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REGULAR ARTICLE

Methamphetamine induces endoplasmic reticulum stress related gene CHOP/Gadd153/ddit3 in dopaminergic cells

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Abstract We examined the toxicity of methamphetamine and dopamine in CATH.a cells, which were derived from mouse dopamine-producing neural cells in the central nervous system. Use of the quantitative real-time polymerase chain reaction revealed that transcripts of the endoplasmic reticulum stress related gene (CHOP/Gadd153/ddit3) were considerably induced at 24–48 h after methamphetamine administration (but only under apoptotic conditions), whereas dopamine slightly induced CHOP/Gadd153/ddit3 transcripts at an early stage. We also found that dopamine and methamphetamine weakly induced transcripts for the glucose-regulated protein 78 gene (Grp78/Bip) at the early stage. Analysis by immunofluorescence microscopy demonstrated an increase of CHOP/Gadd153/ddit3 and Grp78/Bip proteins at 24 h after methamphetamine administration. Treatment of CATH.a cells with methamphetamine caused a re-distribution of dopamine inside the cells, which mimicked the presynaptic activity of neurons with cell bodies located in the ventral tegmental area or the substantia nigra. Thus, we have demonstrated the existence of endoplasmic reticulum stress in a model of presynaptic dopaminergic neurons for the first time. Together with the recent evidence suggesting the importance of presynaptic toxicity, our findings provide new insights into the mechanisms of dopamine toxicity, which might represent one of the most important mechanisms of methamphetamine toxicity and addiction.

Keywords Methamphetamine · Dopamine toxicity · Endoplasmic reticulum stress · CHOP/Gadd153/ddit3 · Grp78/Bip · CATH.a cells · Cell culture

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Introduction

The problem of the drug abuse of methamphetamine (METH) has recently spread over many countries of the world. Acute application of this psychostimulant induces euphoria, increased activity, and decreased appetite. Psychostimulants might also induce anxiety, irritability, and paranoid psychosis at a higher dose. Furthermore, chronic administration of METH or cocaine can produce long-term behavioral changes (Barnett et al. 1987; Gawin and Ellinwood (1988); Klawans et al. 1975; Segal and Mandell 1974). Unlike many other drugs, repetitive

administration of these drugs progressively induces greater behavioral effects such as behavioral sensitization (Robinson et al. 1988; Sato et al. 1983). Eventually, chronic administration of psychostimulants results in a profound state of dependence.

Dependency on psychostimulants is established after repetitive administration of drugs, and once this is established, it lasts for a long period. These phenomena suggest that long-term drug administration can play a critical role in the alternation of gene expression (Berke and Hyman 2000; Nestler 2005). METH is reported to induce many genes such as Arc, an immediate-early gene, which contributes to the maintenance of long-term potentiation and the consolidation of long-term memory (Fosnaugh et al. 1995; Lyford et al. 1995; Yamagata et al. 2000). We have reported that Arc interacts with Amida, which is involved in apoptosis and the cell cycle (Gan et al. 2001, 2003; Irie et al. 2000), and we speculate that the apoptotic mechanisms related to Amida might be involved in the development of METH toxicity, as for many other molecules (Cadet and Brannock 1998; Cadet et al. 2003, 2005), and in the degeneration of dopaminergic terminals (Kita et al. 2003).

Perturbation of endoplasmic reticulum (ER) homeostasis is called ER stress (Welihinda et al. 1999). ER stress has been implicated in a variety of diseases such as diabetes, ischemia, and Parkinson's disease (Oyadomari and Mori 2004), up-regulates chaperone genes such as glucose-regulated protein 78 (Grp78/Bip), and induces the degradation of unfolded proteins. However, when ER function is severely impaired, the organelle elicits apoptotic signals. This apoptotic event is mediated by a transcriptional activation of the CCAAT/enhancer binding protein (C/EBP) family member CHOP/Gadd153/ddit3 (hereinafter referred to as CHOP) and by the activation of ER-associated caspase-12 (Nakagawa et al. 2000; Oyadomari and Mori 2004; Wang et al. 1996). Recently, Jayanthi et al. (2004, 2009) have shown the involvement of ER stress in METH toxicity, but the detailed mechanism of ER-stress-mediated toxicity remains to be elucidated.

In this study, we have investigated whether ER stress is involved in the mechanism underlying the dopaminergic toxicity induced by METH. We have examined the expression of CHOP and Grp78/Bip in METH-treated CATH.a cells derived from mouse dopamine (DA)-producing neural cells of the central nervous system (Suri et al. 1993).

Materials and methods

Materials

CellTiter-Glo Luminescent Cell Viability assay reagent was from Promega (Madison, Wis., USA). ABsolute SYBR Green Mixes was from ABgene (Surrey, UK). Oligonucleotide primers were synthesized by Greiner (Frickenhausen, Germany). TRIzol reagent, SuperScript III, and horse serum was from Invitrogen (Carlsbad, Calif., USA). Fetal calf serum was from Hyclone Laboratories (Logan, Utah, USA). RPMI 1640 medium was from Sigma (St. Louis, Mo., USA). Rabbit anti-Grp78/Bip antibody was purchased from Stressgen (Victoria, BC, Canada). Rabbit anti-procaspase-12 antibody was from Calbiochem (La Jolla, Calif., USA). Anti-CHOP/Gadd153/ddit3 (R-20) antibody and anti-Grp78/Bip antibody were purchased from Santa Cruz. A secondary antibody conjugated with Alexa Fluor 488 was from Molecular Probes (Eugene, Ore., USA). Methamphetamine HCl (METH) was purchased from Dainippon Pharmaceutical (Osaka, Japan). Throughout the study, the Student *t*-test was used for statistical analysis.

Cell culture and treatments

CATH.a cells (ATCC no. CRL-11179) were maintained in RPMI 1640 supplemented with 8% horse serum, 5% fetal calf serum. Cells were treated with METH dissolved in dimethylsulfoxide (DMSO) or DA dissolved in phosphate-buffered saline for 24 h, unless otherwise described. The final concentration of DMSO did not exceed 0.1%, a dose that had no apparent effect on these cells.

Drug cytotoxicity in vitro

For the measurement of cell toxicity, cells were seeded in 96-well culture plates (Nunc, Roskilde, Denmark). The effect of the studied compounds on cell toxicity was determined by using a CellTiter-Glo Luminescent Cell Viability assay according to the manufacturer's protocol, as based on quantification of the ATP level (Lovborg et al. 2002). Luminescent signals were measured in LB96P, a microplate luminometer. Each point represents the mean±SD (bars) of eight values from one representative experiment.

Reverse transcription and quantitative real-time polymerase chain reaction

Cells were harvested in TRIzol. Then, total RNA was isolated and subjected to reverse transcription by using SuperScript III. cDNAs were amplified by quantitative real-time polymerase chain reaction (RT-PCR) by using ABsolute SYBR Green Mixes according to manufacturer's protocols. A 7900HT thermal cycler (Applied Biosystems, Foster City, Calif., USA) was utilized to detect amplification. Oligonucleotide pairs used to amplify mouse cDNA sequences were as follows: chop/gadd153 forward primer, 5′-GGAAGTGCATCTTCATACACCACC and reverse primer, 5′-TGACTGGAATCTGGAGAGCGAGGGC;

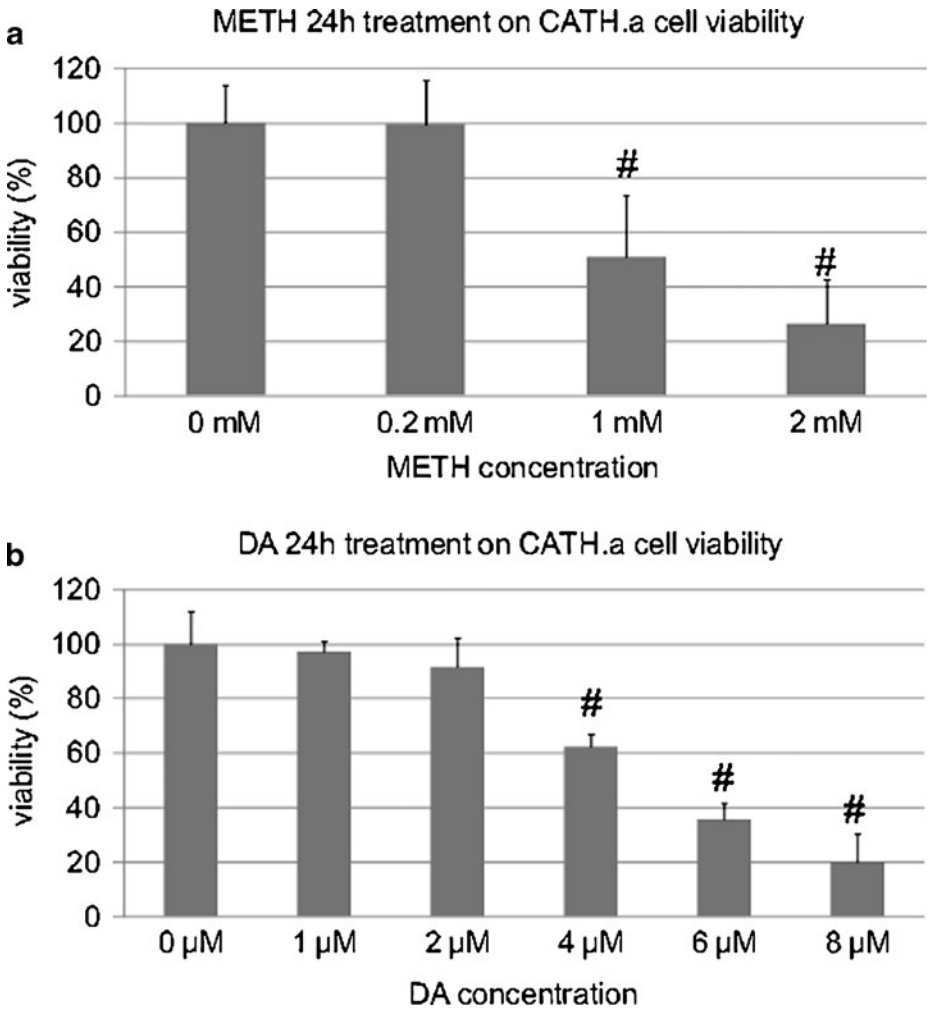

Fig. 1 Cell toxicity of methamphetamine (*METH*) or dopamine (*DA*). **a** Viability of CATH.a cells treated with various concentrations of METH. After 24 h of treatment, the ATP content of each culture (a value proportional to the extent of cell viability) was measured by luminescence. **b** Viability of CATH.a cells treated with various concentration of DA. The same assay was employed as for the METH-treated cells. Data shown in **a**, **b** are mean±SE of eight experiments ($^{\#}P<0.01$). Similar sets of experiments were repeated at least three times

Grp78/Bip forward primer, 5′-CAGAGACCCTTACTCG, and reverse primer, 5′-GTTTATGCCACGGGAT; Hprt1 forward primer, 5′-GCCTAAGATGAGCGCAAGTTGAA, and reverse primer, 5′-ACTAGGCAGATGGCCACAGGAC, as previously described (Jayanthi et al. 2004). To ensure the amplification of a single product, a dissociation curve was produced for each amplification. The relative concentration of CHOP or Grp78 in the samples was determined by normalizing the level of expression to that of Hprt1 (hypoxanthine guanine phosphoribosyl transferase 1) in each of the samples by using standard curves for the respective amplifications (SYBR Green PCR mix and quantitative RT-PCR protocol, Applied Biosystems).

Immunofluorescent and immunoblotting analysis

CATH.a cells were seeded on gelatin-coated coverslips. On the next day, 1 mM METH or the same concentration of vehicle (0.1% DMSO) was used to treat the cells. After 24 h, the cells were fixed in 4% paraformaldehyde and visualized by either an anti-CHOP/GADD153 (R-20) antibody or an anti-Grp78/Bip antibody and a secondary antibody conjugated with Alexa Fluor 488. The morphology of the nuclei was visualized with 4,6-diamidino-2-phenylindole nuclear counter-staining. Immunoblotting was performed according to methods described previously (Irie et al. 2003). Immunoblot results were developed on X-ray film and scanned into image files; relative band intensities were determined with ImageJ software.

Results

Toxicity analysis for CATH.a cells

CATH.a cells are reported to undergo apoptosis when treated with METH or DA (Choi et al. 2002; Masserano et al. 1996). To determine a condition for analyzing METH toxicity, cells were treated with several concentrations of METH or DA for 24 h, and their viability was assessed (Fig. 1). Almost half of the cells died in 1 mM METH, whereas few cells died in

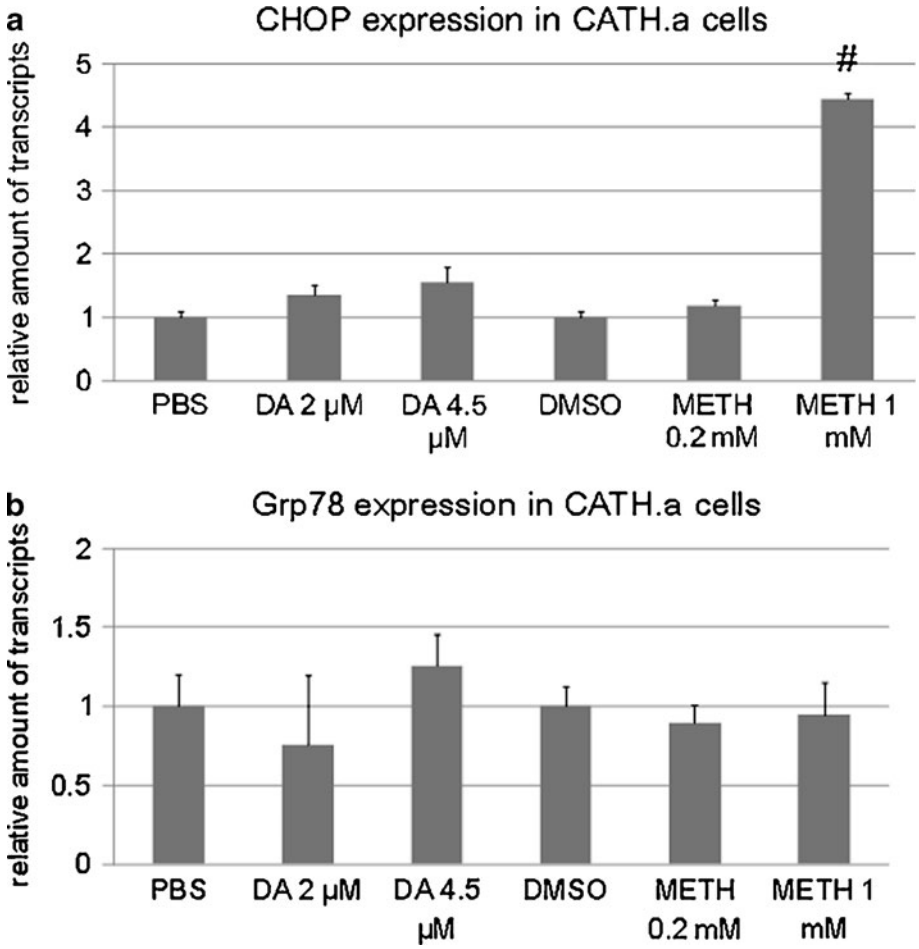

Fig. 2 Expression of endoplasmic reticulum stress related gene (*CHOP*) and glucose-regulated protein 78 gene (*Grp78*) in CATH.a cells treated with METH or DA. CATH.a cells were treated with the indicated concentrations of METH or DA for 24 h. Total RNA was isolated from each sample and subsequently reverse-transcribed. The resultant cDNA samples were subjected to quantitative real-time polymerase chain reaction (RT-PCR) to quantify CHOP (**a**) or Grp78 (**b**) gene expression. Results are expressed as relative amount (fold) of transcripts to control samples treated with solvent, normalized to Hprt1 transcripts. The entire set of experiments was repeated at least three times with RNA samples obtained independently from separate cultures. Each value represents the mean of four measurements of the sample from a representative experiment (*error bars* standard deviations; $^{\#}P<0.01$)

0.2 mM METH. Nearly half of cells died when treated with 4 μM DA, whereas only a limited number of the cells died in 2 μM DA. We decided to employ these concentrations to assess gene expression in cells killed by METH, since we assumed that 0.2 mM METH caused METH-related changes, whereas 1 mM METH additionally induced apoptosis-specific changes. The same was considered to apply at 2 μM DA and 4.5 μM DA, respectively.

METH causes CHOP induction in dose-dependent manner

To investigate whether ER stress is involved in METH-induced apoptosis of CATH.a cells, the expression of CHOP and Grp78/Bip mRNA were assessed by quantitative RT-PCR. By using primers specific for each mRNA, the signals from PCR products showed a dissociation curve with a single peak, which assured the proper and reliable condition for PCR-based quantification. Housekeeping Hprt1 mRNA was used as an internal control. All genes showed sufficient correlation between the quantity of cDNA and cycles threshold (Ct). All of the resultant Ct values fell into the range of standard curves.

We found that CHOP mRNA was induced by 24 h of METH treatment in a dose-dependent manner (Fig. 2a). Notably, 1 mM METH induced CHOP expression at a significance level ($P<0.01$), whereas 0.2 mM METH did not. In contrast, DA induced a slight induction of CHOP mRNA at the lower concentration than the IC50. Meanwhile, at 24 h after treatment, Grp78/Bip mRNA was not significantly induced under any conditions that we examined.

METH induces CHOP in the later phase

We examined the time course of METH and DA effects on the expression of CHOP and Grp78/Bip in CATH.a cells, because METH injection was reported to cause rapid induction of CHOP transcript in the mouse striatum by Jayanthi et al. (2004); in their experiment, the peak of CHOP expression occurred within 2 h after METH treatment, and the maximum expression level was about two-fold compared with the untreated striatum. We found that METH treatment of CATH.a cells caused a robust induction of CHOP expression at 48 h after METH

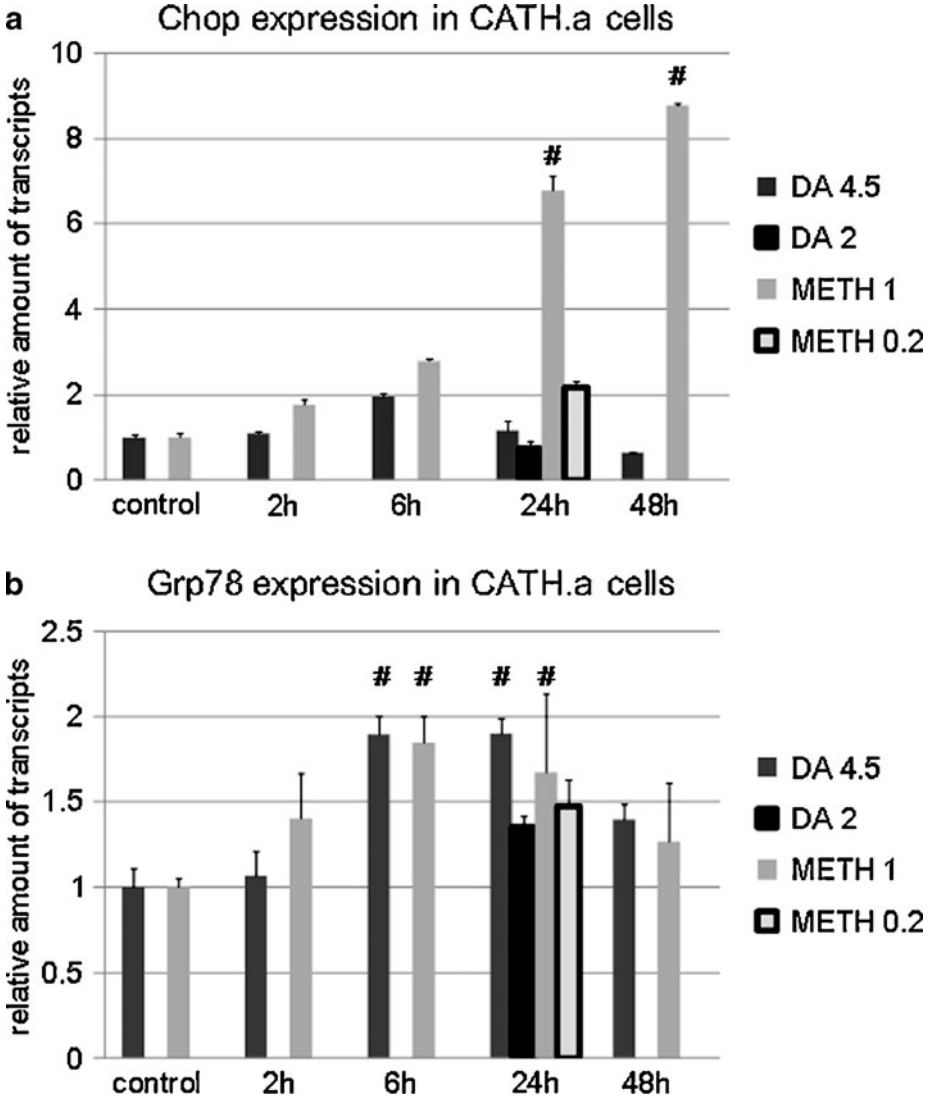

Fig. 3 Time course analysis of CHOP or Grp78 expression in CATH.a cells treated by METH or DA. CATH.a cells were treated with 0.2 mM METH, 1 mM METH, 2.0 μM DA, or 4.5 μM DA and harvested at the indicated time after treatment. Total RNA was isolated from each sample and subsequently reverse-transcribed. The resultant cDNA samples were subjected to quantitative RT-PCR to quantify CHOP (**a**) or Grp78 (**b**) gene expression. Results are expressed as relative amount (fold) of transcripts to control samples treated with solvent, normalized to Hprt1 transcripts. The entire set of experiments was repeated at least three times with RNA samples obtained independently from separate cultures. Each value represents the mean of four measurements of the sample from a representative experiment (*error bars* standard deviations; $^{\#}P<0.01$)

treatment. On the other hand, weak induction was observed in response to DA treatment in the early phase (Fig. 3a). We noted that Grp78/Bip, another marker gene related to ER stress, was only modestly induced by either METH or DA at 6 h after treatment (Fig. 3b).

METH increases ER stress marker proteins

In order to verify the ER stress to these proteins, we examined the effect of METH administration on CHOP and Grp78/Bip expression by using immunofluorescence microscopy. Vehicle-treated CATH.a cells were negative for CHOP expression, whereas treatment with 1 mM METH resulted in fluorescence signals located in the nucleus, indicating that CHOP expression was induced by ER stress (Fig. 4). At the time points of 24 h or 48 h after METH treatment, dying CHOP-positive cells with fragmented nuclei were observed (Fig. 4b). The expression of Grp78/Bip was similarly induced by treatment with METH (Fig. 4c). These results parallel the findings from the RNA analysis, i.e., that ER stress was induced by METH treatment.

Furthermore, we analyzed ER stress marker proteins by immunoblotting. The expression of Grp78/Bip protein was induced by treatment with METH (Fig. 5a, c). Concomitantly, the cleavage of caspase-12 (Wootz et al. 2004) was shown by the reduction in procaspase-12 levels (Fig. 5b, d).

Discussion

In the present study, we have shown that METH causes the induction of CHOP transcripts in CATH.a cells around 24–48 h after treatment. This induction has been observed

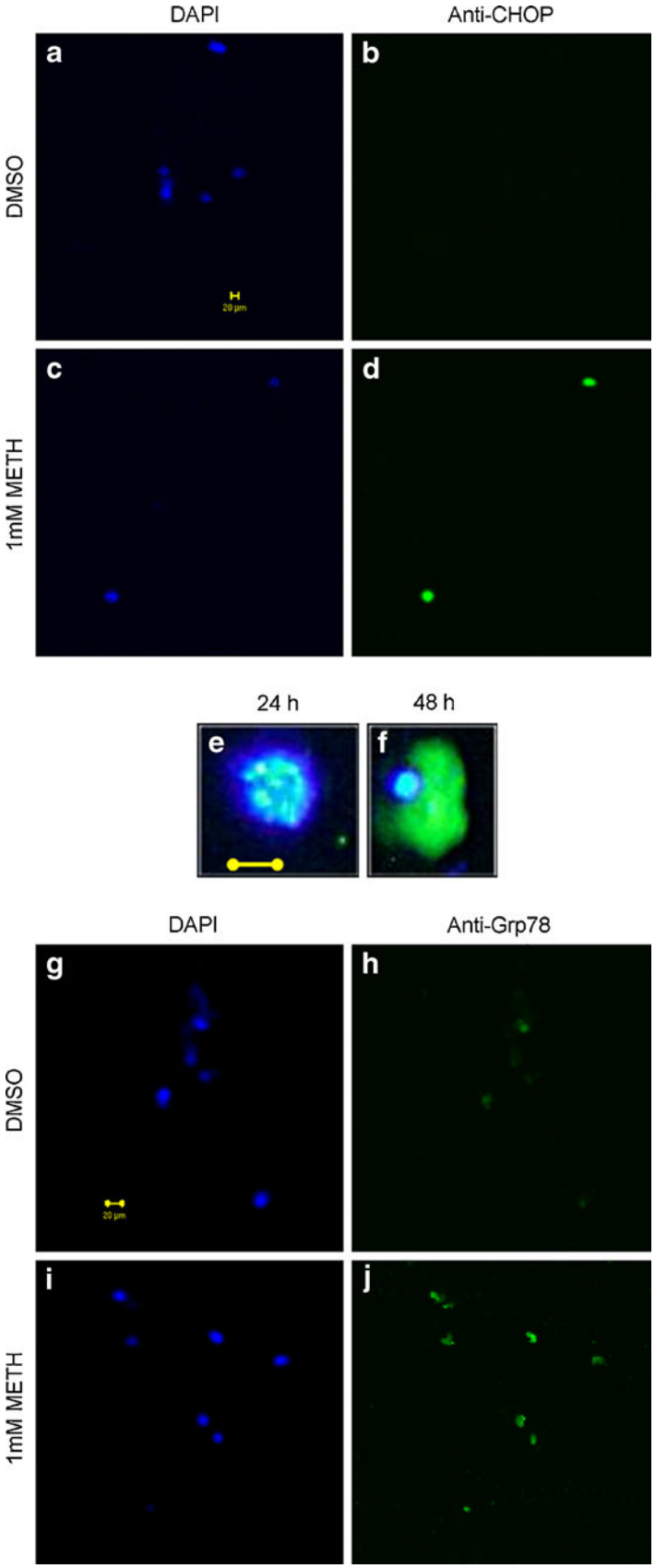

Fig. 4 METH–induced expression of ER stress marker proteins in CATH.a cells. CATH a cells were treated by 1 mM METH for 24 h unless indicated (*DMSO* dimethylsulfoxide). Subsequently, the cells were fixed and immunostained for CHOP or Grp78 followed by counter-staining with 4,6-diamidino-2-phenylindole (*DAPI*). Representative microphotographs showing the induction of CHOP (**b**, **d**–**f**) or Grp78 (**h**, **j**) in CATH.a cells treated with 1 mM METH. Note that dying cells expressed CHOP proteins (**e**, **f**). *Bar* 20 μm (**a**–**d**), (**g**–**j**), 10 μm (**e**, **f**)

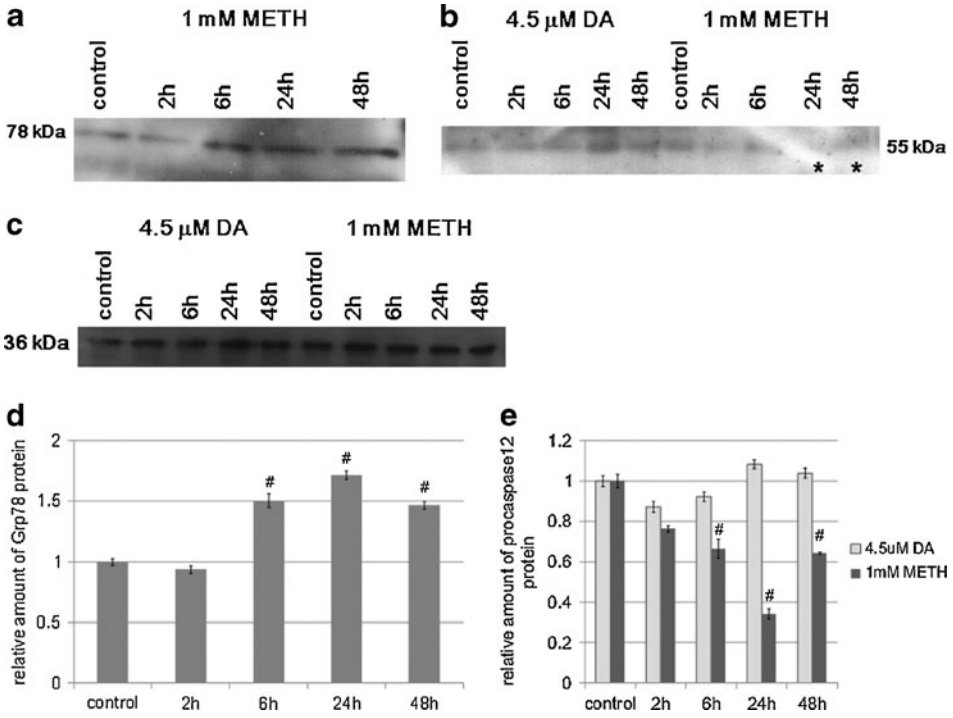

Fig. 5 METH–induced expression of Grp78/Bip and activation of caspase-12 in CATH.a cells. Immunoblotting of ER-stress-related proteins. Similar sets of experiments were repeated at least three times, and representative data are shown. **a** CATH.a cells were treated with 1 mM METH for the indicated times and analyzed by immunoblotting for Grp78/Bip. **b** CATH.a cells were treated with either 4.5 μM dopamine or 1 mM METH for the indicated times and analyzed by immunoblotting for procaspase-12 (*stars* reduction of procaspase-12 band representing the activation of caspase-12). **c** To monitor protein loading, the same amount of samples as in **a**, **b** were analyzed by immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **d**, **e** Relative band intensities were determined with ImageJ software. The quantified signals for Grp78/Bip (**d**) or caspase-12 (**e**) were normalized to that for GAPDH. A statistical analysis was performed; significant changes are marked ($^{\#}P<0.01$)

under conditions that cause cell death in a dose-dependent manner. Moreover, the amounts of both CHOP and Grp78/Bip proteins also increase after METH treatment. These findings suggested ER stress in CATH.a cells is caused by METH administration.

METH induces the release of DA from synaptic vesicles in the dopaminergic neuron in the brain (Fig. 6a). Dopamine excessively released in the synaptic cleft is oxidized outside the neuron. This oxidized DA causes the dysfunction of postsynaptic neurons. At the same time, METH causes a leakage of DA from synaptic vesicles, thereby eliciting a redistribution inside the neuron. Ectopically leaked DA is also quickly oxidized and triggers toxicity in the DA terminal, causing a dysfunction of presynaptic neurons. These two mechanisms, namely the presynaptic dysfunction and the postsynaptic dysfunction, eventually lead to a rise in the loss of DA synapses, which is currently recognized as one of the most important mechanisms of METH dependency.

We have also compared CATH.a cells treated with METH or DA (Fig. 6b). METH induces the release of DA from vesicles into the culture medium and causes the dysfunction of the CATH.a cells. This process serves as a model for the disturbance of the postsynaptic neuron. At the same time, the intracellularly leaked DA is also quickly oxidized and triggers toxicity in the CATH.a cells. This process mimics METH-induced disturbance of the presynaptic neuron. Therefore, METH treatment of CATH.a cells represents a complex model for the disturbance of the presynaptic and postsynaptic neurons. On the other hand, DA treatment of CATH.a cells causes a dysfunction of the cells via extracellularly oxidized DA. This represents a simple model for the disturbance of postsynaptic neurons. Consequently, a comparison of METH and DA treatment provides insights into the respective mechanisms of presynaptic and postsynaptic dysfunction in the process of METH addiction.

The excessively secreted DA into the synaptic cleft is thought to be oxidized and to cause toxicity in vivo, since the systemic administration of METH is reported to induce the apoptosis of striatum postsynaptic neurons (Cadet et al. 2005; Jayanthi et al. 2005). METH might cause the excessive secretion of DA in the stratum, and the secreted DA will be oxidized, producing reactive oxygen species

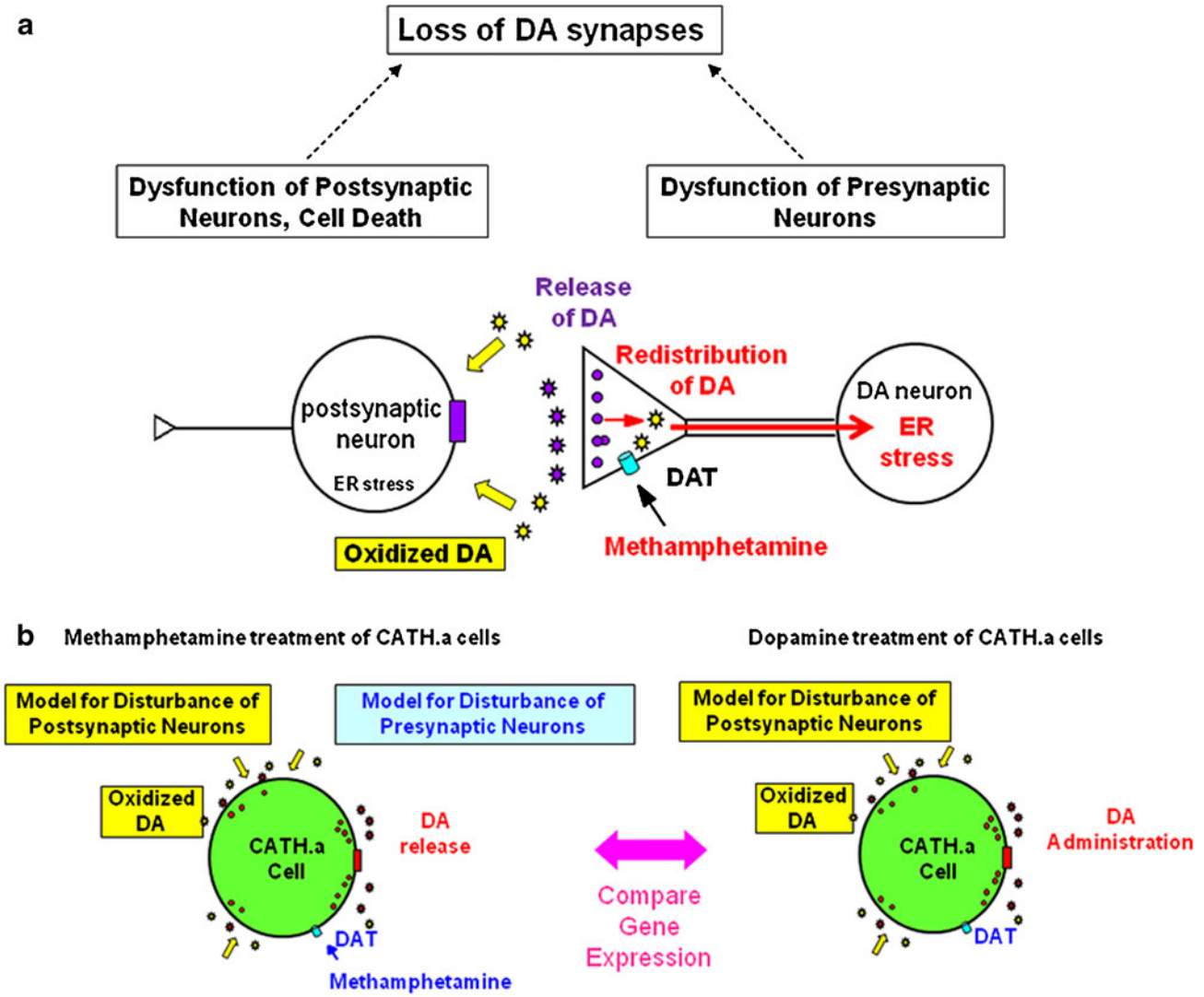

Fig. 6 Schematic model for dopamine (*DA*) terminal toxicity. **a** Represented model for METH toxicity on a DA terminal. METH induces the release of DA from synaptic vesicles (*purple circles*) in the dopaminergic neuron (*DA neuron*). Excessively released DA in the synaptic cleft (*purple stars*) is readily oxidized outside the neuron (*yellow stars outside* neuron). This oxidized DA causes the dysfunction of postsynaptic neurons. At the same time, METH encourages leakage of DA from synaptic vesicles eliciting redistribution inside the neuron (*small red arrow*). The ectopically leaked DA is also quickly oxidized and triggers ER stress in the DA neurons (*yellow stars inside* neuron) leadinng to the dysfunction of presynaptic neurons (*DA neuron*). These two mechanisms, namely the presynaptic dysfunction and the postsynaptic dysfunction, gradually increase the loss of DA synapses, one of the most important mechanisms of METH addiction. **b** Overview of current study as a comparison of METH and DA treatment. METH induces the release of DA from vesicles (*red circles*) in CATH.a cells. Excessively released DA in the culture medium (*red stars*) is readily oxidized outside the cell (*yellow stars*). This oxidized DA causes the dysfunction of the CATH.a cells (model for disturbance of postsynaptic neuron). At the same time, METH leads to the leakage of DA from synaptic vesicles eliciting redistribution inside the cell. The ectopically leaked DA is also quickly oxidized and triggers ER stress in the CATH.a cells causing the dysfunction of the cell (model for disturbance of presynaptic neuron). Therefore, METH treatment represents a complex model for disturbance of presynaptic and postsynaptic neurons. On the other hand, DA treatment of CATH.a cells causes the dysfunction of the cell via extracellularly oxidized DA (*yellow stars*). This represents a simple model for the disturbance of the postsynaptic neuron

(ROS) and other toxic materials. This might be the reason that METH induces the apoptosis of striatum neurons.

On the other hand, CATH.a cells treated with METH cause a redistribution of DA storage inside the cells, thereby mimicking presynaptic neurons (Fumagalli et al. 1999; LaVoie and Hastings 1999; Sulzer et al. 2005). Notably, the gene expression in response to DA redistribution occurs in the presynaptic neurons whose cell bodies are located in the ventral tegmental area (VTA) or the substantia nigra. In the present study, although both DA

and METH induce CHOP transcripts at the concentration of IC50, the time course and the magnitude of induction are different. The peak of CHOP induction occurs later than 24 h when CATH.a cells are treated by METH, whereas DA causes an earlier induction with a peak at 6 h after treatment. The magnitude of the induction by METH compared to that of a vehicle is more than six times, whereas it is no more than two-fold on treatment with DA (Fig. 3a). As the concentrations of DA and METH are their respective IC50s, METH probably causes apoptosis in the CATH.a cells via ER stress, and the cell death induced by DA might contain other mechanism of cytotoxicity in parallel. Collectively, our pivotal finding is that we have clearly shown the existence of ER stress in the model of dopaminergic presynaptic neurons for the first time (Fig. 6). Moreover, we have found that the CHOP protein is also up-regulated in CATH.a cells treated by METH and with Grp78/Bip protein (Figs. 4, 6). Furthermore, METH treatment causes the activation of Caspase-12 in CATH.a cells (Fig. 5). These findings also imply the generation of ER stress in CATH.a cells after METH administration. Although the effects of CHOP and Grp78/Bip on apoptotic cells are contradictory, the effect of CHOP seems predominant at higher concentrations than 1 mM as observed, because 1 mM METH caused 50% of CATH.a cells to die (Fig. 1a), and the CHOP-positive dying cells are observed after 24 h of treatment with 1 mM METH.

The probable trigger for ER stress generation by METH might be the redistribution of DA from vesicles to the cytoplasm. Auto-oxidation of cytoplasmic DA and the consequent generation of ROS have been reported to be involved in METH-induced neurotoxicity in dopaminergic neurons (Cadet and Brannock 1998; Kita et al. 2003). Recently, Miyazaki et al. (2006) have demonstrated that protein-bound quinone is increased in CATH.a cells after METH treatment, and that this phenomenon is correlated with cell death. This finding suggests the possibility that quinoprotein formation is one of the factors contributing to a generation of ER stress. Other factors might also trigger the formation of improperly folded proteins, such as the nitration of tyrosine residues increases after METH administration (Imam et al. 1999, 2001). This modification causes the alteration of protein function, enzymatic activity, and accordingly physiological process (Adewuya et al. 2003; Kuhn et al. 2004; Marcondes et al. 2001, 2006; Turko et al. 2001). Although the existence of activity to remove this potentially hazardous protein modification has been suggested, the accumulation of nitrated protein causes the death of dopaminergic neurons under certain conditions (Giasson et al. 2000; Irie et al. 2003; Kamisaki et al. 1998). Indeed, a powerful nitrating agent (peroxynitrite) is reported to cause ER stress (Dickhout et al. 2005).

Several genes mutated in familial Parkinson's disease have been shown to have functions linked to the ubiquitin–proteasomal pathway. For example, Parkin is one of the ubiquitinating enzymes (E3), whereas UchL1 is a deubiquitinating enzyme (Dawson and Dawson 2003). Furthermore, an increase of the ER stress response can promote the aggregation of wild-type α-synuclein, which forms inclusions that reproduce many morphological and biochemical characteristics of Lewy bodies (Jiang et al. 2010). Many previous studies (Wang and Takahashi 2007) suggest that ER stress induced by aberrant protein degradation is involved in Parkinson's disease. Yamamuro et al. (2006) have shown the involvement of ER stress in the cell death of SH-SY5Y neuroblastoma cells induced by 6-hydroxydopamine, an oxidized derivative of DA, which has been extensively used for the preparation of animal models of Parkinson's disease. Meanwhile, METH has been utilized to prepare animal models of Parkinson's disease (Betarbet et al. 2002). These studies suggest that CATH.a cells treated by METH will provide an in vitro model of Parkinson's disease.

At present, studies of METH toxicity are mainly focused on the apoptotic mechanism of post-synaptic neurons. However, we can assume that the degradation of the postsynaptic neuron precedes the degradation of the DA terminal on the basis of our present study and also of other recent evidence suggesting the importance of presynaptic toxicity, which consists of the auto-oxidation of cytosolic DA and the consequent generation of ROS (Cadet and Brannock 1998; Fumagalli et al. 1999; Kita et al. 2003; LaVoie and Hastings 1999); the degradation of the presynaptic terminal of the dopaminergic synapse might be the principal event of DA toxicity.

In conclusion, the present study has explicitly demonstrated the existence of ER stress in the model of dopaminergic presynaptic neurons for the first time. This finding should provide a new insight into the mechanisms of DA toxicity, which is currently accepted as being one of the most important mechanisms of methamphetamine toxicity and addiction.

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