Involvement of autophagy upregulation in 3,4-methylenedioxymethamphetamine (‘ecstasy’)-induced serotonergic neurotoxicity

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ABSTRACT

It has been suggested that autophagy plays pathogenetic roles in cerebral ischemia, brain trauma, and neurodegenerative disorders. 3,4-Methylenedioxymethamphetamine (MDMA or ecstasy) is an illicit drug that causes long-term serotonergic neurotoxicity in the brain. Apoptosis and necrosis have been implicated in MDMA-induced neurotoxicity, but the role of autophagy in MDMA-elicited serotonergic toxicity has not been investigated. The present study aimed to examine the contribution of autophagy to neurotoxicity in serotonergic neurons in vitro and in vivo animal models challenged with MDMA. Here, we demonstrated that in cultured rat serotonergic neurons, MDMA exposure induced LC3B-densely stained autophagosome formation, accompanying by a decrease in neurite outgrowth. Autophagy inhibitor 3-methyladenine (3-MA) significantly attenuated MDMA-induced autophagosome accumulation, and ameliorated MDMA-triggered serotonergic neurite damage and neuron death. In contrast, enhanced autophagy flux by rapamycin or impaired autophagosome clearance by bafilomycin A1 led to more autophagosome accumulation in serotonergic neurons and aggravated neurite degeneration. In addition, MDMA-induced autophagy activation in cultured serotonergic neurons might be mediated by serotonin transporter (SERT). In an in vivo animal model administered MDMA, neuroimaging showed that 3-MA protected the serotonin system against MDMA-induced downregulation of SERT evaluated by animal-PET with 4-[18F]-ADAM, a SERT radioligand. Taken together, our results demonstrated that MDMA triggers upregulation of autophagy in serotonergic neurons, which appears to be detrimental to neuronal growth.

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Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; LC3, microtubule-associated protein 1 light chain 3; SERT, serotonin transporter; TPH, tryptophan hydroxylase; SSRI, selective serotonin reuptake inhibitors; 5-HT, 5-hydroxytryptamine (serotonin); 3-MA, 3-methyladenine; PI3K, phosphatidylinositol 3-kinase; PE, phosphatidylethanolamine; ROS, reactive oxygen species; CNS, central nervous system; PET/SPECT, positron emission computed tomography/single photo emission computed tomography; VOIs, volumes of interest; SURs, specific uptake ratios.

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1. Introduction

Autophagy is a highly conserved, regulated process by which intracellular constituents are delivered to lysosomes for degradation. Depending on the mode of delivery, there are three different types of autophagy: macroautophagy, chaperone-mediated autophagy, and microautophagy. Macroautophagy, simply referred to hereafter as autophagy, has been extensively investigated (Ghavami et al., 2014; Nixon, 2013). Upon initiation of autophagy, a small vesicular sac (“phagophore”) elongates and subsequently encloses a portion of cytoplasm, which results in formation of an autophagosome. This is a multistep process in which the formation of an autophagosome necessitates the sequential modification of microtubule-associated protein 1 light chain 3 (LC3). After...
synthesis, the C-terminal of LC3 is first cleaved by a cysteine protease, ATG4, to produce LC3-I with a molecular weight (MW) of 16 kDa. The product is localized diffusely throughout the entire cytoplasm. Upon induction of autophagy, LC3 is lipidated. A fraction of LC3-I is transferred to phosphatidylethanolamine (PE) to produce a new molecule, LC3-PE conjugate (also known as LC3-II), with an apparent MW of 14 kDa. LC3-II is associated with the autophagosome and thus the amount of LC3-II and the formation of LC3 puncta are thought to be a faithful marker of the autophagosome (Kesidou et al., 2013; Yang et al., 2013). The autophagosome then fuses with a lysosome to form an autolysosome, leading to degradation of the enclosed materials by acid hydrolases. The basal level of autophagy helps to control the cellular quality of proteins and organelles, and protects cells from protein aggregation or damaged organelles (Yang et al., 2013).

Autophagy can be activated in response to various cellular and environmental stress conditions to promote cell survival (e.g., starvation, oxidative stress), or to act as a mode of cell death (e.g., cerebral ischemia) (Abounit et al., 2012; Baehrecke, 2005; Din et al., 2012). Autophagic cell death (also called type II programmed cell death) is characterized by the massive accumulation of autophagic vacuoles in the cytoplasm of cells as they die (Din et al., 2012; Shen and Codogno, 2011). Defective autophagy has been connected to many human diseases including cancer, myopathies, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Ghavami et al., 2014; Kwon et al., 2011; Lee, 2012; Nixon and Yang, 2012).

3,4-Methylenedioxymethamphetamine (MDMA or Ecstasy), which is a central nervous system (CNS) psychostimulant, is one of the most popular recreational drugs that is abused by adolescents. Because of its stimulant properties, repeated or high-dose MDMA/ecstasy use can produce a variety of neurological disorders including cognitive impairments and mood disturbances (Kirilly, 2010). A combination of MDMA, its metabolites, oxidative stress and hyperthermia contributes to MDMA's neurotoxicity (Barbosa et al., 2014a; Mueller et al., 2013; Puerta et al., 2010). In the CNS, MDMA is toxic to serotonergic neurons in rodent and human, and also damage to dopaminergic system in mice (O'Shea et al., 2001).

2. Materials and methods

2.1. Antibodies and reagents

The following antibodies were used: rabbit anti-LC3B (Sigma Chemical, St. Louis, MO), mouse anti-SERT (Millipore, Temecula, CA), mouse anti-TPH (Sigma), mouse anti-MAP2 (Sigma), mouse anti-B-actin (Sigma), FITC-conjugated goat anti-rabbit IgG (Sigma), and Texas red-conjugated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA). 3-Methylenedehydroxyamine, rapamycin, bafilomycin A1 (Sigma) and Torin-1 (Tocris Bioscience, Ellisville, MO) were used in our autophagy flux studies. Citalopram was obtained from Abcam (Cambridge, United Kingdom). MDMA (purity, 98%) was obtained from the Investment Bureau of Taiwan.

2.2. Primary culture of serotonergic neurons from embryonic rat brain stems

Primary cultured brain stem and serotonergic neurons were established from embryonic Sprague–Dawley rats according to a published procedure with modifications (Kivell et al., 2000). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center (Taipei, Taiwan). E14 embryos were removed from the uterus and their brain stems were removed. Meninges and blood vessels were carefully removed. The brain stems were placed in ice-cold calcium and magnesium-free Hank's balanced saline solution (HBSS, Invitrogen, Carlsbad, CA), minced, digested with 0.0625% trypsin and 0.05% DNase for 10 min at 37 °C, suspended in DMEM with 10% fetal bovine serum (FBS) to inactivate the trypsin, and pelleted by centrifugation at 1000 rpm for 10 min. After suspending the cell pellet in medium and adjusting the cell density to 1 × 10⁶ cells/ml, neurons were seeded on coverslips or culture dishes precoated with poly-D-lysine and cultured with growth medium (1:1 mixture of DMEM/F-12 and neurobasal medium [Invitrogen], 5% FBS, supplemented with 0.5 mM l-glutamine, 1% penicillin–streptomycin, and 2% B–27 serum-free supplement [Invitrogen]). On the third day post-plating, non-neuronal cell proliferation was suppressed by treatment with 5 μM cytosine-d-arabinofuranoside for 24 h. On the fifth day of culture, the cells were used for the experiment. Serotonergic neurons were identified by positive staining with anti-TPH antibody.

2.3. Primary rat cortical neuron culture

Rat primary cortical neuron culture was performed according to a published procedure with modifications (Kindlundh-Hogberg et al., 2010). E18 Sprague–Dawley rats were removed from the uterus and their cortex were removed. Meninges and blood vessels were carefully removed. The cerebral cortex was placed in ice-cold calcium and magnesium-free Hank's balanced saline solution (HBSS), minced, digested with 0.0625% trypsin and 0.05% DNase for 10 min at 37 °C, suspended in DMEM with 10% FBS to inactivate the trypsin, and pelleted by centrifugation at 1000 rpm for 10 min. After suspending the cell pellet in medium and adjusting the cell density to 1 × 10⁶ cells/ml, neurons were seeded on coverslips or culture dishes precoated with poly-D-lysine and cultured with neurobasal medium (Invitrogen), supplemented with 0.5 mM l-glutamine, 1% penicillin–streptomycin and 2% B–27 serum-free supplement (Invitrogen). On the third day post-plating, non-neuronal cell proliferation was suppressed by treatment with...
5 μM cytosine-β-arabinofuranoside for 24 h. On the fifth day of culture, the cells were used for the experiment. Neurons were identified by positive staining with neuron-specific MAP2 antibody and the percentage of MAP2-immunostained neurons was over 90%.

2.4. Administration of MDMA and autophagy drug

Cells were pretreated with 3-MA, rapamycin, Torin-1, bafilomycin A1 and citalopram for 30 min prior to the treatment with MDMA.

2.5. Lactate dehydrogenase (LDH) assay

As an index of cell death, release into extracellular medium was measured using the LDH assay. Briefly, supernatants were collected at the times indicated and intact cells were lysed using Triton X-100-containing lysis buffer. The amount of LDH release was determined spectrophotometrically at 492 nm using the LDH-Cytotoxicity Assay Kit (Sigma). Percent cell death was calculated using the formula: % cytotoxicity = LDH release (OD492)/maximum LDH release (OD492x), after correcting for baseline absorbance of the LDH release at 492 nm.

2.6. Western blot assay

Serotonergic neurons were cultured in 6-well plates with a coverslip (18 × 18 mm) per well. For double immunofluorescence, coverslips were fixed in methanol for 5 min, washed 3 times in PBS (5 min for each), simultaneously incubated with anti-rabbit and anti-mouse primary antibodies for 1 h, treated with FITC-conjugated goat anti-rabbit IgG and Texas red-conjugated goat anti-mouse IgG at room temperature for 1 h, washed with PBS, mounted with 3% n-propyl gallate and 50% glycerol in PBS, and viewed under confocal microscope (Zeiss LSM510, Germany) or a fluorescence microscope (Nikon, Japan). The total number of TPH-positive cells was counted from at least 3 coverslips per experimental condition, and the average number of TPH-positive cells per coverslip was determined for each condition tested and compared to a control group. At least n = 3 independent experiments for each condition.

2.7. Neurite outgrowth assay

Neurite outgrowth visible in TPH-positive neuron images was quantified as the total length of neurites radiated from a single neuron. Images were acquired from randomly selected fields (n = 8–12) under a fluorescence microscope. The length of the total neurites of 40–50 neurons per condition was determined using ImageJ software (version 1.46r; NIH, Bethesda, MD). The outcome of neurite outgrowth assay were administered by an investigator blinded to treated group. Each experimental condition was done in duplicate wells, and at least three independent experiments were conducted to acquire the final results.

2.9. Animals and drug administration

The animals in this study were cared for according to the National Institutes of Health guidelines (National Academy Press, Washington, D.C., 1996). Approval for the protocol was obtained from the Institutional Animal Care and Use Committee of the National Defense Medical Center (Taipei, Taiwan). Adult male Sprague–Dawley rats (BioLASCO Taiwan Co., Ltd., Taipei, Taiwan) weighing between 250 and 300 g were randomly assigned to four groups: control group that received 1 ml/kg vehicle (normal saline, s.c. and DMSO, i.p.); MDMA group that received MDMA (10 mg/kg, s.c.) and DMSO (1 ml/kg, i.p.); 3-MA group that received saline (1 ml/kg, s.c.) and 3-MA (15 mg/kg, i.p.); and MDMA+3-MA group that received MDMA (10 mg/kg, s.c.) and 3-MA (15 mg/kg, i.p.). All drugs were administered twice a day for 4 consecutive days. For subcutaneous injection, MDMA was dissolved in saline (SAL, 0.9% NaCl) for intraperitoneal injection. 3-MA was prepared in dimethyl sulfoxide (DMSO) and administered prior to each injection of MDMA.

2.10. Radiopharmaceuticals and animal-PET imaging

The radiotracer 4-[18F]-ADAM was synthesized as previously described (Ma et al., 2009). After 4 consecutive days of MDMA administration, animal-PET image acquisition was performed as previously described with minor modifications (Li et al., 2010). The rats were anesthetized by passive inhalation of a mixture of isoflurane/oxygen (5% isoflurane for induction and 2% for maintenance). The 4-[18F]-ADAM (14.8–18.5 MBq; 0.4–0.5 mCi) was injected via tail vein. After 60 min, the PET images were acquired by BIOPET105 (Bioscan, Inc., Washington, DC, USA) for 30 min and the energy window was set at 250–700 keV. The 3D-OSEM was employed to reconstruct the images. The above imaging procedure was performed at National Defense Medical Center Laboratory Animal Center. Quantification of PET signal in specific anatomical regions was performed by drawing 3D ROIs using AMIDE software version 1.0.4 (http://amide.sourceforge.net/)(Loening and Gambhir, 2003). The volumes of interest (VOIs) within the frontal cortex, striatum, thalamus, hypothalamus, midbrain, and cerebellum were drawn manually on the reconstructed PET images according to the MR images and a rat brain atlas. The reconstructed PET images were fused and co-localized with the MR images in order to measure the activities in various brain regions. Binding of 4-[18F]-ADAM to SERT was analyzed using the ratio of specific to non-specific binding, with the cerebellum as a reference region for background radioactivity. The final data were expressed as specific uptake ratios (SURs) where, SUR = (target region – cerebellum)/cerebellum (Ma et al., 2009).

2.11. Statistical analysis

The quantitative data are presented as mean ± SEM. Statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. Difference were considered to be significant at P value <0.05 defined as statistically significant and a P value <0.01 as highly statistically significant.
3. Results

3.1. Characteristics of cultured serotonergic neurons

To characterize the cultured serotonergic neurons, the cells were double immunofluorescence staining with antibodies against serotonin transporter (SERT) and tryptophan hydroxylase (TPH), both specific proteins expressed in serotonergic neurons. As shown in Fig. 1A–C, the cells co-expressed SERT and TPH, indicating that the cultures contained functional serotonergic neurons. Negative control images (Fig. 1D–F) supported that antibody is specific for the antigen.

3.2. Dose dependence of MDMA-induced neurotoxicity in cultured brain stem neurons and cultured serotonergic neurons

We examined the dose effect of MDMA on neurotoxicity in primary culture of embryonic brain stem neurons. Cultured cells were treated with different concentration of MDMA (0.5, 1, 1.5, 2 mM) for 48 h, then the neuronal death was determined by LDH assay. As shown in Fig. 2, MDMA produced a concentration-dependent increase in neuronal death (Fig. 2A). Similar results were observed in serotonergic neuron cultures. Cultured serotonergic cells were treated with different concentrations of MDMA (0.5, 1, 1.2, 1.5, 1.8 and 2 mM) for 48 h, and then cell viability was determined by TPH immunostaining. MDMA produced a concentration-dependent decrease in cell viability (Fig. 2B). Treatment with 0.5 and 1 mM MDMA for 48 h did not have an obvious effect on cell viability, as approximately 97% and 95%, respectively, compared to normal control. When cells were treated with higher concentration of MMDA (1.2, 1.5, 1.8, and 2 mM), cell viability was significantly decreased to 83%, 58%, 42% and 14%, respectively.

3.3. MDMA-induced autophagy and neurite degeneration in a dose- and time-dependent manner

We investigated the dose-dependent effect of MDMA on autophagy activity and neurite outgrowth in cultured serotonergic neurons. Autophagy activity was determined by immunofluorescence to detect the essential autophagy protein LC3. Upon autophagy activation, the LC3-I protein localized in the cytoplasm is cleaved, lipidated, and inserted as LC3-II into autophagosome membranes. Thus, autophagy upregulation is correlated with an increased number of autophagosomes. LC3-II is a faithful marker of autophagosomes, and its punctate staining has been used...
quantitatively for this purpose in vitro (Kabeya et al., 2000). In the present study, the cells were treated with different concentrations (1 mM, 1.5 mM, and 2 mM) of MDMA for 48 h and then double immunofluorescence followed by confocal microscopy was performed using TPH and LC3B antibody. As shown in Fig. 3, in non-treated control neurons, the pattern of LC3B immunoreactivity was light, diffuse, and cytoplasmic within the cell body (Fig. 3A). After exposure to 1 mM MDMA, typical cytoplasmic LC3B puncta were formed, notably in almost all TPH-positive neurons with shortening of all neurites compared to control cells. At higher concentrations of MDMA (1.5 and 2 mM), more LC3 punctate spots were observed (arrows in Fig. 3A and C) and were accompanied by dose-dependent neurite degeneration (Fig. 3A and D). Western blot analysis showed that a dose-dependent reduction in protein level of TPH in MDMA-treated cells (Fig. 3B). Immunofluorescence data demonstrated that MDMA triggers dose-dependent autophagosome formation accompanied by reduction of neurite outgrowth in cultured neuronal cells. Concomitantly, we found that LC3 punctate staining was not limited to TPH-positive neurons; it also occurred in TPH-negative neurons in brain stem cultures (Fig. 3A, arrowhead), suggesting autophagy activation may be a common biological event occurring in cultured neurons.

Although 1 mM MDMA had no obvious effect on cell viability, pharmacological actions significantly caused a decrease in neurite outgrowth and upregulation of autophagy. Therefore, 1 mM MDMA was applied to cultures in the subsequent experiments. We then examined the time-dependent effect of MDMA on autophagy activation. The cells were treated with 1 mM MDMA for 18 h, 24 h, and 48 h and subjected to double immunofluorescence using anti-TPH and anti-LC3B antibody. Double immunofluorescence staining showed that LC3B punctate staining was not observed within 13 h of MDMA treatment (data not shown), whereas positive aggregations increased rapidly within 18 h and even more punctate staining was observed after 24 h and 48 h of MDMA treatment (arrows in Fig. 4A and B). Moreover, neurite outgrowth was significantly reduced in a time-dependent manner (Fig. 4A and C). MDMA removal for 48 h enhanced regrowth of neurites and reduced autophagosome accumulation almost to the baseline level (Fig. 4, recovery), suggesting that this process is reversible.

3.4. Autophagy inhibitor 3-MA decreases MDMA-induced autophagy upregulation, and protects serotonergic neurons against neurite degeneration and neuronal death

The autophagy inhibitor 3-MA is commonly used to define the role of autophagy under various physiological conditions (Seglen and Gordon, 1982). To further address the role of MDMA in

![Fig. 3. Dose dependence of MDMA-induced autophagy and neurite degeneration in serotonergic neuron cultures. (A) Cultured cells were treated with the indicated concentrations of MDMA for 48 h, and then double immunostained with anti-TPH and anti-LC3B antibodies. The right panels are merged images. Bar = 20 μm. The confocal images indicated that MDMA induced formation of LC3B-labeled puncta in TPH-positive neurons (white arrow) in a dose-dependent manner. LC3 punctate staining is also seen in TPH-negative neurons (white arrowhead). (B) Western blot analysis of TPH expression. β-actin was used as the internal control. Quantitative data are expressed as intensity relative to the control mean value (mean ± SEM). n = 3. *P < 0.05, **P < 0.01 vs. control. (C) Number of LC3-labeled puncta per TPH+ neuron was quantified. The data were mean ± SEM from three independent experiments. *P < 0.01 vs. control group. (D) Quantitation of neurite outgrowth. The total neurite length of each TPH+ neuron was measured and presented as mean ± SEM from three independent experiments. *P < 0.01 vs. control group.]
autophagy induction, cells were pre-incubated with 3-MA for 30 min prior to induction of autophagy by 1 mM MDMA for 48 h and then double immunostained followed by confocal microscopy was performed with anti-TPH and anti-LC3B. As shown in Fig. 5, 3-MA significantly reduced LC3 punctate number (Fig. 5A and C) and significantly prevented nerve fiber degeneration (Fig. 5A and D). Cells were treated as the same above, and Western blot analysis showed that 3-MA significantly prevented MDMA-induced ratio of LC3B-II/LC3B-I from 2.5-fold to 1.6-fold of control value (Fig. 5B). Interestingly, treatment with 1 mM 3-MA alone significantly promoted neurite outgrowth (Fig. 5A and D). We further investigated the protective effect of 3-MA against MDMA-induced neuronal cell death. Cells were pre-incubated with 3-MA for 30 min prior to 1.8 mM MDMA treatment for 48 h, then the serotonergic neuron survival was determined by TPH immunostaining. As shown in Fig. 5E, morphological data by anti-TPH immunostaining confirmed that 3-MA significantly attenuated MDMA-induced serotonergic neuronal cell loss. Therefore, MDMA-induced neuronal death is at least partly autophagy-dependent.

3.5. Autophagosome accumulation is associated with MDMA-induced serotonergic degeneration

To further clarify the detrimental role of autophagosome accumulation in MDMA-induced neurotoxicity, we employed rapamycin and bafilomycin A1 to affect autophagy flux. Rapamycin
is an mTOR inhibitor commonly used to activate/enhance autophagosome formation. Bafilomycin A1, a specific inhibitor of vacuolar H\(^+\) ATPase (V-ATPase), prevents autophagy at a late stage by inhibiting fusion between autophagosomes and lysosomes (Yamamoto et al., 1998), resulting in autophagosome accumulation. Cells were treated with MDMA in the presence or absence of rapamycin or bafilomycin A1 for 48 h and double immunostained with anti-TPH and anti-LC3B antibodies. As shown in Fig. 6, rapamycin and bafilomycin A1 in the presence of MDMA acted synergistically with MDMA to enhance LC3B puncta formation (Fig. 6A and B) and neurite degeneration (Fig. 6A and C), indicating that autophagosome accumulation is associated with MDMA-induced reduction of neurite outgrowth. However, rapamycin or bafilomycin A1 alone weakly induced autophagy and less effect on neurite outgrowth in cultured serotonergic neurons (data not shown). Another mTOR inhibitor Torin-1 directly inhibits both mTORC1 and mTORC2 (Thoreen et al., 2009). Cotreatment of MDMA with Torin-1 (100 nM) also synergistically resulted in LC3B-labeled autophagosome accumulation and aggravated neurite degeneration, whereas Torin-1 alone increased LC3B immunostaining intensity slightly without affecting neurite outgrowth (Fig. S1). This result was similar to that observed in treatment with rapamycin.

3.6 Citalopram, a selective serotonin reuptake inhibitor, downregulates autophagy and protects against MDMA-induced serotonergic neuron degeneration

Accumulating evidence has indicated that MDMA mediates neurotoxicity via SERT (Fox et al., 2007). SERT is a protein on the membrane of serotonergic neurons that functions to “recycle” released 5-HT by transferring it back into the serotonergic neuron.
The MDMA molecule binds with high affinity to SERT or likely crosses membranes into serotonergic neurons due to highly lipophilic property, which may damage neurons partly by mitochondria dysfunction and oxidative stress (Quinton and Yamamoto, 2006). For that reason, selective serotonin reuptake inhibitors (SSRIs), such as citalopram, can block MDMA-induced damage via competing for SERT binding sites (Liechti et al., 2000). To further address the autophagy in response to citalopram-mediated neuroprotection against MDMA toxicity, cultured cells were pre-incubated with citalopram (5 μM) for 30 min prior to induction of autophagy by MDMA for 48 h, and then double immunostaining was performed with anti-TPH and anti-LC3B. As shown in Fig. 7, citalopram significantly reduced LC3 punctate staining (Fig. 7A and B), and significantly prevented nerve fiber degeneration (Fig. 7A and C). To evaluate whether the neuroprotective effect of citalopram extended to non-TPH positive neurons, cultured cortical neurons were exposed to 1 mM MDMA for 48 h in the presence or absence of 5 μM citalopram, and then subjected to double immunofluorescence staining with anti-MAP2 and anti-LC3B antibodies. The result indicated that citalopram failed to protect cortical neurons against MDMA-induced neurite degeneration and LC3B upregulation (Fig. S2). This result suggests that the neuroprotective effect of citalopram restricted to TPH-positive neurons and MDMA-induced autophagy and subsequent neurite damage may mediated by SERT.
3.7. 3-MA protects against MDMA-induced neurotoxicity in rat brain assessed by 4-[18F]-ADAM animal-PET

The density of SERT is considered to be one of the markers of the integrity of serotonin neurons (Laakso and Hietala, 2000), and has been validated in animal models of MDMA neurotoxicity (Li et al., 2010). It is well established that SERT density can be also determined by SERT radiotracer using positron emission computed tomography (PET) (Laakso and Hietala, 2000) and single photon emission computed tomography (SPECT) (Buchert et al., 2003). This noninvasive assay has been validated for directly evaluating the neurotoxicity of MDMA in the living brain and for monitoring the neuroprotective effect. A SERT radioligand, 4-[18F]-ADAM [N,N-dimethyl-2-[(2-amino-4-[18F]-fluorophenylthio) benzylamine], has a high affinity and high specificity for SERT (Chen et al., 2012; Ma et al., 2009; Peng et al., 2008), and has been demonstrated to reflect brain SERT density. In this study, we evaluated the neuroprotective effect of 3-MA against MDMA-induced neurotoxicity on the rat brain SERT availability assessed with 4-[18F]-ADAM animal PET. As shown in Fig. 8, in the control group, high SERT density was observed at the level of midbrain nuclei, reflecting the presence of serotonergic cell bodies (raphe nuclei). High SERT density was also observed in the hypothalamus, thalamus, and striatum (Fig. 8B). Intermediate levels were found in...
the frontal cortex and very low to negligible levels have been reported in the cerebellum. In MDMA-treated rats, administration of MDMA significantly decreased 4-[^18F]-ADAM uptake in these SERT-rich brain regions including the midbrain, hypothalamus, thalamus, striatum, and cortex (Fig. 8B). Interestingly, 3-MA pretreatment prevented MDMA-induced loss of 4-[^18F]-ADAM uptake in these brain regions. However, treatment with 3-MA alone did not affect the 4-[^18F]-ADAM uptake, which is a similar result to that observed in the control group.

4. Discussion

In the present study, we examined the role of autophagy in MDMA-induced serotonergic neurotoxicity. Our data provide the first evidence that MDMA elicits a robust autophagic response in primary rat serotonergic neurons, as judged by an increase in LC3-labeled autophagosome formation. The autophagy inhibitor 3-MA pretreatment prevented MDMA-induced loss of 4-[^18F]-ADAM uptake in these brain regions. However, treatment with 3-MA alone did not affect the 4-[^18F]-ADAM uptake, which is a similar result to that observed in the control group.

MDMA-induced autophagosome accumulation and neurite degeneration, indicating that excessive autophagy may play a detrimental role in MDMA-triggered neurotoxicity. In addition, citalopram, an SSRI, decreased MDMA-induced autophagosome formation and was protective against serotonergic neurite degeneration by MDMA, suggesting that MDMA-induced autophagy activation occurs via an SERT-dependent mechanism. Furthermore, in vivo data also demonstrated that 3-MA prevented MDMA-induced serotonergic toxicity in MDMA challenged rat using animal-PET analysis.

Inhibition of autophagy may contribute to neurite extension and neuroprotection. Previous study have shown that 3-MA may promote neurite extension in cortical neurons (Ban et al., 2013). Administration of 3-MA, or downregulation of Atg7, prevents neurite degeneration of cultured superior cervical ganglion neurons following transection (Yang et al., 2007). Conditional deletion of the essential autophagy gene Atg7 in adult mice achieves striking axon protection in this acute model of retrograde degeneration induced by the neurotoxin 6-hydroxydopamine (6-OHDA) (Cheng et al., 2011). Inhibition of autophagy by 3-MA prevented MDMA against neurite degeneration in serotonergic neurons, suggesting that autophagy may in fact contribute to neurite degeneration. Autophagic flux implies a balance between

Fig. 8. Animal-PET analysis of the neuroprotective effect of 3-MA on serotonergic system in MDMA animal model. Rats were administered saline (control group), MDMA, MDMA + 3MA, and 3-MA alone, respectively. After 28 days, the rat serotonergic system was evaluated by 4-[^18F]-ADAM uptake using animal-PET. (A) Representative 4-[^18F]-ADAM/animal-PET images of transverse sections of rat brain. The rat brain MRI provides orientation to anatomical position of brain regions as indicated. (B) Specific uptake ratios of 4-[^18F]-ADAM/animal-PET on day 28 in rat brain regions, including midbrain, hypothalamus, thalamus, striatum, and frontal cortex. Values were expressed as the mean ± SEM with 6 rats in each group. ***P < 0.001 vs. control group; ###P < 0.001 vs. MDMA administration group.
autophagosome formation and autophagic degradation. Either increased autophagosome formation in the early stage or defective degradation in the late stage can lead to accumulation of autophagic vacuoles. MDMA-induced autophagosome accumulation could be a result of either increased autophagosome generation or defects in autophagosome degradation. In this study, 3-MA, an autophagy inhibitor at the early stage of autophagosome biogenesis, prevented MDMA-induced autophagosome vacuole accumulation, suggesting that MDMA may activate signaling cascades that initiate autophagosome generation. However, it cannot be completely excluded that autophagosome accumulation might occur because of organelle turnover inhibition. In addition, we found that induction of autophagy by MDMA is not limited to serotonergic neurons. Indeed, MDMA-induced autophagy also occurred in cultured TPH-negative neurons from brain stem, suggesting that autophagy induction by MDMA is a common signaling event in neurons. There are kinds of serotonin (5-hydroxytryptamine, 5-HT) receptors responsible for different signaling transmission in pre- and post-synaptic neurons (Filip and Bader, 2009).

Numerous studies have demonstrated that MDMA may alter serotonergic transmission in brain through direct action at 5-HT recognition sites (Capela et al., 2009). Therefore, we speculate that the autophagic pathway induced by MDMA may be modulated by different neuronal receptors.

In the present study, we further confirmed that sustained autophagy by higher concentration of MDMA is associated with neuron loss. Indeed, a important limitation of this study is the higher concentration of MDMA used in our cultured model compared to plasma or brain level of MDMA in rodent and human. Although millimolar concentration of MDMA is required compared to plasma or brain level of MDMA in rodent and higher concentration of MDMA used in our cultured model, rapamycin or its analogus, everolimus weakly induced autophagy in neurons (Thoren et al., 2009; Tsvetkov et al., 2010). In yeast, rapamycin is a potent activator of autophagy (Noda and Ohsumi, 1998), whereas the situation is less clear in mammalian systems, where rapamycin alone is an inconsistent activator of autophagy and highly dependent on the cell type and treatment conditions (Thoren et al., 2009). Rapamycin frequently requires combination with other PI3K/mTOR inhibitors, such as LY294002 to enhance autophagy activity (Takeuchi et al., 2005). PI3K-Akt pathway is upstream of mTOR (Heras-Sandoval et al., 2014). Therefore, we suspected that synergistic autophagy effect of MDMA plus mTOR inhibitor might dysregulated PI3K/Akt/mTOR signaling. However, detailed molecular mechanism remains further investigation.

Numerous studies have revealed that MDMA induces neurotoxicity via a receptor-mediated mechanism (Lizarraga et al., 2014). MDMA can bind to SERT and enters the presynaptic axon terminal, which may result in downstream adverse effects, including Ca²⁺ overload and reactive oxygen species (ROS) production (Baumgarten and Lachmayer, 2004). Selective SERT inhibitors, also known as SSRIs, have been known to prevent MDMA-induced ROS production, 5-HT release, and 5-HT fiber degeneration through blocking the SERT binding sites (Jones et al., 2004; Li et al., 2010). Recently, in SERT-knockout mice, MDMA-mediated thermogenesis, hyperactivity, and neurotoxicity effects were abolished (Lizarraga et al., 2014), suggesting that SERT may constitute a key target in the development of new treatment regimens for MDMA abuse. In the present study, we further confirmed that an SSRI, citalopram, prevents MDMA-induced neurotoxicity via autophagy inhibition, whereas this neuroprotective effect of citalopram did not extend to non-TPH neurons. In other words, SERT-mediated autophagy upregulation is required for MDMA-induced neurotoxicity in serotonergic neurons. Some other studies have reported that ROS production correlates with autophagy upregulation (Lin and Kuang, 2014; Wang et al., 2010). Therefore, ROS-mediated autophagy activity in MDMA-treated neurons will be further investigated.

SERT density may provide a useful biomarker for 5-HT dysfunction, which is especially applicable to depression (Staley et al., 1998) and drug abuse (Li et al., 2010). SERT density can be determined by immunocytochemistry (Kovacs et al., 2007; Sur et al., 1996) and PET/SPECT assay (Buchert et al., 2003; Scheffel et al., 1994; Urban et al., 2012). Noninvasive imaging modalities [e.g., PET or SPECT] in conjunction with SERT radioligand (e.g., [11C]-MCN5652, [11C]-DASB, [I123]-ADAM, and 4-[18F]-ADAM) have been used successfully in animals to visualize SERT in living brain tissues and monitor the response to therapy in cases of MDMA-induced neurotoxicity (Li et al., 2010; Szabo et al., 2002). As an important milestone for such studies the potent fluoride-18 labeled PET tracer, 4-[18F]-ADAM, has been developed and characterized in preclinical as well as clinical studies with regard to its high affinity and selectivity for SERT in various brain regions including the midbrain, hypothalamus, striatum, hippocampus, and cortex in rats, monkeys, and humans (Huang et al., 2013). In our study, 4-[18F]-ADAM with PET imaging data demonstrated that administration of MDMA reduced uptake of 4-[18F]-ADAM in midbrain, hypothalamus, thalamus, striatum, and cerebral cortex in rat. This result of reduction in SERT density is consistent with previous results (Li et al., 2010). We then examined the neuroprotective effect of 3-MA on MDMA-induced toxicity in rats in vivo using 4-[18F]-ADAM animal-PET. We provided the first evidence that 3-MA protected serotonergic neurons against the decrease in SERT density in various brain regions, and the long-term protective effects remained for days (up to 28 days) suggesting that autophagy activation may occur in 5-HT neurons of MDMA-treated rat. However, a detailed molecular mechanism requires further investigation.
5. Conclusion

We propose that MDMA neurotoxic stress triggers autophagy in serotonergic neurons, which appears to be detrimental to cell survival. In other words, autophagy under the MDMA toxicity paradigm in fact contributes to the neuronal cell injury/cell death process. Abnormal accumulation of autophagic vacuoles is a critical hallmark feature of autophagic cell death. Thus, specific modulation of autophagy might be a novel neuroprotective strategy to attenuate MDMA-induced serotonergic neurotoxicity.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.neuro.2015.11.009.

References


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