

Evaluation of brain SERT occupancy by resveratrol against MDMA-induced neurobiological and behavioral changes in rats: A 4- $[^{18}\text{F}]$ -ADAM/small-animal PET study

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Abstract

The misuse of 3,4-methylenedioxymethamphetamine (MDMA) has drawn a growing concern worldwide for its psychophysiological impacts on humans. MDMA abusers are often accompanied by long-term serotonergic neurotoxicity, which is associated with reduced density of cerebral serotonin transporters (SERT) and depressive disorders. Resveratrol (RSV) is a natural polyphenolic phytoalexin that has been known for its antidepressant and neuroprotective effects. However, biological targets of RSV as well as its neuroprotective effects against MDMA

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remained largely unknown. In this study, we examined binding potency of RSV and MDMA to SERT using small-animal positron emission tomography (PET) with the SERT radioligand, N,N-dimethyl-2-(2-amino-4-[^{18}F]fluorophenylthio)benzylamine (4-[^{18}F]-ADAM) and investigated the protection of RSV against the acute and long-term adverse effects of MDMA. We found that RSV exhibit binding potentials to SERT *in vivo* in a dose-dependent manner with variation among brain regions. When the MDMA-treated rats (10 mg/kg, s.c.) were co-injected with RSV (20 mg/kg, i.p.) twice daily for 4 consecutive days, MDMA-induced acute elevation in plasma corticosterone was significantly reduced. Further, 4-[^{18}F]-ADAM PET imaging revealed that RSV protected against the MDMA-induced decrease in SERT availability in the midbrain and the thalamus 2 weeks following the co-treatment. The PET data were comparable to the observation from the forced swim test that RSV sufficiently ameliorated the depressive-like behaviors of the MDMA-treated rats. Together, these findings suggest that RSV is a potential antidepressant and may confer protection against neurobiological and behavioral changes induced by MDMA.

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1. Introduction

3,4-methylenedioxymethamphetamine (MDMA) known as ecstasy is a ring-substituted amphetamine analog which is commonly used as a recreational drug. MDMA abuse is a growing concern around the world because its short-term euphoric effects have been shown to be associated with long-term serotonergic neurotoxicity (Lanteri et al., 2014; McCann et al., 1998; Parrott, 2013). Human and animal studies suggest that MDMA may cause a long-term decrease in serotonin transporter (SERT) density and consequently promote depressive-like behaviors (Curran and Travill, 1997; Erritzoe et al., 2011; Kish et al., 2010; Li et al., 2010; McCann et al., 2008; Thompson et al., 2004). These effects of MDMA are suggested to be attributed to the alteration in monoaminergic systems, especially changes involving serotonin (5-HT), 5-HT receptors, and SERT (Battaglia et al., 1988; Han and Gu, 2006; Schmidt et al., 1987). The usage of MDMA could cause acute symptoms such as weight loss and elevation in levels of the stress hormone corticosterone, due to the suppression of appetite and the activation of hypothalamic-pituitary-adrenal (HPA) axis (Francis et al., 2011; Nash et al., 1988).

SERT is a member of the sodium/neurotransmitter symporter family that transports 5-HT from the synapse to the presynaptic neuron. This protein is also the main target of many antidepressant medications, including selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (Immadisetty et al., 2013). The involvement of SERT in the mechanism of MDMA-induced neurotoxicity has been well demonstrated (Li et al., 2010; Renoir et al., 2008; Sanchez et al., 2001; Schmidt and Taylor, 1990; Shankaran et al., 1999). MDMA can bind to SERT and enter 5-HT nerve terminal via the transporter that facilitate pre-synaptic release of 5-HT from the storage vesicles (Partilla et al., 2006). This acute increase in 5-HT levels in the nerve terminal triggers a rapid accumulation of hydrogen peroxide, a by-product of 5-HT metabolism by monoamine oxidase B (MAO-B), which is converted into hydroxyl radical to induce oxidative stress in mitochondria of serotonergic neurons (Alves et al., 2007). Oxidative damage to the mitochondria can initiate the intracellular cascade leading to neurotoxicity.

One potential therapeutic mechanism by which a SSRI inhibits MDMA-induced serotonergic neurotoxicity is preventing the entry of MDMA and its free radical-generating reactive metabolites into serotonergic nerve terminals (Capela et al., 2009).

Positron emission tomography (PET) in conjunction with ^{11}C -labeled radioligands that bind to SERT has been used to detect serotonergic neurotoxicity induced by MDMA *in vivo* (Buchert et al., 2007; Cumming et al., 2007; McCann et al., 2005; Urban et al., 2012). In addition to ^{11}C -labeled radioligands (e.g. [^{11}C]-(+)-McN5652 and [^{11}C]-DASB), our group has recently developed a ^{18}F -labeled ligand, N,N-dimethyl-2-(2-amino-4-[^{18}F]fluorophenylthio)benzylamine (4-[^{18}F]-ADAM), which has specific binding to SERT and has longer half-lives than ^{11}C -labeled ligands. 4-[^{18}F]-ADAM has been used to visualize SERT in living brain of rats, monkeys, and humans (Chen et al., 2012; Huang et al., 2013; Ma et al., 2009; Yeh et al., 2015). Our group has also demonstrated that, in the use of small-animal PET and 4-[^{18}F]-ADAM, a single dose of fluoxetine (a SSRI) provides long-lasting protection against MDMA-induced loss of SERT of the rat brain (Li et al., 2010).

Resveratrol (3,5,4'-trihydroxy-trans-stilbene; RSV) is a natural polyphenolic phytoalexin which is well known for its antioxidant, anti-apoptotic, anti-inflammatory, anti-aging, and anti-angiogenic properties (Li et al., 2014; Lin et al., 2014). It has been shown that RSV has an *ex-vivo* inhibitory effect on the uptake of [^3H]5-Hydroxytryptamine ([^3H]-5-HT) by synaptosomes from rat brain (Yanez et al., 2006), suggesting a probable binding affinity between RSV and SERT. However, it is still unknown whether RSV can exert neuroprotection against MDMA, possibly via blocking the entry of MDMA into 5-HT neuronal terminals.

In light of these findings, we hypothesized that RSV may have the potential to protect against MDMA-induced neurotoxicity, possibly through competing binding sites of SERT with MDMA. To test this, binding relationship between RSV and SERT was first evaluated by measuring SERT occupancy by RSV using the PET scans. We then tested whether the RSV treatment is effective against MDMA-induced acute changes in body weight and plasma corticosterone levels as well as long-term changes in cerebral SERT availability and depressive-like behaviors from the rats.

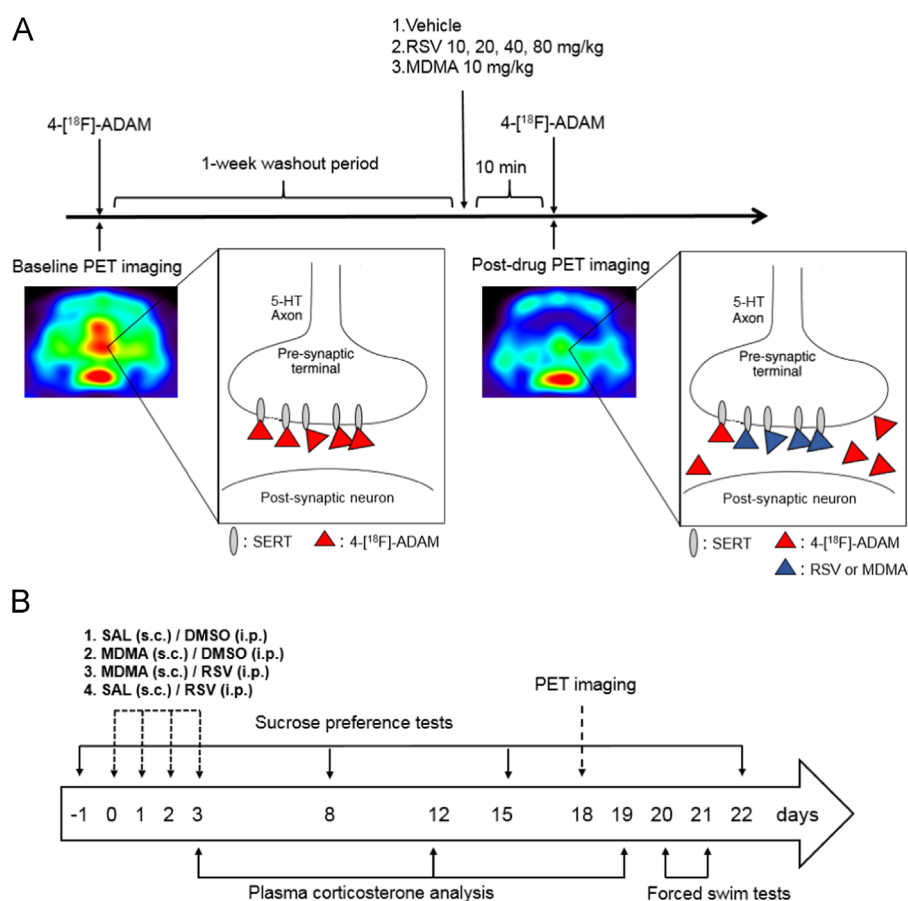


Figure 1 (A) Design of SERT occupancy using 4-[¹⁸F]-ADAM PET imaging to evaluate test drugs including RSV and MDMA. (B) The protocol for Experiment 2. Rats received subcutaneous injections of SAL or MDMA and intraperitoneal injections of DMSO or RSV twice daily on days 0, 1, 2, and 3. Sucrose preference was evaluated on days -1 (baseline), 8, 15, and 22. PET imaging with was performed on day 18. Plasma corticosterone levels were measured on days 3, 12, and 19. Forced swim tests were evaluated on days 20 (pretest) and 21.

2. Experimental procedures

2.1. Reagents

MDMA (purity, 98%) was obtained from the Investigation Bureau of Taiwan. RSV and (2-hydroxypropyl)- β -cyclodextrin (average Mw \sim 1460) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Corticosterone was purchased from Sigma-Aldrich (St. Louis, MO, USA) and corticosterone-d8 as internal standard was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). HPLC grade acetonitrile and methanol were obtained from Tedia Company, Inc. (Fairfield, OH, USA) and Merck KGaA (Darmstadt, Germany), respectively. HPLC grade glacial acetic acid was from JT Baker (Phillipsburg, NJ, USA). All the other chemicals were of analytical grade and commercially available. Water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Animals

Eight-week old male Sprague-Dawley (SD) rats (250–300 g, BioLASCO Taiwan Co., Ltd., Taipei, Taiwan) were housed two per cage in a 12-h light/dark temperature-controlled

environment with free access to water and food. They were allowed to acclimatize to the environment for 5 days before any experiment. All experimental procedures were in compliance with the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan, R.O.C.

2.3. Radiopharmaceuticals and small-animal PET imaging

Small-animal PET imaging was conducted at the National Defense Medical Center Laboratory Animal Center. The radioligand 4-[¹⁸F]-ADAM (purity, $>95\%$) was synthesized in an automated synthesis module (Peng et al., 2008) and obtained from Department of Nuclear Medicine at Tri-Service General Hospital (Taipei, Taiwan). The rats were first anesthetized by passive inhalation of a mixture of isoflurane/oxygen (5% isoflurane for induction, and 2% for maintenance). After deep anesthesia was confirmed, 4-[¹⁸F]-ADAM (18.5–22.2 MBq; 0.5–0.6 mCi) was injected into the animal via the tail vein. 60 min after 4-[¹⁸F]-ADAM injection, the PET images were acquired by BIOPET 105 imager (Bioscan, Inc., Washington, DC, USA) for 30 min, with the energy window set at 250–700 keV. Image

acquisition procedure was similar to those of the previous reports (Li et al., 2010; Ma et al., 2009). Three-dimensional ordered subsets expectation maximization was employed to reconstruct the images. Imaging data were analyzed by open-source AMIDE software version 1.0.4. A volume of interest (VOI) was defined for each brain region that was of particular interest. These brain regions include midbrain, amygdala, hypothalamus, thalamus, striatum, and frontal cortex. To minimize inconsistencies in VOI placement among the animals, the MR image was obtained from a typical SD rat brain and fused manually with 6 reconstructed 4-[¹⁸F]-ADAM PET images of normal SD rats to draw VOIs according to a rat brain atlas. These VOIs and the typical MR image were saved as a template for further analysis. The 4-[¹⁸F]-ADAM image of each individual animal was co-registered manually to the MR template image with VOIs using AMIDE software for measuring the activities in a number of brain regions. As SERT availability in a specific brain region correlates to 4-[¹⁸F]-ADAM uptake in a VOI, the specific uptake ratio (SUR) of 4-[¹⁸F]-ADAM in each brain region was analyzed as: (region VOI uptake-cerebellum VOI uptake) / cerebellum VOI uptake (Ma et al., 2009). The cerebellum was chosen as the reference tissue for its negligible SERT density (Lin et al., 2004) that generates individual-specific background 4-[¹⁸F]-ADAM uptakes.

2.4. Experiment 1: SERT occupancy by RSV or MDMA measured using 4-[¹⁸F]-ADAM PET

Experiment 1 was conducted, using 4-[¹⁸F]-ADAM PET imaging, to measure SERT occupancy [O (%)] by RSV and MDMA, as a method to gauge *in-vivo* SERT binding of RSV and MDMA. The experimental design is schematically shown in Figure 1A. Baseline 4-[¹⁸F]-ADAM PET scans were done when the animals were free from any drug treatment. 1 week after the baseline scans, we performed post-drug scans, which were initiated 10 minutes after the drug injection. Eighteen rats were randomly assigned to 6 treatment groups that were injected intravenously (i.v.) with either vehicle (20% (2-hydroxypropyl)- β -cyclodextrin, average Mw ~1460) (Marier et al., 2002) or RSV (10, 20, 40, 80 mg/kg), or subcutaneously (s.c.) with MDMA (10 mg/kg). SERT

occupancy represents the reduction of 4-[¹⁸F]-ADAM binding from the baseline when the animal was under a given dose of vehicle, RSV or MDMA and was calculated using the formula: $O (\%) = 100 \times (SUR_{baseline} - SUR_{post-drug}) / SUR_{baseline}$. This equation was modified from: $O (\%) = 100 \times (BP_{baseline} - BP_{post-drug}) / BP_{baseline}$, where BP is the binding potential of the transporter and its ligand (Passchier et al., 2002). This transformation is in accordance with the simplified reference tissue model (Zhang and Fox, 2012).

Occupancy values by different RSV doses were plotted and the dose-response curves were generated in a region-specific manner (Supplemental Figure 1). ED₅₀ (drug dose for 50% of maximum occupancy of SERT) values of RSV in the selected regions were generated based upon the sigmoidal Emax model. The ED₅₀ in the midbrain and the amygdala cannot be generated because these two dose-response curves did not fit the sigmoidal Emax function. Average occupancy by a given dose of the test drug was the average of the regional occupancy values weighted by the size of the VOI.

2.5. Experiment 2: Acute and long-term effects of MDMA in the presence of RSV

MDMA was dissolved in saline (SAL, 0.9% NaCl) for subcutaneous injection. RSV was prepared in dimethyl sulfoxide (DMSO) for intraperitoneal injection. Twenty four rats were randomly assigned to 4 groups. The SAL/DMSO group received 1 mL/kg of SAL (s.c.) and 1 mL/kg DMSO (i.p.) vehicles and served as a control; the MDMA/DMSO group received MDMA (10 mg/kg, s.c.) and DMSO (1 mL/kg, i.p.); the MDMA/RSV group received MDMA (10 mg/kg, s.c.) and RSV (20 mg/kg, i.p.); and the SAL/RSV group received saline (1 mL/kg, s.c.) and RSV (20 mg/kg, i.p.). All drugs were administered twice daily for 4 consecutive days. This administration schedule for MDMA has been shown in the previous experiments to produce persistent alterations in serotonergic function (Li et al., 2010), and the dose of RSV used in this study both based on the similar ED₅₀ values in three regions including thalamus, striatum and frontal cortex in Experiment 1 (Supplemental Figure 1) and followed the study described previously for serotonergic

Table 1 SERT occupancy (%) by RSV and MDMA.

Region	Vehicle ^a	RSV (i.v.)					MDMA (s.c.)
		10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	ED ₅₀ (mg/kg)	
Midbrain	0.6 ± 4.1	5.4 ± 4.9	13.0 ± 10.5	12.9 ± 6.5	40.5 ± 0.9	NC	90.1 ± 2.8
Amygdala	-8.9 ± 9.5	3.1 ± 12.2	14.4 ± 13.8	13.1 ± 6.0	23.5 ± 6.3	NC	78.9 ± 5.5
Hypothalamus	7.6 ± 4.0	4.1 ± 12.0	9.9 ± 11.1	20.1 ± 9.0	28.9 ± 2.2	34.8	88.3 ± 0.7
Thalamus	7.9 ± 5.5	-5.4 ± 12.0	14.8 ± 11.7	31.8 ± 7.8	27.0 ± 7.8	19.7	81.6 ± 3.8
Striatum	-4.1 ± 8.5	1.4 ± 6.5	3.9 ± 10.5	35.4 ± 12.1	21.2 ± 14.7	21.5	80.3 ± 3.7
Frontal cortex	-4.7 ± 11.0	6.6 ± 7.7	11.2 ± 10.9	19.2 ± 3.5	13.3 ± 9.6	20.1	70.0 ± 2.6
Average ^b	0.7 ± 2.7	3.4 ± .3	11.2 ± 10.6	22.9 ± 6.7	29.4 ± 3.1	26.5	83.2 ± 1.3

^a(2-Hydroxypropyl)- β -cyclodextrin was used as vehicle. SERT occupancy was calculated as follows: occupancy (%) = $100 \times (SUR_{baseline} - SUR_{post-drug}) / SUR_{baseline}$.

^bAverage occupancy by a given dose of the test drug was the average of the regional occupancy values weighted by the size of the VOI. NC stands for Not Calculated because the least-square curve did not fit the sigmoidal Emax function.

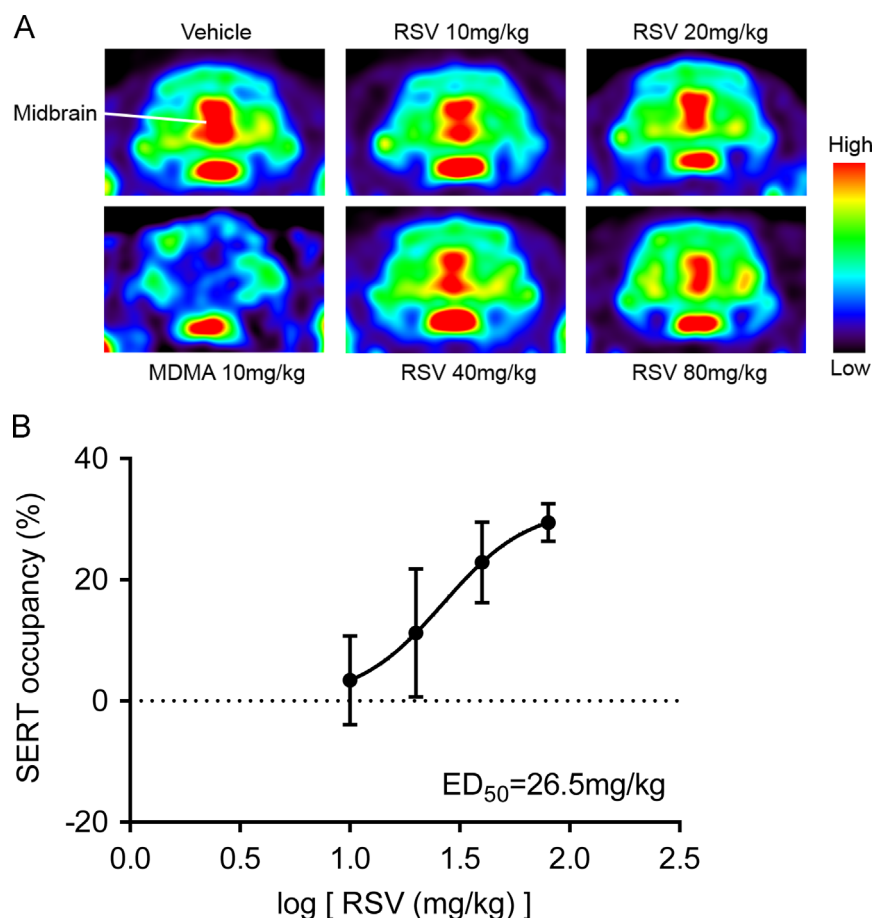


Figure 2 RSV inhibited 4- $[^{18}\text{F}]$ -ADAM binding to the target sites (SERT). (A) 4- $[^{18}\text{F}]$ -ADAM distribution in the transverse section of rat midbrain after intravenous administration of RSV at different doses (vehicle, 10, 20, 40, 80 mg/kg) compared to subcutaneous MDMA at 10 mg/kg. RSV produced significant, dose-dependent inhibition of 4- $[^{18}\text{F}]$ -ADAM binding to SERT. (B) Relationship between i.v. dose of RSV and average SERT occupancy was plotted for calculating ED_{50} . Closed circles represent mean value with the errors ($n=3$). Solid line represents least-square curve fit using sigmoidal Emax function. ED_{50} , drug dose for 50% of maximum occupancy of SERT.

activation and antidepressant-like effects (Xu et al., 2010). The schedule for Experiment 2 is shown in Figure 1B. Body weight and plasma corticosterone levels were measured to assess the acute effects of MDMA. In order to confirm changes in body weight that were not influenced by anhedonia, the sucrose preference test was also monitored. To evaluate the long-term effects of MDMA, SERT imaging and performance in the forced swim test were measured 2 weeks after drug treatment. Because the half-life of RSV ($t_{1/2}$, 0.1–2.0 h) and MDMA ($t_{1/2}$, 0.9–2.0 h) is much shorter than 24 h in the rats (Concheiro et al., 2014; Liang et al., 2013; Marier et al., 2002), we would not expect residual RSV or MDMA, if there was any, could bind competitively against 4- $[^{18}\text{F}]$ -ADAM at SERT binding sites 2 weeks after the treatment. Therefore, the SUR of 4- $[^{18}\text{F}]$ -ADAM in the brain regions can be regarded as a surrogate indicator of the SERT availability.

2.6. Blood sampling procedures

Blood samples were collected from the rats in the evening (6–8 p.m.) on days 3 (one hour after the last treatment), 12 and 19 for analyzing plasma corticosterone levels (Figure 1B). The animals were moved to the testing room

1 h before blood sampling to prevent arousal effects from a changed environment and then anesthetized with passive inhalation of a mixture of isoflurane/oxygen to reduce stress. 200 μL blood of each rat was collected via the tail vein into 1-mL insulin syringes, transferred to 1.5-mL plastic tubes on ice, and spun for 5 min at 13000 rpm; plasma was decanted and stored at -80°C until assay.

2.7. Plasma corticosterone quantified using UPLC-tandem mass

All rat plasma samples were thawed at room temperature before analysis. 200 μL of acetonitrile was added to 50- μL aliquot of plasma and mixed thoroughly for 3 min to deproteinate the plasma. 10 μL of internal standard solution was added in each sample. After 30 s of vortex mixing, all the samples were centrifuged at 13,000 rpm for 5 min at 4°C . The supernatants were evaporated to dryness under a nitrogen stream and reconstituted with 100 μL 50% methanol. The reconstituted solutions were transferred to the autosampler vials, and 10 μL were injected into an ultrahigh performance liquid chromatography (UPLC) tandem mass system. A Waters Acquity UPLC (Waters, Milford, MA, USA)

coupled to an Applied Biosystems API 3000 (Foster City, CA, USA) triple-quadrupole mass spectrometer was used for plasma corticosterone analysis. The ionization was conducted using an electrospray ionization interface in positive mode, and analytes were quantified using multiple reaction monitoring mode. The MS/MS ion transitions monitored were m/z 355.4 \rightarrow 337.4 for corticosterone-d8 and 347.3 \rightarrow 329.3 for corticosterone. The dwell time of each ion was set at 300 ms. Ion source temperature was maintained at 450 °C, and ion spray voltage was 5500 V. High purity nitrogen gas was used as the collision-induced dissociation gas (12 psi), curtain gas (13 psi), and nebulizer gas (10 psi). Chromatographic separation was performed on an Acquity UPLC BEH C18 column (100 mm length \times 2.1 mm inner diameter; 1.7 μ m particle size; Waters, Milford, MA, USA) maintained at 35 °C. A gradient elution scheme was employed using 2 mobile phases: A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile). The flow rate was set at 0.3 mL/min. In the gradient program the pump was ramped from 10% phase B to 90% phase B in 3.5 min, and then back to 10% phase B in 0.5 minutes, where it remained for 2 min. Data processing was performed using Analyst 1.4.1 software package.

2.8. Sucrose preference test

The sucrose preference test was employed as a measure of anhedonia. Rats were habituated to drinking a 2% sucrose solution by replacing normal water with sucrose solution for 48 h, during which they had free access to food. On the following day, baseline sucrose preference was measured. In this procedure, all rats were deprived of food and water for 14 h, starting at the beginning of the dark phase of the light/dark cycle. After the 14-h deprivation period, rats were given free access for one hour to two drinking bottles, containing either water or 2% sucrose, placed side-by-side at the rear of the cage. Sucrose preference was calculated using the following formula: sucrose preference = sucrose solution intake (g) / total fluid intake (g).

2.9. Forced swim test

The forced swim test employed was similar to the paradigm described previously (Porsolt et al., 1978). Briefly, rats were exposed to a 15-min pretest 24 h prior to the 6-min swim test. The pretest facilitates the development of immobility during the test session and increases the sensitivity for detecting antidepressant behavioral effects. Rats were placed into a glass cylinder (height: 46 cm; diameter: 20 cm) filled to a depth of 30 cm with water at room temperature. A rat was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only small movements necessary to keep its head above the water. The duration of observed immobility was videotaped for scoring later during the last 4 min of the testing period. Following the swimming session, the rats were removed from the cylinders, dried with paper towels, placed in heated cages for 15 min, and then returned to their home cages.

2.10. Statistical analysis

Results were expressed as the mean \pm standard error of the mean (SEM). Data were tested for normality using the Shapiro-Wilk test. Group differences in the sucrose preference test were analyzed using the Kruskal-Wallis test. Spearman's rank correlations were used to determine the degree of association between regional SERT availabilities and behaviors in the forced swim test (e.g., immobility, swimming and climbing). Other data were analyzed statistically using one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA, followed by *post hoc* Bonferroni test honestly significant difference in cases with homogeneity of variance and the Games-Howell test in cases without assuming homoscedasticity. The chance of a type I error (α) was set at 0.05 using 2-tailed tests of significance.

3. Results

3.1. RSV binding to SERT assessed using small-animal PET with 4-[¹⁸F]-ADAM

The goal of the first experiment was to evaluate binding between SERT and RSV. We performed PET imaging with 4-[¹⁸F]-ADAM to measure SERT occupancy by RSV, which can be used to gauge *in-vivo* binding between RSV and SERT (see experimental procedures 2.4). Regional and average occupancy by RSV (with 4 different doses) reveal a dose-dependent relationship (Table 1). The SERT occupancy values of RSV at different doses were found in the midbrain (5.4-40.5%), striatum (1.4-35.4%), thalamus (-5.4-31.8%), hypothalamus (4.1-28.9%), amygdala (3.1-23.5%), frontal cortex (6.6-19.2%), and the weighted average of all regions examined for different doses were from 3.4 to 29.4%. Examples of the distribution of 4-[¹⁸F]-ADAM binding in the rat midbrain after different doses of RSV are shown in Figure 2A. ED₅₀ of RSV in the thalamus, hypothalamus, striatum and frontal cortex range from 19.7 to 34.8 mg/kg (Supplemental Figure 1), which give an average ED₅₀ about 26.5 mg/kg (Figure 2B). Difference in ED₅₀ among the brain regions may reflect variability in serotonin innervation of the brain. Occupancy patterns induced by chemicals with minute BP (vehicle) and strong BP (MDMA) to SERT can be seen as the references for RSV binding. Our data showed that MDMA at 10 mg/kg, s.c. was capable of occupying an average of 83.2% of available SERT of the rat's brain. In contrast, even at 80 mg/kg i.v., RSV may occupy no more than 30% of available SERT, suggesting that RSV may only bind partially to SERT.

3.2. RSV ameliorates weight loss induced by MDMA during post-treatment

The effects of MDMA and RSV on body weight are shown in Figure 3A. During drug treatment, two-way repeated measures ANOVA revealed a significant main effect of drug treatment ($F_{(3, 15)}=3.852$, $p=0.032$) and drug by day interaction ($F_{(3, 15)}=6.528$, $p=0.005$), but no significant effect of day ($F_{(1, 5)}=1.438$, $p=0.284$). Since drug by day interaction was significant, the simple main effect on day 4 was further analyzed by a one-way ANOVA to compare the difference of body weight among the 4 groups. This indicated a significant

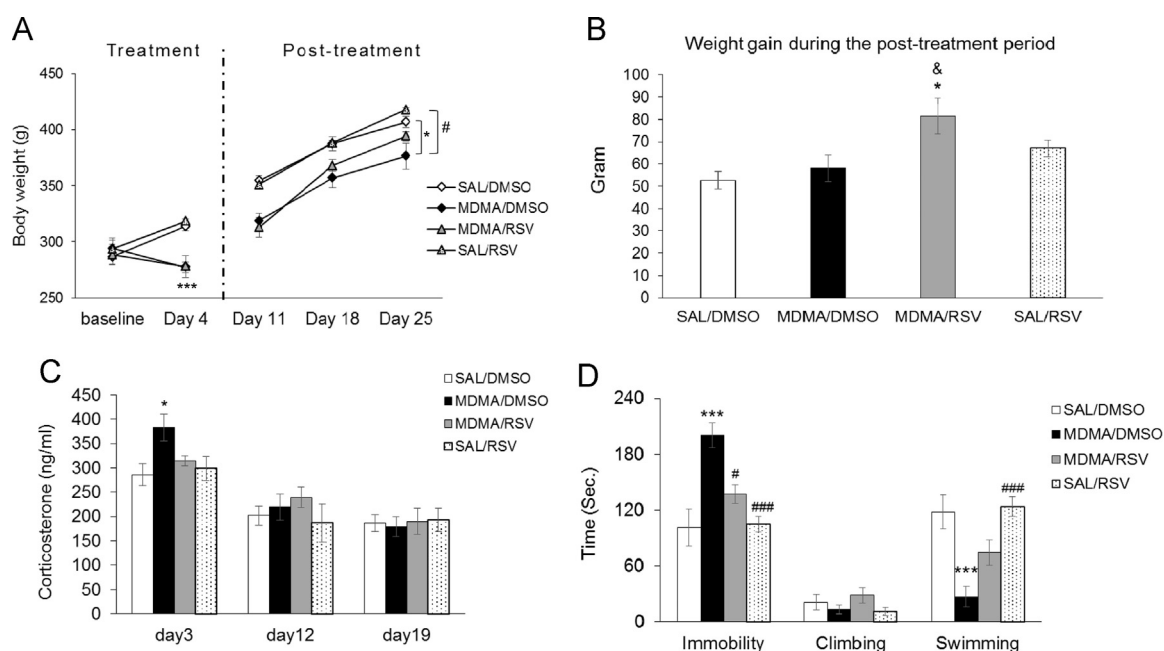


Figure 3 Effects of RSV on body weight, plasma corticosterone levels and forced swim tests. (A) During the 4 days of treatment, RSV had a neutral effect on body weight, and MDMA significantly inhibited weight gain. (B) Body weight of MDMA/RSV rats increased significantly compared to SAL/DMSO rats during the post-treatment period. (C) RSV inhibits MDMA-induced elevation of plasma corticosterone levels. Plasma corticosterone levels (ng/mL) were measured in the evening (6–8 p.m.) on days 3 (after the last treatment), 12 and 19. (D) RSV reduces immobility and increases swimming in MDMA-treated rats in forced swim tests. Forced swim tests were evaluated on days 20 (pretest) and 21. Data represent the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus SAL/DMSO. $p < 0.05$, $p < 0.01$ and $p < 0.001$ versus MDMA/DMSO. $^{\&}p = 0.059$ (borderline significant) versus MDMA/DMSO.

difference between the means of the 4 groups on day 4 ($F_{(3, 20)} = 14.134$, $p < 0.001$). The *post hoc* comparisons revealed a significant decrease in body weight for rats in the MDMA/DMSO group relative to SAL/DMSO rats ($p < 0.001$). After treatment withdrawal, two-way repeated measures ANOVA showed a significant overall effect of drug treatment ($F_{(3, 15)} = 11.702$, $p < 0.001$) and day ($F_{(2, 10)} = 319.353$, $p < 0.001$), but no significant drug by day interaction ($F_{(6, 30)} = 2.276$, $p = 0.063$). If we further analyzed weight gain from days 11 to 25 between groups (Figure 3B), one-way ANOVA indicated a significant difference between the means of the 4 groups ($F_{(3, 20)} = 4.697$, $p = 0.012$). Bonferroni *post hoc* comparisons revealed a significant increase in body weight for MDMA/RSV rats relative to SAL/DMSO rats ($p < 0.05$) and a borderline significance for MDMA/RSV rats relative to MDMA/DMSO rats ($p = 0.059$). There were no statistical differences among MDMA/DMSO, SAL/RSV, and SAL/DMSO rats.

Because anhedonia involving brain dopamine has been postulated to play a critical role in incentive motivation associated with food and water (Wise, 2008), we evaluated the sucrose preference test (Supplemental Figure 2). From baseline to day 22, the Kruskal-Wallis test showed that no difference between groups was identified.

3.3. RSV inhibits MDMA-induced elevation of plasma corticosterone levels

To evaluate both acute and withdrawal effects of RSV on MDMA-induced activation of the HPA axis, plasma corticosterone levels were measured on days 3, 12 and 19 (Figure 1B). One-way ANOVA indicated a significant difference between the means of

the 4 groups on day 3 ($F_{(3, 20)} = 3.718$, $p = 0.028$) (Figure 3C). Bonferroni *post hoc* comparisons revealed a significant increase in post-drug plasma corticosterone only for MDMA/DMSO rats relative to SAL/DMSO rats ($p < 0.05$), and there were no statistical differences among MDMA/RSV, SAL/RSV, and SAL/DMSO rats on day 3. In addition, no significant differences were observed between groups on days 12 and 19.

3.4. RSV reduces immobility and increases swimming in MDMA-treated rats

We carried out the forced swim test to explore the effect of RSV on MDMA-evoked long-term depressive behaviors (Figure 3D). There were significant overall group effects for immobility ($F_{(3, 20)} = 10.936$, $p < 0.001$) and swimming ($F_{(3, 20)} = 10.993$, $p < 0.001$), but not climbing ($F_{(3, 20)} = 1.385$, $p = 0.276$). Bonferroni *post hoc* analysis showed that rats in the MDMA/DMSO group displayed greater immobility and less swimming than those in the SAL/DMSO and SAL/RSV groups ($p < 0.001$). Although MDMA/RSV rats showed greater immobility and less swimming than SAL/DMSO or SAL/RSV rats, there were no statistically significant differences. No differences in climbing time were observed between groups.

3.5. RSV protects rats against MDMA-induced loss of SERT and depressive-like behaviors

Previous studies demonstrated that MDMA could produce long-lasting changes in the density of SERT (Li et al., 2010).

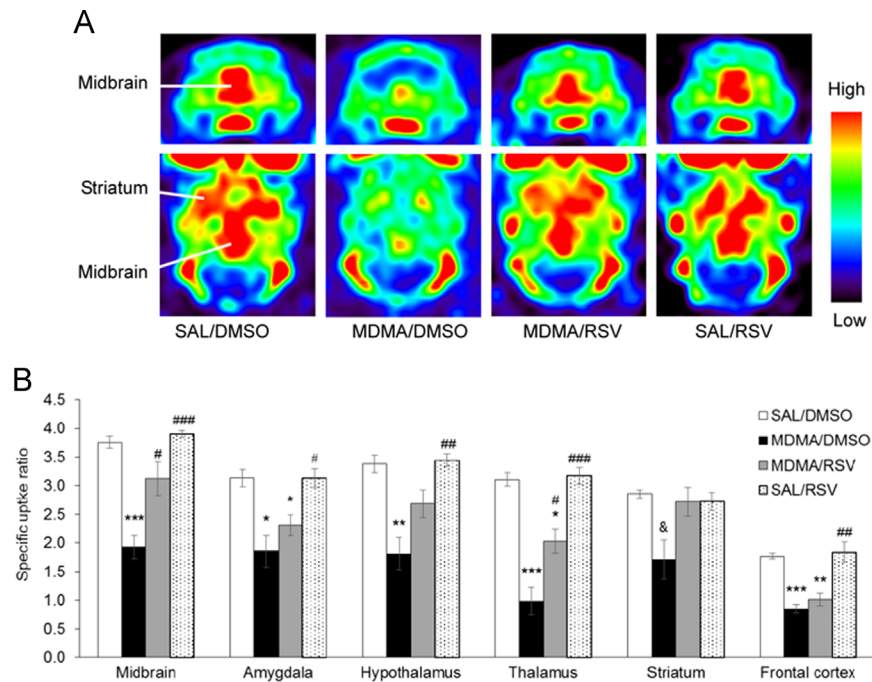


Figure 4 RSV protected the rats against the MDMA-induced decrease in SERT availability. (A) Example of the 4- $[^{18}\text{F}]$ -ADAM/small-animal PET image showing transverse and coronal sections of the rat brain was acquired on day18. (B) SUR values of 4- $[^{18}\text{F}]$ -ADAM in multiple brain regions ($n=6$ per group). Data represent the mean \pm SEM. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ versus SAL/DMSO. $p<0.05$, $p<0.01$ and $p<0.001$ versus MDMA/DMSO. $^{\alpha}p=0.069$ (borderline significant) versus SAL/DMSO.

Table 2 Correlation matrix of the forced swim test and regional SERT availabilities.

	Forced swim test			SERT availabilities					
	Immobility	Swimming	Climbing	Midbrain	Hypothalamus	Thalamus	Amygdala	Striatum	Frontal cortex
Forced swim test									
Immobility	1								
Swimming	-0.72***	1							
Climbing	-0.24	-0.10	1						
SERT availabilities									
Midbrain	-0.82***	0.60**	0.09	1					
Hypothalamus	-0.87***	0.69***	0.15	0.86***	1				
Thalamus	-0.75***	0.73***	-0.05	0.78***	0.81***	1			
Amygdala	-0.69***	0.52**	0.07	0.80***	0.87***	0.78***	1		
Striatum	-0.42*	0.25	0.12	0.64***	0.66***	0.66***	0.68***	1	
Frontal cortex	-0.69***	0.62**	-0.02	0.74***	0.81***	0.87***	0.87***	0.60**	1

* $p<0.05$.

** $p<0.01$.

*** $p<0.001$.

We therefore would like to determine whether RSV is a potential drug that helps to protect against MDMA-induced SERT loss, which can be revealed using small-animal PET with 4- $[^{18}\text{F}]$ -ADAM. The PET images of MDMA/DMSO treated rats showed significantly reduced 4- $[^{18}\text{F}]$ -ADAM uptakes in all the brain regions examined (except the striatum at borderline significance) compared to the SAL/DMSO group (Figure 4). The extent of 4- $[^{18}\text{F}]$ -ADAM uptake in the SAL/RSV group was similar to that in the SAL/DMSO group. In the

MDMA/RSV group, the uptake of 4- $[^{18}\text{F}]$ -ADAM in the regions examined was moderately higher than that in the MDMA/DMSO group (Figure 4A). The SUR values of 4- $[^{18}\text{F}]$ -ADAM in the rat brain were further calculated for quantification (Figure 4B). In the MDMA/DMSO group, the average SUR values for SERT in the brain regions examined were significantly reduced, by 40-69%, relative to those in the SAL/DMSO group. In addition, the SUR values of the MDMA/RSV group were markedly higher (approximately 37-52% higher)

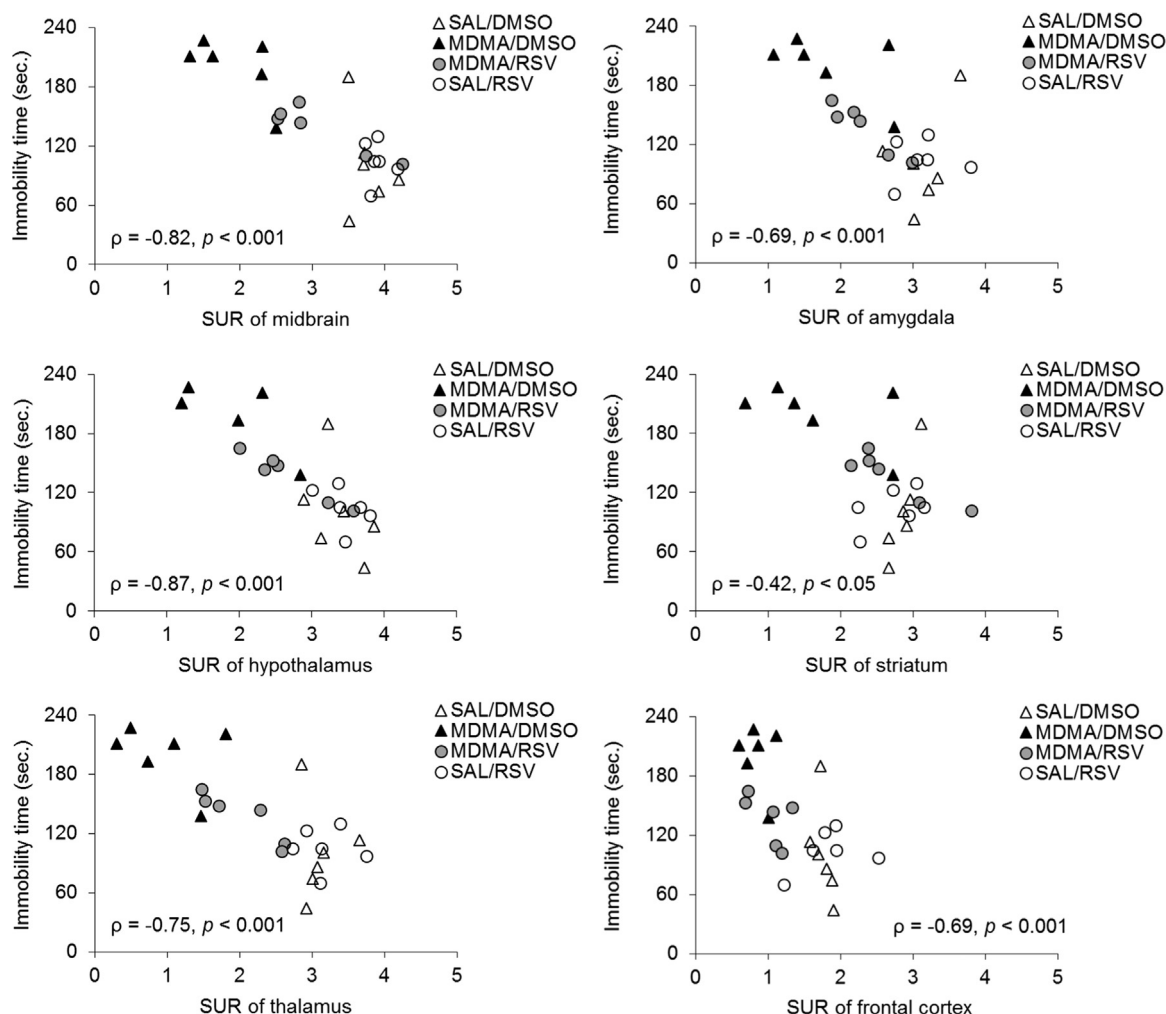


Figure 5 Scatter plots of SUR values of brain regions examined plotted against immobility times. Spearman's rank correlations were used to determine the degree of association between regional SERT availability in experiment 2 and immobility time in the forced swim test. ρ represents the Spearman correlation coefficient.

than those in the MDMA/DMSO group, especially in midbrain and thalamus ($p < 0.05$).

Spearman's rank correlations were evaluated to determine the degree of relationship between regional SERT availabilities, immobility times, swimming times, and climbing times (Table 2). Immobility time was highly negatively related to SERT availabilities in midbrain, hypothalamus, and thalamus ($\rho = -0.75 \sim -0.87, p < 0.001$). Swimming time was moderately positively related to SERT availabilities in midbrain, hypothalamus, thalamus, amygdala and frontal cortex ($\rho = 0.52 \sim 0.73, p < 0.01$). However, no significant correlation was uncovered between climbing time and SERT availability in any region. The correlations for SERT availabilities between the brain regions examined were moderate to high ($\rho = 0.60 \sim 0.87, p < 0.01$). Scatter plots of SUR of brain regions examined plotted against immobility times (Figure 5).

4. Discussion

Using small-animal PET with 4- ^{18}F -ADAM, SERT was found to be a potential biological target of RSV with direct

interaction, indicating a possible role of RSV in interfering uptake of MDMA into serotonergic nerve terminals and in reducing MDMA neurotoxicity. In this study, we found that RSV ameliorated MDMA-induced neurobiological and behavioral effects including HPA axis activation, reductions in SERT availability, and depressive-like behaviors.

Previous *ex-vivo* studies have demonstrated the inhibitory effects of MDMA and RSV on [^3H]5-HT uptake by synaptosomes from rat brain. IC_{50} values for the inhibition of [^3H]5-HT uptake by MDMA and RSV in synaptosomes are $0.4 \sim 1.7 \mu\text{M}$ and $32.5 \sim 51.6 \mu\text{M}$, respectively, indicating that MDMA is at least 19-fold more potent than RSV (Johnson et al., 1991; Steele et al., 1987; Yanez et al., 2006). However, the *ex-vivo* uptake process of [^3H]5-HT by synaptosomes fails to distinguish between SERT transporting and diffusion. In the present study, by contrast, we were able to collect *in-vivo* data of SERT occupancy of the brain, which was subsequently used to calculate SERT binding potency under different pharmacological scenarios. In addition, our data indicated that the average potency of MDMA (at 10 mg/kg s.c.) is 24-fold greater than that of RSV (at 10 mg/kg i.v.) (Table 1) and this is at large in agreement with the previous *ex-vivo* results.

The low binding potency of RSV may be explained by the fact that the concentrations of RSV and its metabolites are lower in the rat brain due to rapid first-pass metabolism, in which 40% of the parent compound is metabolized to glucuronide and sulfate conjugates in the first minute after intravenous administration and only 12% of the parent compound is detectable 20 minutes later (Juan et al., 2010; Wenzel and Somoza, 2005). Moreover, 87% of intravenous RSV is rapidly eliminated via the kidney after metabolism by the glucuronidation pathway (Marier et al., 2002), and glucuronide conjugates may also be too polar to traverse the blood-brain barrier. Furthermore, the brain concentration of MDMA after subcutaneous administration at 20 mg/kg is far higher than that for RSV after intravenous dosing at 20 mg/kg (43.7 nmol/g versus <0.1 nmol/g, respectively) (Chu et al., 1996; Lou et al., 2014), which favors the binding of MDMA to SERT.

As SERT occupancy is partially generated by calculating the reduction in 4-[¹⁸F]-ADAM binding from the baseline after the test drug was given, the values of SERT occupancy by the test drug are in general positive values. In the vehicle group, however, we observed several negative values for SERT occupancy, which indicated an increase instead of a decrease in SERT availability after the vehicle was given to the animals (Table 1). This phenomenon may be due to a steady increase in SERT availability from puberty until adulthood (Supplemental Figure 3), as described in the previous studies (Moll et al., 2000; Ulloa et al., 2014). Additionally, SERT occupancy by RSV and MDMA in this study (Table 1) revealed that the greatest SERT occupancy values were found in the midbrain at 80 mg/kg RSV and 10 mg/kg MDMA. This observation may be due to that SERT availability in the midbrain is the highest among the brain regions examined in accord with the previous studies (Park et al., 2014; Parsey et al., 2000).

Owing to the partial SERT occupancy by RSV, it is of great interest how RSV affords protection against the acute and long-term adverse effects of MDMA. We found that RSV had a neutral effect on body weight during treatment (Figure 3A), which is in agreement with the previous studies (Juan et al., 2002; Turner et al., 1999). However, RSV treatment significantly facilitated weight gain of MDMA/RSV rats during the 14-day post-treatment period (Figure 3B). This effect is particularly interesting while the underlying mechanism is still unclear. One possible explanation is that the partial occupancy of SERT by RSV during treatment may prevent the MDMA-induced long-lasting reduction in SERT expression. In addition, RSV may help to prevent MDMA-induced damage on serotonergic neurons. SERT proteins have been shown to be appropriate markers for the integrity and density of serotonergic innervation (Li et al., 2010; Nielsen et al., 2006). The higher SERT availability found in MDMA/RSV rats compared to MDMA/DMSO rats in the midbrain and the thalamus may indicate a better serotonergic innervation (Figure 4). Besides, higher serotonergic innervation in the frontal cortex has been associated with higher body weights from infancy to adulthood (Himpel et al., 2006). However, our data showed that RSV afforded negligible protection against MDMA-induced loss of SERT in the frontal cortex (Figure 4B).

MDMA has been well demonstrated to induce an immediate elevation in plasma corticosterone or cortisol level (Downey et al., 2015; Johnson and Yamamoto, 2010; Parrott et al.,

2013; Parrott et al., 2014), likely resulting from the release of 5-HT and activation of 5-HT₂ receptor on hypothalamic neurons that regulate pituitary-adrenocortical function (Fuller, 1981; Holmes et al., 1982; Koenig et al., 1987). The current data confirmed these previous findings (Figure 3C), although this MDMA-induced effect was ceased upon drug withdrawal. Also, we found that RSV appeared to inhibit MDMA-induced elevation in the corticosterone level, although this inhibitory effect was not statistically significant (Figure 3C). Even though RSV has been reported to suppress corticosterone production in primary rat adrenocortical cell cultures (Supornsilchai et al., 2005), our results revealed no differences in plasma corticosterone levels between SAL/DMSO and SAL/RSV rats.

The forced swim test is one of the most commonly used behavioral animal models for assessing antidepressant-like activity of drugs. It is well accepted that increased time of swimming behavior in rats relates to an elevation of brain 5-HT levels, whereas an increase in climbing behavior indicates an upregulated release of norepinephrine or dopamine (Bogdanova et al., 2013; Javelot et al., 2014; Vieira et al., 2008). Our results that MDMA-treated rats displayed significantly less swimming and greater immobility is in agreement with previous studies (Thompson et al., 2004), suggesting that this dosage regimen of MDMA leads to long-term depressive-like symptoms in this experimental setting. Interestingly, no significant difference between groups in the climbing time was observed, implying that catecholaminergic neurons may not be influenced by MDMA in the present study. We demonstrated that RSV treatment significantly reduced immobility and increased swimming in the MDMA-treated rats. These results are consistent with previous researches reporting anti-depressive effects of RSV (Ali et al., 2015; Ge et al., 2013; Pang et al., 2015; Wang et al., 2013; Xu et al., 2010; Yu et al., 2013). Moreover, we found that immobility time was negatively associated with SERT availabilities in the brain regions examined of the rats (Figure 5). Taken together, these findings provide evidence that co-treatment of RSV in the MDMA administration could prevent a long-term decrease in SERT availabilities in multiple brain regions of the rats that potentially helps to ameliorate the MDMA-induced depressive-like behaviors in the forced swim test.

In conclusion, using small-animal PET with 4-[¹⁸F]-ADAM, a novel and potentially important finding was that SERT is a biological target of RSV. In addition, that SERT availabilities in several key regions of the rat brain were negatively correlated with immobility time in the forced swim test implies that low cerebral SERT availability is strongly associated with depressive behaviors. These findings highlight a therapeutic potential of RSV for depression and anxiety disorders, as well as a neuroprotective potential for recreationally abused substances including MDMA, cocaine and methamphetamine.

Role of funding source

Tri-Service General Hospital and Ministry of Science and Technology had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

JHS conducted experiments, performed analyses and wrote the manuscript. CYS and CYC designed and offered 4 -[18 F]-ADAM, respectively. SJW operated the small-animal PET. LHP measured plasma corticosterone levels using UPLC-MS/MS. CFC, YSH, MKY and KHM provided guidance and edited the paper. IHL supervised the entire project. All authors critically reviewed content and approved final version for publication.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.euroneuro.2015.11.001>.

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