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Tunicamycin-induced Unfolded Protein Response in the Developing Mouse Brain

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Abstract

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) causes ER stress, resulting in the activation of the unfolded protein response (UPR). ER stress and UPR are associated with many neurodevelopmental and neurodegenerative disorders. The developing brain is particularly susceptible to environmental insults which may cause ER stress. We evaluated the UPR in the brain of postnatal mice. Tunicamycin, a commonly used ER stress inducer, was administered subcutaneously to mice of postnatal day (PD) 4, 12 and 25. Tunicamycin caused UPR in the cerebral cortex, hippocampus and cerebellum of mice of PD4 and PD12, which was evident by the upregulation of ATF6, XBP1s, p-eIF2 α , GRP78, GRP94 and MANF, but failed to induce UPR in the brain of PD25 mice. Tunicamycin-induced UPR in the liver was observed at all stages. In PD4 mice, tunicamycin-induced caspase-3 activation was observed in layer II of the parietal and optical cortex, CA1-CA3 and the subiculum of the hippocampus, the cerebellar external germinal layer and the superior/inferior colliculus. Tunicamycin-induced caspase-3 activation was also shown on PD12 but to a much lesser degree and mainly located in the dentate gyrus of the hippocampus, deep cerebellar nuclei and pons. Tunicamycin did not activate caspase-3 in the brain of PD25 mice and the liver of all stages. Similarly, immature cerebellar neurons were sensitive to tunicamycin-induced cell death in culture, but became resistant as they matured *in vitro*. These results suggest that the UPR is developmentally regulated and the immature brain is more susceptible to ER stress.

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Keywords

Apoptosis; development; endoplasmic reticulum stress; protein degradation

Introduction

The endoplasmic reticulum (ER) is a subcellular organelle responsible for posttranslational protein processing and transport. Approximately one third of all cellular proteins are translocated into the lumen of the ER where posttranslational modification, folding and oligomerization occur. The ER is also the site for biosynthesis of steroids, cholesterol and other lipids. Cellular stress conditions, such as perturbed calcium homeostasis or redox status, elevated secretory protein synthesis rates, altered glycosylation levels and cholesterol overloading, can interfere with oxidative protein folding, leading to the accumulation of unfolded or misfolded proteins in the ER lumen. This causes ER stress and activates a compensatory mechanism, called the unfolded protein response (UPR) (Wang and Kaufman, 2012; Hetz *et al.*, 2013). UPR attempts to relieve ER stress by two major pathways: the first is to halt the translation of unfolded proteins and enhance endoplasmic reticulum-associated degradation (ERAD) of unfolded or misfolded proteins; the second is to increase the expression of molecular chaperones to facilitate proper protein folding (Walter and Ron, 2011; Logue *et al.*, 2013). However, when sustained or severe ER stress surpasses the capacity of UPR, apoptotic cell death occurs (Hetz *et al.*, 2013; Logue *et al.*, 2013).

ER stress and UPR participate in various physiological processes such as lipid and cholesterol metabolism, energy homeostasis, circadian function, cell surface signaling, development and cell differentiation (Rutkowski and Hegde, 2010; Walter and Ron, 2011; Hetz, 2012). ER stress and UPR are also involved in many human diseases and disorders, such as inflammation, metabolic disorders, cardiovascular diseases, diabetes, obesity and cancer (Yamamoto *et al.*, 2010; Wang and Kaufman, 2012; Hetz *et al.*, 2013). ER stress has been shown to play an important role in the pathogenesis of various neurological diseases (DeGracia and Montie, 2004; Scheper and Hoozemans, 2009; Vidal *et al.*, 2011; Xu and Zhu, 2012; Cornejo and Hetz, 2013; Endres and Reinhardt, 2013) and have been implicated in neurodegenerative processes in brain ischemia (Tajiri *et al.*, 2004), Alzheimer's disease (AD) (Katayama *et al.*, 2004), Parkinson's disease (PD) (Chen *et al.*, 2004; Silva *et al.*, 2005; Smith *et al.*, 2005), Huntington's disease (HD) (Hirabayashi *et al.*, 2001) and amyotrophic lateral sclerosis (ALS) (Turner and Atkin, 2006).

The developing brain is particularly susceptible to various environmental insults, e.g., exposure to pollutants, infectious pathogens, heavy metals, drugs, alcohol, physical stress and malnutrition. These environmental factors often cause ER stress and induce UPR (Qian and Tiffany-Castiglioni, 2003; Shin *et al.*, 2007; Hettiarachchi *et al.*, 2008; Ke *et al.*, 2011; Oh *et al.*, 2012; Kitamura, 2013; Pavlovsky *et al.*, 2013; Ji, 2014; Kalinec *et al.*, 2014) and ER stress may account for some of their detrimental effects. However, the mechanisms underlying CNS damage caused by these environmental insults are complex; it may be mediated by the interplay of multiple factors, such as direct toxicity, oxidative stress or disruption of cellular metabolism. To evaluate the impact of ER stress on the developing

brain, we need a model system which allows more specific induction of ER stress. Tunicamycin is an N-linked glycosylation inhibitor and is commonly used to induce ER stress experimentally. In this study, we evaluated tunicamycin-induced ER stress in the postnatal development of the mouse brain. We also studied tunicamycin-mediated neuroapoptosis.

Materials and Methods

Materials

Tunicamycin and mouse anti-glial fibrillary acidic protein (GFAP) antibody were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-ATF6 antibody was purchased from LifeSpan Biosciences (Seattle, WA). Rabbit anti-Xbp1s antibody was purchased from Biolegend (San Diego, CA). Rabbit anti-p-eIF2 α and cleavedcaspase-3 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Rabbit anti-GRP78 antibody was obtained from Santa Cruz Biotechnology (Dallas, Texas). Rat anti-GRP94 antibody was obtained from Enzo Life Sciences (Farmingdale, NY 11735). Rabbit anti-MANF antibody was purchased from Abcam (Cambridge, MA). Mouse antineuronal nuclei (NeuN) antibody was obtained from Millipore Corporate (Billerica, MA). Mouse anti-tubulin, HRP-conjugated anti-rabbit, anti-mouse and anti-rat secondary antibodies were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Biotin-conjugated anti-rabbit secondary antibodies and ABC kit were obtained from Vector Laboratories (Burlingame, CA). Alexa-488 conjugated anti-rabbit and Alexa-594 conjugated anti-mouse antibodies were obtained from Life Technologies (Grand Island, NY). Ketamine/xylazine was obtained from Butler Schein Animal Health (Dublin, OH). Other chemicals and reagents were purchased either from Sigma Chemical or Life Technologies.

Animals and tunicamycin treatment

C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN) and maintained in the Division of Laboratory Animal Resources of the University of Kentucky Medical Center. All procedures were performed in accordance with the guidelines set by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky. Tunicamycin was administered on postnatal day 4 (PD4), PD12 and PD25. The mice received two subcutaneous injections of tunicamycin at 3 $\mu\text{g/g}$ each and the injections were two hours apart. Tunicamycin was diluted in 150 mM dextrose at a concentration of 0.3 $\mu\text{g}/\mu\text{l}$. The concentration was selected based on previous studies of tunicamycin injection in adult animals (Krokowski, *et al.*, 2013; Lee, *et al.*, 2012; Puthalakath, *et al.*, 2007; Rosenbaum, *et al.*, 2014; Sammeta and McClintock, 2010; Yamamoto, *et al.*, 2010). Mice in the control group were injected with the same amount of dextrose without tunicamycin. Mice were weighed at 0 and 24 hours after the injection. Twenty four hours after the first injection, the mice were sacrificed, and the brains and livers were dissected and processed for further analysis.

Tissue preparation and immunoblotting

Mice were anesthetized by an intraperitoneal (IP) injection of ketamine/xylazine (100 mg/kg/10 mg/kg) and the brain and liver were dissected and immediately frozen in liquid nitrogen and then stored in -80°C . The protein was extracted and subjected to immunoblotting analysis as previously described (Ke *et al.*, 2011). Briefly, tissues were homogenized in an ice cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5% NP-40, 0.25% SDS, 5 $\mu\text{g/ml}$ leupeptin, and 5 $\mu\text{g/ml}$ aprotinin. Homogenates were centrifuged at 20,000g for 30 min at 4°C and the supernatant fraction was collected. After determining protein concentration, aliquots of the protein samples (30 μg) were separated on a SDS-polyacrylamide gel by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for one hour. Subsequently, the membranes were probed with primary antibodies overnight at 4°C . After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. The immune complexes were detected by the enhanced chemiluminescence substrate (PerkinElmer, Waltham, MA). In some cases, the blots were stripped and re-probed with an anti-tubulin antibody. The density of immunoblotting was quantified with the software of Quantity One (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry

The procedure for immunohistochemistry (IHC) has been previously described with some modifications (Ke *et al.*, 2011). Briefly, animals were deeply anesthetized with intraperitoneal injection of ketamine/xylazine and then intracardially perfused with PBS followed by 4% paraformaldehyde in PBS (pH 7.4). The brain tissues were removed, post fixed in 4% paraformaldehyde for an additional 24 hours and then transferred to 30% sucrose in PBS until the tissues sunk to the bottom. The tissues were frozen in OCT compound and sectioned at 40 μm in a sagittal plane using a freezing sliding microtome (Leica Microsystems, Wetzlar, Germany). Floating sections were incubated in 0.3% H_2O_2 /30% methanol in PBS for 10 min. After washing with PBS, sections were mounted on slides and dried. The slides were then blocked with 5% goat serum and 0.5% TritonX-100 in PBS for 1 hour at room temperature. After blocking, the slides were treated with a rabbit anti-cleaved caspase-3 antibody (1:8,000) overnight at 4°C . After washing with PBS, slides were incubated with biotin conjugated goat anti-rabbit secondary antibody (1:1,000) for 1 hour at room temperature and followed by washing with PBS. Avidin-biotin-peroxidase complex was prepared according to the manufacturer's instructions. The slides were incubated in the complex for 1 hour at room temperature. After rinsing, the slides were developed in 0.05% 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, Inc.) containing 0.003% H_2O_2 in PBS.

Immunofluorescent staining

The procedure for immunofluorescent staining has been previously described with some modifications (Wang *et al.*, 2007). Briefly, mice were anesthetized and perfused as described above. The brain sections were prepared at 15–20 μm thickness, mounted and

dried. After blocking with 5% goat serum and 0.5% TritonX-100 in PBS for 1 hour at room temperature, the slides were incubated with a rabbit anti-cleaved caspase-3 antibody together with anti-NeuN, anti-Iba-1 or anti-GFAP overnight at 4°C. After rinsing in PBS, the sections were incubated with Alexa Fluor488-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-mouse IgG in a dark at room temperature for 1 hour. For examining nuclear morphology, the tissues were incubated with a DNA dye, 4,6-diamidino-phenylindole (DAPI, 1 µg/ml in PBS) for 30 min. After a brief wash, the slides were covered with anti-fade mounting medium (Life Technologies, Grand Island, NY) and examined/recorded with a fluorescence microscopy (IX81, Olympus).

Culture and treatment of cerebellar granule neurons

Cultures of cerebellar granule neurons (CGNs) were generated from PD4 C57BL/6J mice using a previously described method (Wang *et al.*, 2007). Briefly, the pups were decapitated under deep anesthesia and cerebella were removed. The cerebella were suspended in 10 ml of 0.25% trypsin solution at 37°C. After incubation for 15 min, an equal volume of solution containing DNase (130 Kunitz units/ml) and trypsin inhibitor (0.75 mg/ml) were added. The tissue was dissociated by trituration, and the cell suspension was mixed with 4% bovine serum albumin and centrifuged. The cell pellet was resuspended in Neurobasal/B27 medium containing B27 (2%), KCL (25 mmol/L), glutamine (1 mmol/L), penicillin (100 units/mL) and streptomycin (100 µg/mL). Cells were plated into poly-D-lysine (50 µg/mL)-coated 96-well plates, and maintained at 37°C in a humidified environment containing 5% CO₂. We have previously used this culture system to investigate the *in vitro* development of neurons (Chen *et al.*, 2006; Wang *et al.*, 2007; Ke *et al.*, 2009). The neuronal population in this culture system was greater than 95%. After cultured in Neurobasal/B27 medium for 4, 7, or 15 days in vitro (DIV), CGNs were treated with 0.5 µg/ml tunicamycin for 24 hours. Cell viability was determined and quantified by 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Wang *et al.*, 2007).

Statistical Analysis

Quantitative data were presented as the means ± SD. Statistical comparisons were analyzed using ANOVA followed by Dunnett's test. The results with $p < 0.05$ were considered statistically significant.

Results

Tunicamycin induces ER stress in the developing mouse brain

Tunicamycin inhibits protein glycosylation and is a commonly used ER stress inducer. In this study, we injected tunicamycin subcutaneously on the back of mouse pups. Subcutaneous injection was selected over intraperitoneal injection (IP) to avoid the leaking of injected drugs. We did not observe any obvious changes in general behaviors within 24 hours of tunicamycin injection. However, there was a slight but statistically significant reduction of body weight in tunicamycin-treated mice in comparison to control mice (dextrose-treated group) (Fig. 1).

We examined the expression of UPR-related proteins in the cerebral cortex and cerebellum of mouse pups of from postnatal day 4 (PD4) to PD25 by immunoblotting (Figs 2–5). On PD4, tunicamycin induced a significant increase in the expression of XBP1s, p-eIF2 α , GRP78/BIP and GRP94 in the cerebral cortex and cerebellum. Tunicamycin also significantly increased the expression of MANF in the cortex but not in the cerebellum (Fig. 5F). On PD12, tunicamycin significantly increased the expression of XBP1s, p-eIF2 α , GRP78/BIP and GRP94 in the cerebral cortex and cerebellum. Tunicamycin increased the expression of processed ATF6 (50 KDa) in the cerebellum but not in the cortex (Fig. 5A). Generally, tunicamycin-induced an increase of UPR markers on PD12 was significantly less or tended to be less than that on PD4. For example, the tunicamycin-induced expression of XBP1s, GRP94 and MANF was significantly less on PD12 than PD4. Tunicamycin did not alter the expression of any of these UPR markers on PD25. The liver is susceptible to ER stress and was used as a positive control for UPR. Tunicamycin significantly increased all UPR markers tested except ATF6. Generally, tunicamycin-induced upregulation of UPR markers on PD25 was lower than that on PD4 and PD12 in the liver.

Tunicamycin induces caspase-3 activation in the developing brain

To determine whether tunicamycin caused apoptosis in the developing brain, we examined the expression of cleaved caspase-3 (active) by immunoblotting in the developing brain. We have previously demonstrated that cleaved caspase-3 is a reliable indicator for neuroapoptosis in the developing mouse brain (Liu *et al.*, 2009; Ke *et al.*, 2011; Alimov *et al.*, 2013). Our immunoblotting data indicated that tunicamycin increased the expression of cleaved caspase-3 only in the brain of PD4 mice (Figs. 2 and 5G). In the liver of all ages and the brain of PD12 or later, tunicamycin treatment did not alter the expression of cleaved caspase-3 (Figs 3, 4). Consistent with the immunoblotting data, IHC showed much more intensive immunoreactivity of cleaved caspase-3 in the brain of PD4 following tunicamycin treatment (Fig. 6). The tunicamycin-induced increase in caspase-3 immunoreactivity was observed in the cerebral cortex, cerebellum, hippocampus, and superior/inferior colliculus (SC/IC). In the cerebral cortex, tunicamycin-induced caspase-3 activation was mainly concentrated in the layer II of the parietal and optical cortex (Fig. 7A and 7B). We sought to determine the identity of cells undergoing apoptosis following tunicamycin injection and labeled neurons, astrocytes, microglia with anti-NeuN, GFAP and Iba1 antibodies, respectively. With available antibodies, we were unable to double-label NeuN and active caspase-3. We therefore used DAPI to examine nuclear morphology. DAPI showed condensed or fragmented nuclei in tunicamycin-treated animals, indicative of apoptosis. The condensed or fragmented nuclei were observed in NeuN positive cells (Fig. 7C), but not GFAP or Iba1-positive cells (data not shown), indicating that tunicamycin causes apoptosis in neurons but not in astrocytes or microglia. In the hippocampus, tunicamycin induced caspase-3 activation in the subiculum (SUB) and CA1-CA3 of PD4 mice (Fig. 8). In the cerebellum, tunicamycin-induced caspase-3 activation was mainly located in the external germinal layer (EGL) with some caspase-3 positive cells scattered in the internal granule layer (IGL) (Fig. 9).

Interestingly, despite the immunoblotting data shown, no difference was found in the expression of cleaved caspase-3 between control and tunicamycin-treated mice on PD12, the

IHC study demonstrated more intense immunoreactivity of active caspase-3 in the dentate gyrus (DG) of the hippocampus, deep cerebellar nucleus (DCN) and pons (Figs 8, 9 and 10). There was not difference in the immunoreactivity of caspase-3 on PD25 between tunicamycin-treated and dextrose-treated groups. At all developmental stages, tunicamycin did not cause caspase-3 activation in the liver. To verify that immature neurons were more susceptible to tunicamycin-induced cell death, we evaluated the effect of tunicamycin on cultured cerebellar neurons of different maturity. The cerebellar granule neurons (CGNs) were isolated from the cerebella of mice of PD4 and maintained for 4 days in vitro (DIV), 7 DIV and 15 DIV. As shown in Fig. 11, CGNs of 4 DIV were most susceptible to tunicamycin (0.5 µg/ml)-induced cell death, while CGNs of 15 DIV were resistant to tunicamycin-induced cell death. Therefore, the *in vitro* finding supports our *in vivo* observation that the susceptibility of neurons to tunicamycin diminishes as the brain matures.

Discussion

In this study, we developed a method to induce UPR in the brain of early postnatal mice by subcutaneous injection of tunicamycin. Tunicamycin has been previously used to induce ER stress in adult mice and rats (Reimertz *et al.*, 2003; Iwawaki *et al.*, 2004; Puthalakath *et al.*, 2007; Sammeta and McClintock, 2010; Yamamoto *et al.*, 2010; Lee *et al.*, 2012; Krokowski *et al.*, 2013; Obukuro *et al.*, 2013; Rosenbaum *et al.*, 2014). Intracerebroventricular (ICV) administration of tunicamycin was previously used to evaluate ER stress in the hippocampus and hypothalamus of adult mice (Ono *et al.*, 2012; Brennan *et al.*, 2013; Obukuro *et al.*, 2013). The amount of tunicamycin for ICV administration ranges from 0.04–0.5 µg. The IP injection of tunicamycin was also employed to induce ER stress in various organ systems including the CNS, such as liver, kidney, pancreas, olfactory sensory neurons and cerebral cortex in adult mice and rats (Puthalakath *et al.*, 2007; Sammeta and McClintock, 2010; Yamamoto *et al.*, 2010; Lee *et al.*, 2012; Krokowski *et al.*, 2013; Rosenbaum *et al.*, 2014). The concentration of tunicamycin for IP injection ranges from 0.5–10 µg/g. ICV administration of tunicamycin would be ideal to evaluate the effect of tunicamycin on the developing brain. However, developing mouse brain, especially the brain of PD4 is very small and fragile; ICV injection is difficult and the needle penetration causes damage to the brain, confounding the results. IP injection in mouse pups frequently causes leaking and is difficult to ensure the accuracy of drugs being injected. We therefore use subcutaneous (SC) injection to avoid these shortcomings. We have previously used this technique to administer ethanol to mouse pups and evaluate ethanol-induced ER stress in the developing brain (Ke *et al.*, 2011). We have evaluated various concentrations of tunicamycin (0.5, 1, 2, 3 µg/g) and treatment duration for 8 and 24 hours. The concentration and the duration used in this study (two injections of 3 µg/g, 24 hours) was based on the finding that this concentration causes significant UPR in the brain but does not induce drastic behavioral alteration or death.

Using the paradigm we developed, we demonstrate that tunicamycin induces UPR and region-selective apoptosis in the developing brain. Tunicamycin only induces UPR in the brain of PD4 and PD12 mice, but not in PD25 mice, suggesting that young animals are more susceptible to tunicamycin-induced ER stress in the CNS. Similarly, tunicamycin activates

caspase-3 in the brain of PD4 mice and PD12 mice, but not in PD25 mice. Tunicamycin-induced caspase-3 activation is region-selective. On PD4, tunicamycin-induced caspase-3 activation is mainly observed in cortex layer II of the parietal and occipital cortex. In the hippocampus, tunicamycin-induced caspase-3 activation is shown in CA1-CA3 and SUB. In the cerebellum, tunicamycin-induced caspase-3 activation is mainly localized in the EGL with some scattered caspase-3 positive cells distributed in the IGL. The mechanisms underlying region-specific activation of caspase-3 are currently unknown. It may be caused by region-related difference in maturation process or neuron subtypes. In future studies, it would be interesting to determine whether tunicamycin-induced activation of caspase-3 and UPR-associated proteins co-localize in the same cell population. It is generally thought that the UPR is a protective response to mitigate ER stress-induced cellular injury. Induction of UPR has been used to alleviate ischemia and hemorrhage-induced brain injury (Yan *et al.*, 2014; Zhang *et al.*, 2014). In this regard, cells that display UPR would be more resistant to tunicamycin-induced cell death and apoptosis markers would possibly be located in neurons deficient of UPR. It is also possible that the high UPR is indicative of intensive ER stress which would induce apoptosis in neurons that the UPR is not sufficient to alleviate ER stress.

For PD12 mice, our immunoblotting analysis fails to reveal tunicamycin-induced caspase-3 activation, but IHC data shows caspase-3 activation in some specific brain regions, such as the DG of the hippocampus, pons and DCN. This may be due to weak and more confined tunicamycin-induced caspase-3 activation on PD12, so that immunoblotting analysis was not sensitive enough for detection. On PD25, tunicamycin fails to induce caspase-3 activation. This *in vivo* observation is supported by *in vitro* study which showed that neurons became resistant to tunicamycin-induced cell death as they matured in culture. Interestingly, tunicamycin did not cause apoptosis in the liver of all stages at the concentration applied, suggesting that the liver has a more efficient protective mechanism and more tolerant to ER stress-induced apoptosis than immature brain.

It is now known that ER stress and UPR play an important role in neural development. In the CNS, the expression of UPR-related proteins is developmentally regulated and ER stress may affect neural development (Zhang *et al.*, 2007; Alimov *et al.*, 2013). ER stress causes aberrant neuronal differentiation and inhibition of dendrite outgrowth in neural stem cells (NSCs) (Kawada *et al.*, 2014). Wolfram Syndrome (WFS) is a rare autosomal recessive disease characterized by insulin-dependent diabetes mellitus, optic nerve atrophy, diabetes insipidus, deafness, and neurological dysfunction leading to death in mid-adulthood. WFS is caused by mutations in the WFS1 gene, which leads to disturbances of ER calcium homeostasis and ER stress-mediated cell death. People with WFS have a pronounced impact on early brain development in addition to later neurodegeneration (Hershey *et al.*, 2012). ER chaperones regulate the survival and vulnerability of Purkinje cells of the cerebellum during development, suggesting that ER stress affects Purkinje cell development (Kitao *et al.*, 2004). Zhang *et al.* (2007) reported that several ER chaperones and UPR related proteins are expressed at higher levels in the embryonic brain and retina than in adult tissues. ER stress-related apoptotic pathway components, such as caspase-7 and caspase-12, are more abundant in embryonic brains than in adult brains. ER stress-like mechanisms may

participate in naturally occurring neuroapoptosis and regulate embryonic development of the CNS (Zhang *et al.*, 2007).

Our findings that the developing brain may be more susceptible to ER stress have several implications. First, it suggests that ER stress may be accountable for at least some of the brain damage caused by developmental exposure to pollutants, infectious pathogens, heavy metals, drugs, alcohol, physical stress and malnutrition. For example, the developing CNS is particularly susceptible to ethanol exposure; developmental ethanol exposure induces a spectrum of disorders called fetal alcohol spectrum disorders (FASD). Ethanol-induced neurodegeneration underlies many of the behavioral deficits observed in FASD (Luo, 2012). Ethanol exposure causes drastic neuroapoptosis in the brain of early postnatal mice (between PD4–12) (Liu *et al.*, 2009; Alimov *et al.*, 2013); this is the same period that mice are sensitive to tunicamycin-induced UPR and caspase-3 activation. Similarly, immature CGNs are sensitive to ethanol-induced cell death but become resistant to ethanol as they mature *in vitro* (Luo *et al.*, 1997). Ethanol induces ER stress in neurons *in vitro* and in the developing brain (Ke *et al.*, 2011). It is therefore possible that ER stress contributes to ethanol-induced neurodegeneration in the developing CNS. Second, since tunicamycin-induced UPR and neuroapoptosis exhibit spatiotemporal specificity in the developing brain, this offers an excellent model system to investigate the mechanisms of the UPR in neurons. Third, if UPR is a protective mechanism for CNS injury, by systematic analysis of the dosage and the timing of tunicamycin administration, this model system can be optimized to evaluate neuroprotection by UPR in the developing brain.

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Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
CGNs	cerebellar granule neurons
DAB	3,3'-diaminobenzidine
DAPI	4,6-diamidino-phenylindole
DCN	deep cerebellar nucleus
DG	dentate gyrus
DIV	days in vitro
EGL	external germinal layer
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation

FASD	fetal alcohol spectrum disorders
GFAP	glial fibrillary acidic protein
HD	Huntington's disease
IACUC	Institutional Animal Care and Use Committee
ICV	Intracerebroventricular
IGL	internal granule layer
IP	intraperitoneal injection
MTT	3-(4,5-dimethyl-thiazol-2yl)-2,5 diphenyltetrazolium bromide
NSCs	neural stem cells
PD	postnatal day
SC	subcutaneous
SUB	subiculum
UPR	unfolded protein response
WFS	Wolfram Syndrome

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Highlights

- Tunicamycin caused a development-dependent UPR in the mouse brain.
- Immature brain was more susceptible to tunicamycin-induced endoplasmic reticulum stress.
- Tunicamycin caused more neuronal death in immature brain than mature brain.
- Tunicamycin-induced neuronal death is region-specific.

Figure 1. Effect of tunicamycin on the body weight of postnatal mice

Mice of postnatal day 4 (PD4), PD12 and PD25 mice were injected subcutaneously with tunicamycin (Tun, 3 μ g/g in 150 mM dextrose) or 150 mM dextrose (Control), at 0 and 24 hours as described in Materials and Methods. Body weight was measured at 0 and 24 hours after first injection. * $p < 0.05$; *** $p < 0.001$ in comparison to controls.

Figure 2. Effect of tunicamycin on the expression of UPR-related proteins and cleaved caspase-3 in mice of PD4

Mice of PD4 were injected subcutaneously with tunicamycin (Tun, 3 μ g/g in 150 mM Dextrose) or 150 mM dextrose (control) at 0 and 2 hours as described in Materials and Methods. At 24 hours after the first injection, the cerebral cortex, cerebellum and liver was dissected for examining the expression of ATF6, Xbp1s, p-eif2 α , GRP78, GRP94, mesencephalic astrocyte-derived neurotrophic factor (MANF) and cleaved caspase-3 were detected by immunoblotting.

Figure 3. Effect of tunicamycin on the expression of UPR-related proteins and cleaved caspase-3 in mice of PD12

Notations are the same as Fig. 1.

Figure 4. Effect of tunicamycin on the expression of UPR-related proteins and cleaved caspase-3 in mice of PD25

Notations are the same as Fig. 1.

Figure 5A

Figure 5B

Figure 5C

Figure 5D

Figure 5E

Figure 5F

Figure 5G

Figure 5. Tunicamycin-induced alterations in UPR-related proteins and cleaved caspase-3
 The expression of ATF6 (A), Xbp1s (B), p-eif2 α (C), GRP78 (D), GRP94 (E), MANF (F) and cleaved caspase-3 (G) analyzed immunoblotting quantified by densitometry and normalized to the expression of tubulin. The results are displayed as mean $\pm$ SD of 3 independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ in comparison to controls.

Figure 6. Tunicamycin-induced activation of cleaved caspase-3 in the brain of postnatal mice Mice of PD4, PD12 and PD25 were injected subcutaneously with tunicamycin (Tun) as described above. Mice were sacrificed 24 hours after the injection. The expression of cleaved caspase-3 in the brain was analyzed by immunohistochemistry (IHC) as described in Materials and Methods. CTX, cortex; CB, cerebellum; Hipp, hippocampus; SC, superior colliculus; IC, inferior colliculus; DCN, deep cerebellar nuclei. Scale bar = 1 mm.

Figure 7A

Figure 7B

Figure 7C

Figure 7. Tunicamycin-induced activation of cleaved caspase-3 in the cerebral cortex of postnatal mice

A: Mice of PD4, PD12 and PD25 were treated as described above and the expression of cleaved caspase-3 in the cerebral cortex was analyzed by IHC. **B:** Images of higher magnification show cleaved caspase-3 positive cells in layer II of parietal cortex of PD4 mice. Scale bar = 100 μ m. **C:** Images show condensed or fragmented nuclei (DAPI) in

neurons (NeuN-positive) of the parietal cortex following tunicamycin treatment. Scale bar = 10 μ m.

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Figure 8. Tunicamycin-induced activation of cleaved caspase-3 in the hippocampus of postnatal mice

Mice of PD4, PD12 and PD25 were treated as described above and the expression of cleaved caspase-3 in the hippocampus was analyzed by IHC. CA1 and CA3, Cornu Ammonis (CA) 1 and 3; SUB, subiculum; DG, dentate gyrus. Scale bar = 100 μ m.

Figure 9A

Figure 9B

Figure 9C

Figure 9. Tunicamycin-induced activation of cleaved caspase-3 in the cerebellum of postnatal mice

A: Mice of PD4, PD12 and PD25 were treated as described above and the expression of cleaved caspase-3 in the cerebellum was analyzed by IHC. IC; Inferior colliculus; CB, cerebellum; DCN, Deep Cerebellar Nuclei. **B:** Images of higher magnification show cleaved caspase-3 positive cells in the cerebellar cortex. EGL, external germinal layer; ML, molecular layer; PCL, Purkinje cells layer; IGL, internal granule layer. **C:** Images of higher magnification show cleaved-caspase 3-positive cells in the DCN. Scale bar = 100 μ m.

Figure 10. Tunicamycin-induced activation of cleaved caspase-3 in the pons of postnatal mice Mice of PD4, PD12 and PD25 were treated as described above and the expression of cleaved caspase-3 in the pons was analyzed by IHC. Scale bar = 100 μ m.

Figure 11. Tunicamycin-induced death of cerebellar neurons in culture

Cerebellum granule neurons (CGNs) were isolated from mice of PD4 and cultured for 4, 7 or 15 days *in vitro* (DIV). After that, cells were exposed to tunicamycin (0.5 $\mu\text{g/ml}$) for 24 hours. Cell viability was determined by MTT as described in the Materials and Methods. The tunicamycin-induced cell death was normalized to age-matched control cultures. Each data point is the mean $\pm$ SD of three independent experiments. * $p < 0.05$; denotes significant difference from age-matched control cultures. # $p < 0.05$; denotes significant difference between 4 and 7 DIV.