labeling were observed after MDMA treatment. The representative distribution in the dorsal raphe is shown in Fig. 7. A higher density of immunoreactivity was found in area CA3 (Fig. 11) than in the dentate gyrus of the hippocampus (data not shown). In contrast, the density of serotonergic fibers and cell bodies clearly decreased after MDMA treatment (Figs. 7B–12B). Also, as expected, the density of SERT-positive fibers within well-defined cerebral structures was similar in the FLU/MDMA (Figs. 7C–12C), FLU/SAL (Figs. 7D–12D), and control groups (Figs. 7A–12A).

## Discussion

In this study, we evaluated the use of 4-[ $^{18}\text{F}$ ]-ADAM and micro-PET in monitoring the effect of fluoxetine on MDMA-induced toxicity in rats *in vivo*. A single dose of fluoxetine before MDMA challenge rapidly blocked the binding of most SERT sites, provided partial protection against the decrease in SERT density, and remained protective from day 4 to day 31, despite multiple doses of MDMA. In addition, the data indicated that 4-[ $^{18}\text{F}$ ]-ADAM micro-PET is a feasible method to evaluate serotonergic damage in the brains of MDMA-treated rats.



**Fig. 8.** Dark-field photomicrograph of SERT immunoreactivity in the lateral hypothalamus. (A) Control group, (B) SAL/MDMA group, (C) FLU/MDMA group, and (D) FLU/SAL group. The serotonergic fiber density was clearly decreased after MDMA treatment (SAL/MDMA vs. FLU/MDMA, FLU/SAL, or control group).



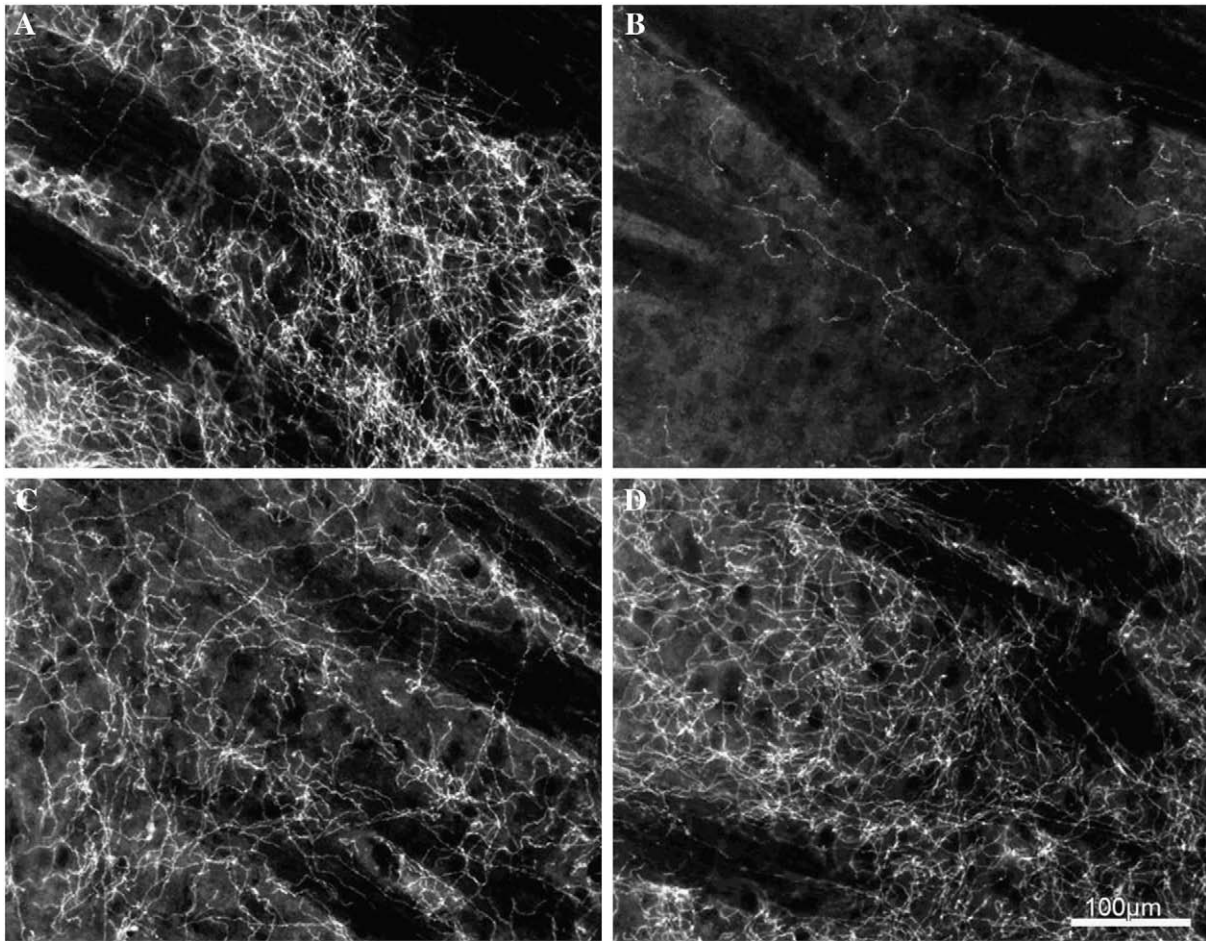
**Fig. 9.** Dark-field photomicrograph of SERT immunoreactivity in the posterior thalamus. (A) Control group, (B) SAL/MDMA group, (C) FLU/MDMA group, and (D) FLU/SAL group. The serotonergic fiber density was clearly decreased after MDMA treatment (SAL/MDMA vs. FLU/MDMA, FLU/SAL, or control group).

The mechanism of MDMA-induced neurotoxicity is not well understood. However, oxidative stress has been implicated in MDMA-induced damage to serotonergic nerve terminals. Previous studies have shown that MDMA induces an acute release of serotonin and dopamine (Sprague et al., 1998) as well as SERT-mediated dopamine uptake into serotonin terminals, resulting in subsequent deamination of dopamine via monoamine oxidase-B. The deamination of dopamine is a possible factor leading to the formation of  $H_2O_2$  and induction of subsequent oxidative stress (de la Torre and Farre, 2004; Hrometz et al., 2004). Other causes, including the formation of neurotoxic metabolites, hyperthermia, and mitochondrial dysfunction, might be involved in the possible serotonergic neurotoxicity (de la Torre and Farre, 2004; Green et al., 2003; Nixdorf et al., 2001). Most serotonin and dopamine release occurs within 4 h after MDMA exposure (Gough et al., 1991). Fluoxetine has a rapid and long-lasting effect on SERT. Schmidt (1987) showed that fluoxetine remained active against MDMA-induced toxicity even when given 3 or 4 h after MDMA exposure. Ginovart et al. (2003) reported 90% SERT occupancy using [ $^3H$ ]-DASB and PET in the striatum, midbrain, and thalamus of cats 30 min after fluoxetine administration. This result is in accordance with our present data showing high fluoxetine binding 90 min after injection (Fig. 5) and suggests that the effect of fluoxetine is rapid in the initial stage. In addition, it has been shown that fluoxetine has lower  $IC_{50}$  values than MDMA for inhibition of bovine and human SERT (Mortensen et al., 2001; Verrico et al., 2007), indicating that fluoxetine is more potent than MDMA. The dose of fluoxetine (5 mg/kg) in the present study was selected on the basis of these pharmacologic considerations and the drug's affinity for SERT.

The effect of CYP450 enzyme inhibitors should also be considered. In addition to MDMA, a CYP450 enzyme inhibitor (Maurer et al., 2000; Tucker et al., 1994), fluoxetine is also a potent inhibitor of the CYP450 2D6 enzyme (CYP450 2D1 in rats; Crewe et al., 1992) and may inhibit MDMA metabolism and substantially increase the toxicity of MDMA (Oesterheld et al., 2004; Upreti and Eddington, 2008). Using a HPLC method, Upreti and Eddington (2008) showed that rats pretreated with fluoxetine (once a day for 4 consecutive days, 10 mg/kg, i.p.) followed by MDMA (1 dose, 10 mg/kg, orally) resulted in increases in both the concentration (approximately 1.4-fold in brain and plasma) and half-life (2.4 h vs. 4.9 h) of MDMA. These pharmacokinetic interactions might be involved in the neuroprotective effect of fluoxetine against MDMA-induced neurotoxicity (Sanchez et al., 2001; Upreti and Eddington, 2008). In addition, it appears reasonable to propose that MDMA in the presence of fluoxetine may also inhibit fluoxetine metabolism, which might increase the fluoxetine concentration, half-life and prolong its pharmacologic action. Further studies will be needed to clarify the exact mechanism, although in the present study on day 31, SURs in various brain regions in the FLU/MDMA group remained significantly decreased compared to those in the control group, and SURs in the FLU/SAL group had returned to baseline values. Owing to these results of pharmacokinetic interactions between both CYP450 enzyme inhibitors described above, we chose a one-dose regimen of fluoxetine not only to protect against the eight doses of MDMA challenge, but also to reduce the adverse effects of fluoxetine–MDMA interactions.

Our pharmacologic results in the FLU/SAL group showed high binding of fluoxetine (i.e., high inhibition of 4-[ $^{18}F$ ]-ADAM uptake) in





**Fig. 10.** Dark-field photomicrograph of SERT immunoreactivity in the caudate putamen. (A) Control group, (B) SAL/MDMA group, (C) FLU/MDMA group, and (D) FLU/SAL group. The serotonergic fiber density was clearly decreased after MDMA treatment (SAL/MDMA vs. FLU/MDMA, FLU/SAL, or control group).

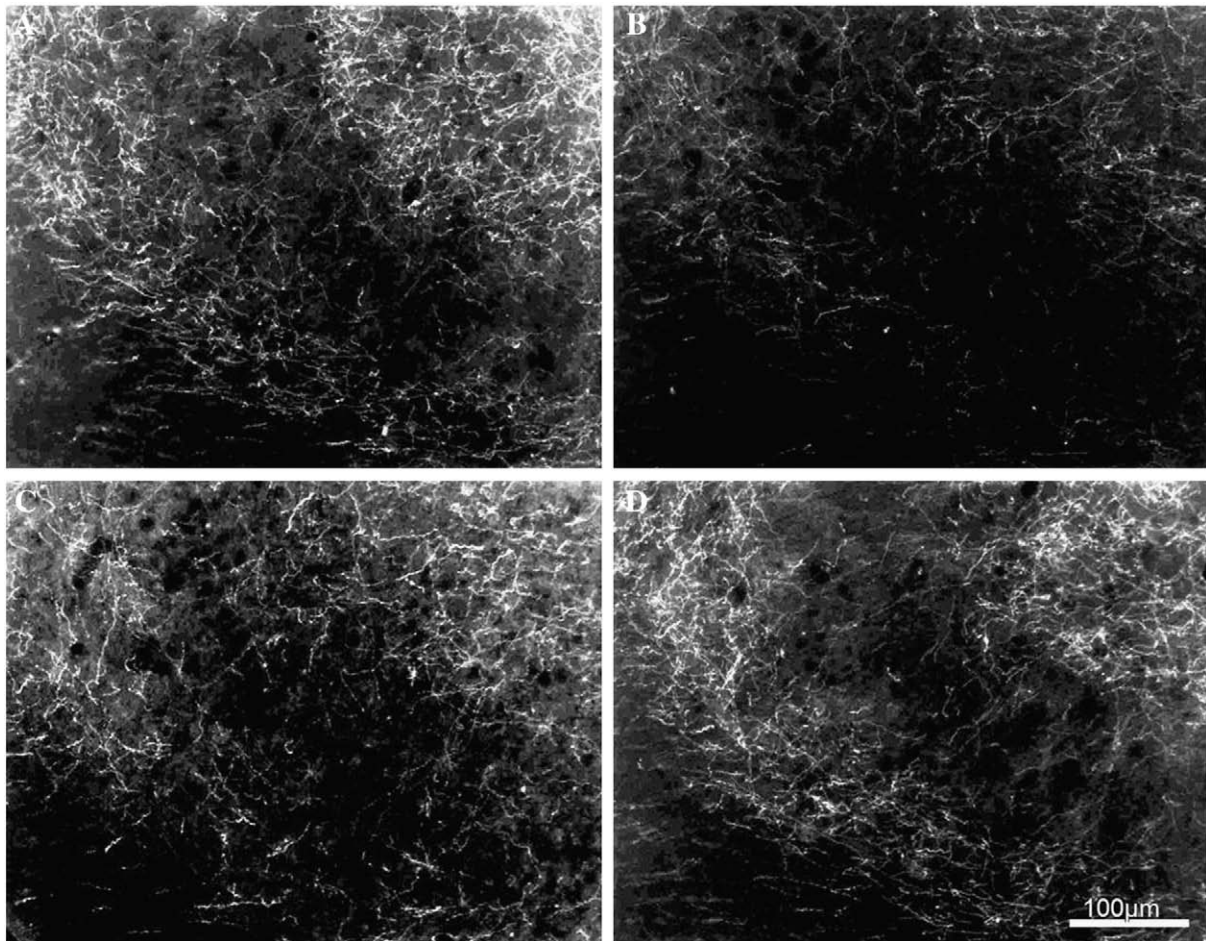
six brain regions 90 min after injection (Fig. 5). In addition, the 4- $^{18}\text{F}$ -ADAM uptake in six brain regions was markedly higher in the FLU/MDMA and FLU/SAL groups than in the SAL/MDMA group. Conceivably, fluoxetine has a higher affinity than MDMA for binding to the SERT protein and can inhibit this binding to produce acute and long-term pharmacologic responses (Berger et al., 1992; Shankaran et al., 1999). The hypothesis that SERT carries MDMA into serotonergic neurons where MDMA has neurotoxic effects (Piper et al., 2008) is thereby supported, at least in part.

Fig. 6 illustrated the long-lasting neuroprotective effect of a single dose of fluoxetine against loss of SERT in response to eight challenges of MDMA in the FLU/MDMA group. Although SURs of 4- $^{18}\text{F}$ -ADAM were lower in the FLU/MDMA group than in the control group at later time points, the dose of fluoxetine may not have been optimal, or the half-life of fluoxetine may be prolonged after co-administration of fluoxetine with MDMA. Therefore, a study design for longer intervals and different doses must be employed in subsequent studies. In addition, the comparable SURs of 4- $^{18}\text{F}$ -ADAM in the FLU/MDMA and FLU/SAL groups may have been due to the presence of fluoxetine. The fluctuation in 4- $^{18}\text{F}$ -ADAM uptake with time in six brain regions may have been due to changes in the fluoxetine or/and norfluoxetine concentrations, given that fluoxetine is demethylated by the hepatic CYP450 enzyme to norfluoxetine (a more potent and selective SERT ligand; Caccia et al., 1990; Lemberger et al., 1985; Sánchez and Hyttel, 1999). In rats, the biological half-lives of fluoxetine and norfluoxetine in plasma are approximately 5 and 15 h, respectively (Caccia et al., 1990). However, our present data showed that fluoxetine alone affected the uptake of 4- $^{18}\text{F}$ -ADAM in all brain regions from day 0 to

day 24 ( $p < 0.05$ ; Fig. 6). Although SERT binding of fluoxetine and norfluoxetine in the brain is not consistent with the plasma concentration of fluoxetine and norfluoxetine in the peripheral region, the high affinity of fluoxetine in rat brain might contribute to the long half-lives of these two active drugs in the brain (Mukherjee et al., 1998). Mukherjee et al. (1998) found that  $^{18}\text{F}$ -fluoxetine binding is markedly high in the mitochondria and synaptosomes and clears out of the cells slowly. This property may account for the unique prolonged pharmacokinetics of fluoxetine. In addition, in rats, fluoxetine and norfluoxetine accumulate in the brain for up to 7 days (Lefebvre et al., 1999; Sanchez et al., 2001). The present time course results of fluoxetine-inhibited 4- $^{18}\text{F}$ -ADAM binding were comparable with those of previous studies of  $^{11}\text{C}$ -DASB in cats from 30 min to 24 days after the same dose (1 dose, 5 mg/kg) of fluoxetine. At 17 days, 12% and 24% SERT occupancy of  $^{11}\text{C}$ -DASB binding was found *in vivo* in the thalamus and midbrain, respectively, of cats (Ginovart et al., 2003). As mentioned above, the residual action of fluoxetine and norfluoxetine may be an important factor contributing to long-lasting neuroprotection against MDMA.

The results from the PET studies were further confirmed by immunohistochemistry studies. In the control group, the degrees of staining were corresponding to the known SERT density in rat brain (Sur et al., 1996). PET studies showed that SERT concentration in the caudate putamen decreased by 50% after MDMA treatment (Fig. 6) while the immunohistochemical studies showed a more marked depletion of SERT in this region (Fig. 10B). There are three possible reasons for this discrepancy: (1) The immunohistochemical study results presented here are qualitative. In addition, the distributions



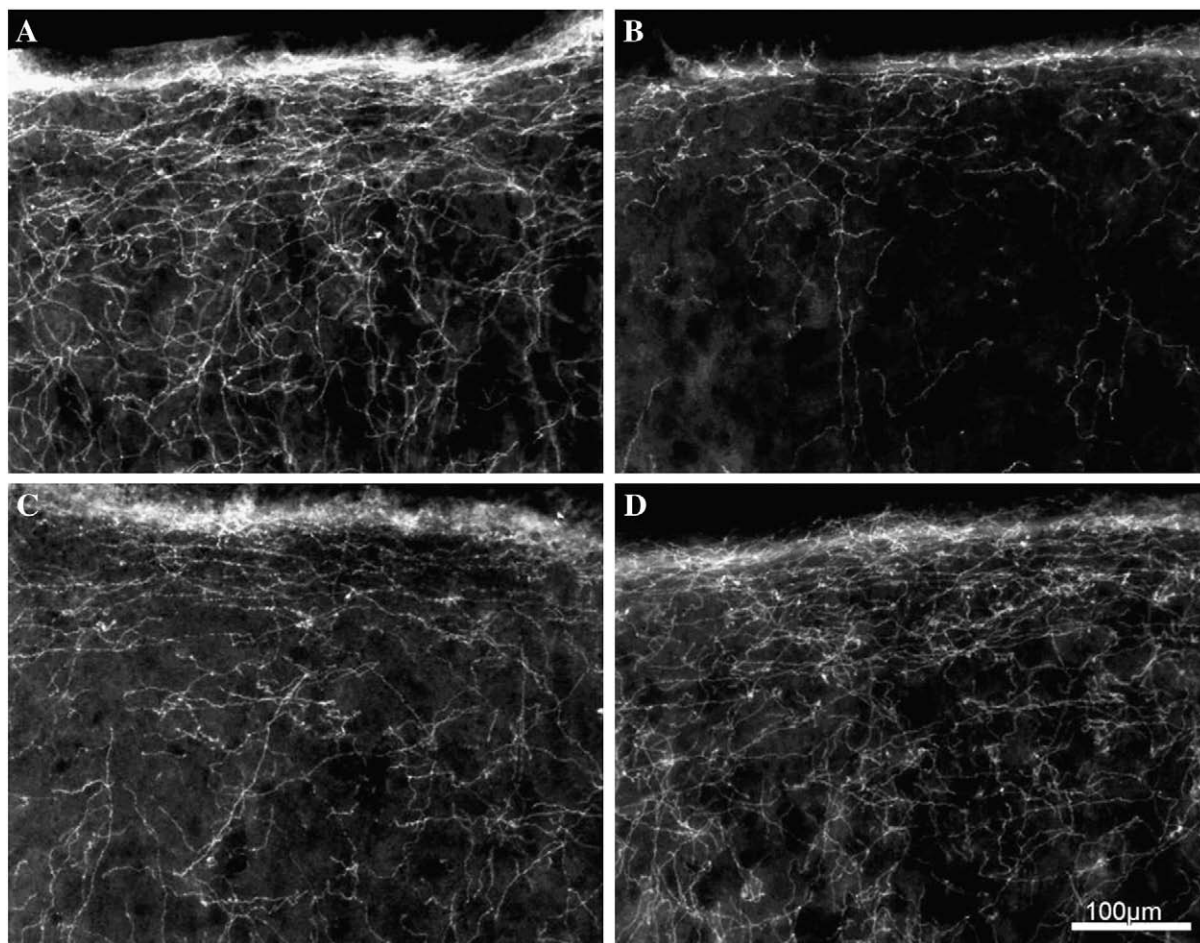


**Fig. 11.** Dark-field photomicrograph of SERT immunoreactivity in area CA3. (A) Control group, (B) SAL/MDMA group, (C) FLU/MDMA group, and (D) FLU/SAL group. The serotonergic fiber density was clearly decreased after MDMA treatment (SAL/MDMA vs. FLU/MDMA, FLU/SAL, or control group).

of SERT in the brain are heterogeneous such that different slice may contain different concentration of SERT. The slice we presented here is only one of around 20 slices, and transects only a small domain of the corresponding VOI in the micro-PET study. (2) The PET data presented here may underestimate the true extent of SERT loss because of biasing effects due to the present use of the cerebellum as a reference region, which contains a small abundance of SERT (de Win et al., 2004; Reneman et al., 2006; Szabo et al., 2002). The cerebellum is not completely devoid of SERT and may have resulted in an overestimation of SERT density and consequently an underestimation of the neurotoxic effects of MDMA (Kish et al., 2005). (3) Partial volume effect and spillover from adjacent tissue with higher uptake, which is still present even in the high resolution micro-PET.

Both *in vivo* 4-[ $^{18}\text{F}$ ]-ADAM micro-PET studies and *ex vivo* immunohistochemical studies have demonstrated that MDMA produces a global decrease in the density of rat brain SERT. Previous immunohistochemical studies have also shown that MDMA causes loss of SERT protein in the cortex (Xie et al., 2006). These results generally agree with those of clinical studies using [ $^{11}\text{C}$ ]-McN5652 and [ $^{11}\text{C}$ ]-DASB in MDMA users (McCann et al., 2005), as well as with those of animal studies using [ $^{11}\text{C}$ ]-DASB in pigs and [ $^{123}\text{I}$ ]- $\beta$ -CIT in rats (Cumming et al., 2007; Reneman et al., 2002). However, there are discrepancies in regional differences in radioligand binding changes between these studies and our present findings. McCann et al. (2005) found that global brain SERTs are reduced in MDMA users, with marked reductions in cortical regions but moderate or no reductions in subcortical regions (e.g. midbrain and caudate putamen). In a study

using [ $^{11}\text{C}$ ]-DASB PET in pig brain, Cumming et al. (2007) reported that a mean total dose of 42 mg/kg MDMA induced a 32% decrease in binding potential for SERT in the mesencephalon and diencephalon and a 53% decrease in telencephalic structures. In the present PET study, the extent of MDMA-induced SUR decreases was uniform across different brain regions. In line with our observations, previous rat studies indicated that SERT was reduced generally evenly among cortical and subcortical brain regions (Ferrington et al., 2006; Fischer et al., 1995; Reneman et al., 2002; Scanzello et al., 1993; Scheffel and Ricaurte, 1990). Such uniform decreases may be explained by changes in the reference region, the cerebellum. Such changes might be caused by an influence of MDMA on the binding of the radioligand to the cerebellar SERT. On the other hand, even small errors in the cerebellar data, i.e., by small changes in the crosstalk from neighboring regions with higher uptake onto the cerebellum would change data of the reference region. In this way all other regions may be concordantly affected. Discrepancies between these rat studies and studies of other species may be attributed to the different dosage of MDMA or to differential vulnerability of the serotonergic system to MDMA between rats and other species, or to differences in analytic methods. In addition, SURs of 4-[ $^{18}\text{F}$ ]-ADAM in the SAL/MDMA group plateaued between day 10 and day 17 in the present study, whereas other studies have suggested that MDMA may induce axonal loss after 1 week of fluctuation in 5-HT level (Linnet et al., 1995; Ramamoorthy and Blakely, 1999). On the other hand, 4-[ $^{18}\text{F}$ ]-ADAM uptake appeared to increase slowly (from day 17 to day 31) in all brain regions in the SAL/MDMA group (Fig. 6), suggesting a partial SERT recovery from MDMA injury. This finding is in accordance with those



**Fig. 12.** Dark-field photomicrograph of SERT immunoreactivity in the frontal cortex. (A) Control group, (B) SAL/MDMA group, (C) FLU/MDMA group, and (D) FLU/SAL group. The serotonergic fiber density was clearly decreased after MDMA treatment (SAL/MDMA vs. FLU/MDMA, FLU/SAL, or control group).

of a previous study demonstrating serotonergic recovery in the cortex, striatum, and hippocampus from 2 to 52 weeks after MDMA challenge (Scanzello et al., 1993). However, that study used an *ex vivo* [ $^3\text{H}$ ]-paroxetine binding assay of 5-HT uptake sites in rats.

The effects of MDMA on SERT *in vivo* have not been well documented. One of the reasons may be lack of suitable techniques for measuring SERT, i.e., suitable radioligands for imaging SERT coupled with SPECT or PET. Both [ $^{11}\text{C}$ ]-McN5652 and [ $^{11}\text{C}$ ]-DASB have been used with PET, and [ $^{123}\text{I}$ ]- $\beta$ -CIT has been used with SPECT to investigate SERT (Reneman et al., 2006). The achievement of image quality in rat brains comparable to that in human brains using small animal PET and SPECT is a challenge (Andringa et al., 2005). To our knowledge, only one relevant MDMA study in rat using [ $^{123}\text{I}$ ]- $\beta$ -CIT SPECT has been reported. In line with our present observations, that study found thalamic uptake of [ $^{123}\text{I}$ ]- $\beta$ -CIT is significantly reduced 10 days after the last MDMA treatment (de Win et al., 2004). However, because [ $^{123}\text{I}$ ]- $\beta$ -CIT binds to both SERT and dopamine transporter (DAT) proteins with high affinity, it is difficult to differentiate SERT binding from DAT binding in regions such as the striatum, where SERT and DAT coexist (Hesse et al., 2004; Laruelle et al., 1993). The use of [ $^{18}\text{F}$ ]-labeled derivatives has some advantages over [ $^{11}\text{C}$ ]-labeled radioligands, including a relatively long half-life (110 min vs. 20 min), allowing for optimization of the scanning protocol, and lower positron energy (0.635 MeV vs. 0.96 MeV), which results in a slight improvement in final resolution of the PET image (Shiue et al., 2003a). As expected, results of the present *in vivo* study showed high uptake of 4-[ $^{18}\text{F}$ ]-ADAM in SERT-rich regions, such as the midbrain, hypothalamus, thalamus, caudate

putamen, hippocampus, and frontal cortex, and sufficient micro-PET image quality to provide acceptable sensitivity and discriminative spatial resolution. However, in this study, volumes of interest were drawn manually in reference to the rat brain atlas and magnetic resonance images by an experienced anatomy specialist. Consequently, it might be a limitation in the precise drawing of subtle rat brain structures. A proper method for image fusion to ensure image registration with PET and magnetic resonance images will be required in the further study.

In conclusion, the present 4-[ $^{18}\text{F}$ ]-ADAM micro-PET study found that a single dose of fluoxetine provided long-lasting protection against MDMA-induced loss of SERT. Neuroimaging may be useful in monitoring the therapeutic responses to fluoxetine against MDMA-induced neurotoxicity. In addition, this method may provide a preclinical screening platform for identifying drugs that are potentially protective against MDMA-induced serotonergic neurotoxicity in animal models and may provide an accelerated path toward clinical trial testing for MDMA abuse.

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