Spatiotemporal analysis of the UPR transition induced by methylmercury in the mouse brain

Hideki Hiraoka[1], Ryosuke Nomura[1], Nobumasa Takasugi[1], Ryoko Akai[2], Takao Iwawaki[2], Yoshito Kumagai[3], Masatake Fujimura[4], and Takashi Uehara[1]

[1]*Department of Medicinal Pharmacology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan*

[2]*Division of Cell Medicine, Department of Life Science, Medical Research Institute, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Kahoku, Ishikawa 920-0293, Japan.*

[3]*Environmental Biology Laboratory, Faculty of Medicine, University of Tsukuba, Ibaraki 306-8575, Japan*

[4]*Department of Basic Medical Science, National Institute for Minamata Disease, Kumamoto 867-0008, Japan.*

*To whom correspondence should be addressed: Takashi Uehara, Ph.D., email: uehara-t@okayama-u.ac.jp;

Abstract

Methylmercury (MeHg), an environmental toxicant, induces neuronal cell death and injures a specific area of the brain. MeHg-mediated neurotoxicity is believed to be caused by oxidative stress and endoplasmic reticulum (ER) stress, but the mechanism by which those stresses lead to neuronal loss is unclear. Here, by utilizing the ER stress-activated indicator (ERAI) system, we investigated the signaling alterations in the unfolded protein response (UPR) prior to neuronal apoptosis in the mouse brain. In ERAI transgenic mice exposed to MeHg (25 mg/kg, S.C.), the ERAI signal, which indicates activation of the cytoprotective pathway of the UPR, was detected in the brain. Interestingly, detailed *ex vivo* analysis showed that the ERAI signal was localized predominantly in neurons. Time course analysis of MeHg exposure (30 ppm in drinking water) showed that whereas the ERAI signal was gradually attenuated at the late phase after increasing at the early phase, activation of the apoptotic pathway of the UPR was enhanced in proportion to the exposure time. These results suggest that MeHg induces not only ER stress but also neuronal cell death *via* a UPR shift. UPR modulation could be a therapeutic target for treating neuropathy caused by electrophiles similar to MeHg.

Keywords

Methylmercury, neuronal cell death, ER stress, UPR, ERAI gene

Introduction

Methylmercury (MeHg), a causative factor of Minamata disease, is a neurotoxic environmental material. Inorganic mercury is released in the air by various sources, especially volcanic activity or gold mining. After deposition in the ocean, mercury is transformed into MeHg by microorganisms. Due to accumulation of MeHg through biocondensation, consumption of edible fishes such as tuna is the main source of MeHg exposure in humans (Mahaffey et al. 2004). MeHg readily crosses the blood-brain barrier and shows selective toxicity to cause neuronal loss, injuring mainly regions of the cerebellum and cerebral cortex, such as the postcentral gyrus, Heschl's gyrus, and calcarine sulcus, in Minamata disease (Eto and Takeuchi 1978; Kerper et al. 1992). Furthermore, several reports suggest that MeHg is involved in the onset of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Fujimura et al. 2009; Weiss et al. 2002). However, the detailed mechanism of the site- and cell-specific neurotoxicity mediated by MeHg is incompletely understood.

With high affinity for nucleophiles, MeHg binds covalently with the sulfhydryl group in cysteine residues of proteins (*S*-mercuration), which leads to dysfunction of the proteins (Kanda et al. 2014). Previous reports suggest that MeHg neurotoxicity is attributed to oxidative stress caused by *S*-mercuration of manganese superoxide dismutase and glutathione (GSH) (Kumagai et al. 1997; Mori et al. 2007). Additionally, several reports suggest that the induction of oxidative stress by MeHg is an initial response, and subsequent endoplasmic reticulum (ER) stress causes the cytotoxicity (Liu et al. 2019; Usuki et al. 2008). ER stress occurs under various abnormal conditions, such as a buildup of misfolded proteins, depletion of Ca^{2+} stores in the ER, and oxidative stress (Bhandary et al. 2013). Previously, we revealed that the chaperoning activity of protein disulfide isomerase (PDI) was reduced by *S*-mercuration, resulting in the accumulation of

misfolded proteins in the ER (Makino et al. 2014).

During ER stress, cells maintain protein homeostasis *via* the unfolded protein response (UPR) (Walter and Ron 2011). The UPR contains three representative pathways mediated by ER membrane proteins; inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Under mild ER stress, the UPR ameliorates ER stress by inhibiting the translation of proteins but enhancing the expression of molecular chaperones and components of ER-associated degradation (ERAD). In contrast, under unresolved ER stress, the UPR initiates apoptosis to induce C/EBP homologous protein (CHOP), which is the main factor in ER stress-mediated apoptosis. As mentioned above, the UPR has bipolar functions; however, the precise switching mechanism from the protective pathway to the apoptotic pathway remains elusive.

By sensing ER stress, IRE1α self-activates by autophosphorylation and splices X-box binding protein 1 (*XBP1*) mRNA independent of the spliceosome. We previously elucidated that electrophiles such as MeHg and nitric oxide cause a decline in *XBP1* mRNA splicing and induce apoptosis *in vitro* (Hiraoka et al. 2017; Nakato et al. 2015). Additionally, UPR dysfunction has been suggested to contribute to the onset of neurodegenerative disease (Remondelli and Renna 2017). Thus, we predicted that functional modulation of the UPR by electrophiles might facilitate neuronal cell death and lead to the development of various nervous system diseases, such as Minamata disease, AD, and PD. However, whether this event occurs *in vivo* is uncertain.

Here, we utilized ER stress-activated indicator (ERAI) transgenic mise, in which induction of ER stress can be monitored precisely *in vivo*, and analyzed the temporal change in UPR pathways activated by MeHg-induced ER stress.

Materials and methods

Animals

Male ERAI-luciferase (LUC) mice and male ERAI-Venus mice were generated as described previously (Iwawaki et al. 2004, 2009) and were maintained on a C57BL/6 background. C57BL6N/Jc1 mice were purchased from Japan Clear (Tokyo, Japan) and housed in the National Institute for Minamata Disease. Ten-week-old ERAI-LUC mice were used for *in vivo* and *ex vivo* imaging. Seven-week-old ERAI-Venus mice were used for immunohistochemistry. All procedures were approved by the animal studies committees at Kanazawa Medical University and the National Institute for Minamata Disease (2017-51 and 310207, respectively).

MeHg administration

ERAI-LUC mice were injected subcutaneously with MeHg chloride (Tokyo Chemical Industry, Tokyo, Japan) dissolved in physiological saline at a dose of 25 mg/kg as previously described (Iwai-Shimada et al. 2016). Tunicamycin (Sigma-Aldrich, St. Louis, USA) was injected subcutaneously at a dose of 0.5 mg/kg as a positive control.

ERAI-Venus mice were randomly divided into the control (n=6) and MeHg-exposed groups (n=5-6 mice/group). Mice were exposed to MeHg *via* drinking water containing 30 ppm MeHg as MeHg-GSH (FUJIFILM Wako Pure Chemical, Osaka, Japan) (1:1) complex as previously described (Fujimura et al. 2009). ERAI-Venus mice in the control group were provided GSH-containing water.

In vivo and *ex vivo* imaging

Detection of ERAI-derived bioluminescence signals was described previously (Iwawaki et al. 2017). ERAI-LUC mice were injected intraperitoneally with luciferin (Summit Pharmaceuticals

International, Tokyo, Japan) (150 g/kg in physiological saline) for 10 min before imaging. Mice were anesthetized with isoflurane and analyzed with an IVIS (Perkin Elmer, Waltham, USA). After *in vivo* imaging, mice were sacrificed, and their brains, hearts, livers, kidneys, pancreas, and muscles were surgically removed immediately. These organs were immersed in 0.3 mg/mL luciferin diluted with phosphate-buffered saline (PBS) and analyzed by IVIS. Bioluminescence signals were measured for 60 sec/sample.

Measurements of mercury deposition

ERAI-Venus mice were sacrificed at the indicated times, and the cerebral cortex was removed from their right brains. Tissues were dissolved with 5 N NaOH (Nacalai Tesque, Kyoto, Japan) solution and boiled at 70 °C for 30 min. After neutralization with 5 N HCl (Nacalai Tesque, Kyoto, Japan), the total concentration of mercury was measured by the oxygen combustion-gold amalgamation method using an MA2000 analyzer (Nippon Instruments Corporation, Tokyo, Japan).

Immunohistochemistry

ERAI-Venus mice were sacrificed, and their left brains were extracted and fixed with 4 % paraformaldehyde (FUJIFILM Wako Pure Chemical, Osaka, Japan) in PBS. After embedding in paraffin, the brains were sliced into 5-μm coronal sections using a microtome. Paraffin-embedded sections were dewaxed with xylenes, rehydrated through successive ethanol washes and boiled for 20 min in pH 6.0 citrate buffer (Genemed Biotechnologies, South San Francisco, USA) for antigen retrieval. Immunofluorescence staining was performed using a M.O.M. immunodetection kit (#FMK-2201, Vector Laboratories, Burlingame, USA) with the following antibodies: anti-green fluorescent protein (GFP) (1:200; #M048-3, Medical & Biological Laboratories, Nagoya,

Japan), anti-neuronal nuclei (NeuN) (1:200; #MAB377, Merck Millipore, Burlington, USA), anti-glial fibrillary acidic protein (GFAP) (1:100; #M0761, Dako, Santa Clara, USA) and anti-CHOP (1:1000; #ab11419, Abcam, Cambridge, UK). Anti-ionized calcium binding adaptor molecule 1 (Iba1) (1:1000; #019-19741, FUJIFILM Wako Pure Chemical, Osaka, Japan) and anti-HMG-CoA reductase degradation enzyme 1 (HRD1) (1:500; #sc-130889, Santa Cruz Biotechnology, Dallas, USA) antibodies were diluted in PBS with 5 % BSA and incubated overnight at 4 ℃. After one wash with PBS, a goat anti-rabbit IgG Alexa Fluor 594 (1:200; #A-11012, Thermo Fisher Scientific, Waltham, USA) secondary antibody was applied and incubated for one hour at room temperature. Nuclei were stained with a mountant containing DAPI (Thermo Fisher Scientific, Waltham, USA). All images were acquired with a BZ-X810 microscope (Keyence, Osaka, Japan) and analyzed with ImageJ software (NIH, Bethesda, USA).

Statistical analysis

All results are expressed as the mean ± S.E.M. values. Statistical comparisons were performed by one-way or two-way ANOVA followed by Dunnett's or Tukey's test. When appropriate, a two-tailed Student's *t*-test was performed. All data were analyzed with GraphPad Prism 8 (GraphPad Software, San Diego, USA). Statistical significance is represented as $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results

Live imaging of acute ER stress induction by MeHg

To perform *in vivo* imaging of ER stress occurrence, we utilized ERAI-LUC mice (Iwawaki et al. 2009). Under ER stress conditions, the *XBP1* region of the ERAI gene is spliced by IRE1α, which causes a frameshift resulting in expression of XBP1 fused to LUC (Fig. S1a). Using this model mouse, we can monitor the tissue-specific occurrence of ER stress *in vivo* by bioluminescence. We confirmed that administration of the ER stressor tunicamycin (0.5 mg/kg, S.C.) induced luminescence signals in several areas in the model mice (Fig. 1a). Consistent with a previous study (Iwawaki et al. 2004), the pancreas and skeletal muscle, in which ER stress always occurred, showed luminescence at baseline (Figs. S1b and c).

To gain insight into the correlation between MeHg and ER stress, we injected MeHg (25 mg/kg, S.C.) into ERAI-LUC mice. After 24 h, we subjected the mice to whole-body imaging and detected increased luminescence signals in several areas, including the head region (Fig. 1a). To identify organs with induced ER stress, we removed several organs from ERAI-LUC mice and analyzed them by *ex vivo* imaging. The ERAI signal in MeHg-exposed mice showed an increasing trend strongly in the liver (3.6-fold) and weakly in the brain (1.3-fold), heart (1.2-fold), and kidney (1.4-fold) (Fig. 1b and Table 1). These organs have high blood circulation and are thus subjected to high exposure to MeHg, which may result in ER stress induction. These *in vivo* data indicate that MeHg induces tissue-specific ER stress.

Time course and spatiotemporal analysis of ER stress induction by MeHg

MeHg has been reported to induce the expression of the ER stress marker BiP in the cerebral cortex (Zhang et al. 2013). However, the regions and types of cells in the cerebral cortex in which MeHg-induced ER stress occurs remain elusive. The requirement for oxygen supply for the

luminescence reaction in ERAI-LUC mice makes performing further detailed *ex vivo* analysis difficult. To overcome this limitation, we used ERAI-Venus mice, in which ER stress is indicated by the XBP1ΔDBD-Venus fluorescence signal (Iwawaki et al. 2004). To exclude artificial transcriptional regulation by XBP1-Venus, the DNA-binding domain of XBP1 is deleted in this model (Iwawaki et al. 2004). As observed in the previous study, we detected cytosolic expression of XBP1ΔDBD-Venus, which lacks a nuclear localization signal, in the somatosensory cortex of ERAI-Venus mice exposed to MeHg for 3 weeks (Fig. S2a).

In a previous study, after exposure to 30 ppm MeHg for 8 weeks *via* drinking water, neuronal cell death was observed in the cerebral cortex of wild-type C57BL6N/Jc1 mice (Fujimura et al. 2009). To clarify whether the occurrence of ER stress is directly linked to neuronal apoptosis, we investigated the temporal response to MeHg over a 6-week period in ERAI-Venus mice (Fig. 2a). No significant difference was observed in body weights between ERAI-Venus mice and wild-type (WT) mice after MeHg exposure (Fig. S2b). Because the most common neurological symptom of MeHg poisoning is sensory dysfunction (Maruyama et al. 2012; Ninomiya et al. 2005), we first focused on the somatosensory cortex. Time course analysis revealed that the increase in the ERAI fluorescence signal induced by MeHg was transient and peaked after 3 weeks of exposure (Figs. 2b and c). These lines of evidence suggest that there is a time lag between the occurrence of MeHg-induced ER stress and the induction of neuronal cell death.

Differential sensitivity to MeHg-induced ER stress across brain areas

MeHg injures several regions of the cerebral cortex, such as the somatosensory cortex, auditory cortex, visual cortex, and motor cortex (Eto and Takeuchi 1978; Fujimura et al. 2009). However, few reports have shown the difference in MeHg susceptibility between these regions. Thus, we further assessed the temporal change in the ERAI fluorescence signal in each region. Interestingly,

the strongest fluorescence signals followed the order of the auditory cortex, somatosensory cortex, and visual cortex or motor cortex, suggesting that these areas exhibit differential sensitivity to MeHg-induced ER stress (Figs. 3a-c and e). Consistent with a previous report indicating that MeHg damages the striatum in mice (National Institute for Minamata Disease (NIMD) 2006), we revealed that the number of ERAI-positive cells was increased in the striatum at the late phase of MeHg exposure (Fig. 3d). These results indicate that MeHg-induced ER stress occurs in several regions of the brain, but those regions have differential susceptibility.

Identification of cell types exhibiting MeHg-induced ER stress

To define the cell types showing an ERAI fluorescence signal induced by MeHg, we used dual immunohistochemical staining. A previous study showed that the number of GFAP-positive astrocytes and Iba1-positive microglia were increased at the late phase of MeHg administration (Fujimura et al. 2009). Moreover, the expression of GFAP and Iba1 was increased in ERAI-Venus mice exposed to MeHg for 6 weeks (Figs. S3a and b). Hence, we collected brain sections from mice exposed to MeHg for 3 weeks or 6 weeks and analyzed the coexpression of ERAI with NeuN, GFAP and Iba1, separately. In ERAI-Venus mice exposed to MeHg, almost all ERAI-positive cells coexpressed NeuN (Figs. 4a and d). On the other hand, a weak ERAI signal was observed in Iba1-positive microglia (Figs. 4b and d); this coexpression may result from phagocytosis of ERAI-positive neurons by microglia. Moreover, the ERAI signal was rarely observed in GFAP-positive astrocytes (Figs. 4c and d). Our results indicate that the increase in the ERAI fluorescence signal induced by MeHg occurs predominantly in neurons.

Activation of UPR branches by MeHg exposure

Spliced XBP1 induces the expression of molecular chaperones or ERAD-related proteins to

alleviate ER stress (Yoshida et al. 2001, 2003). Thus, to confirm whether MeHg exposure activates downstream signaling of the IRE1α-XBP1 axis, we investigated the expression of the ERAD-related E3 ubiquitin ligase HRD1 (Kaneko et al. 2002). Similar to the ERAI signal patterns indicated in Fig. 2c, ERAI-Venus mice showed transient upregulation of HRD1 after exposure to MeHg (Figs. 5a and b). Additionally, HRD1 expression was specifically observed in NeuN-positive neurons (Fig. S4a). In a previous study, we showed that high-dose MeHg blocked *XBP1* mRNA splicing *in vitro* (Hiraoka et al. 2017). Thus, we hypothesized that in the late phase of MeHg exposure, the ERAI signal is decreased due to the decline in *XBP1* mRNA splicing. Indeed, mercury accumulated in the cerebral cortex of ERAI-Venus mice administered MeHg in a time-dependent manner (Fig. S4b). These results suggest the activation of the IRE1α-XBP1 axis at the early phase but not at late phase of MeHg exposure.

If ER stress cannot be resolved, cells activate apoptosis *via* induction of the ER stress mediated-apoptosis inducer CHOP through the PERK or ATF6 branches (Rutkowski and Hegde 2010). To elucidate the contribution of ER stress to neuronal cell death induced by MeHg, we investigated the expression of CHOP. In contrast with the pattern seen in the IRE1α-XBP1 axis, the number of CHOP-positive cells in ERAI-Venus mice exposed to MeHg exhibited a time-dependent increase (Figs. 5c and d). Furthermore, we observed colocalization of CHOP and NeuN, indicating that neurons initiate ER stress-mediated apoptosis (Fig. S4c). Although the difference is nonsignificant, the number of NeuN-positive neurons showed a decreasing trend in ERAI-Venus mice exposed to MeHg for 6 weeks (Fig. S4d).

Our study strongly indicates that MeHg induces ER stress-resistance factors *via* the IRE1-XBP1 axis at the early stage. However, accumulation of MeHg at a later stage switches the response to apoptosis induction *via* the PERK and ATF6 branches.

Discussion

MeHg-induced ER stress has historically been thought to be involved in neuronal cell death, but the mechanism by which the UPR, which has bipolar signaling functions of “cell survival” and “cell death”, is changed by MeHg is unclear. In this study, we observed MeHg-induced ER stress by utilizing the ERAI system (Figs. 1 and 2). The ER-stressed regions in the brain were consistent with the regions reported to be injured by MeHg, which showed differential sensitivity to MeHg (Figs. 2 and 3). Moreover, most brain cells with ER stress were neurons (Fig. 4). These results suggest that this model reflects MeHg poisoning and is useful for evaluating site- and cell type-specific toxicity of MeHg. Interestingly, activation of cytoprotective signaling through the IRE1α-XBP1 axis peaked at 3 weeks of exposure to MeHg and was gradually attenuated (Fig. 5). In contrast, apoptotic signaling in both the PERK and ATF6 branches was activated in a MeHg exposure time-dependent manner (Fig. 5). These results suggest that the signaling function of the UPR is shifted from cell survival to cell death by MeHg accumulation in the mouse brain, which results in neuronal apoptosis.

First, we challenged mice with acute MeHg exposure to confirm whether ER stress induced by MeHg is detected by the ERAI system. The luminescence signals in several organs, such as the brain, heart, liver, and kidney, were increased by MeHg exposure (Fig. 1b). These organs have high sensitivity to MeHg poisoning (Guallar et al. 2002; International Programme on Chemical Safety (IPCS) 1990; Salonen et al. 1995). MeHg is reabsorbed by the enterohepatic circulation and kidneys, resulting in a long half-life in the body (IPCS, 1990). The brain and heart may come in contact with high levels of MeHg due to their exposure to a large volume of circulating blood, and MeHg readily crosses the blood-brain barrier as MeHg-L-cysteine conjugates (Kerper et al.1992). Therefore, these organs may be highly exposed to MeHg, which

may cause ER stress. Although MeHg toxicity is known to cause peripheral neuropathy (Eto 1997; Eto et al. 2002), we could not confirm an increase in MeHg-induced ER stress in peripheral nerves. The induction of ER stress was weak or structurally undetectable.

Patients with MeHg poisoning, such as those with Minamata disease, have been shown to exhibit neuronal damage in the postcentral gyrus (somatosensory cortex), Heschl's gyrus (auditory cortex), and calcarine sulcus (visual cortex) (Eto and Takeuchi 1978). Additionally, MeHg-exposed mice have been reported to show damage to the motor cortex and striatum (Fujimura et al. 2009; NIMD 2006). Hence, to evaluate the occurrence of MeHg-induced ER stress in those regions, we investigated the temporal change in the ERAI signal. We observed an increase in the number of ERAI-positive cells in regions of the striatum and cerebral cortex, such as the somatosensory cortex, auditory cortex, visual cortex, and motor cortex (Figs. 2 and 3), suggesting that this model reflects MeHg neurotoxicity in mice. Moreover, we revealed a difference between the ERAI signal peaks in those regions (Figs. 2 and 3). In a previous study, the site-specific toxicity of MeHg was indicated to arise from the expression levels of antioxidant proteins (Fujimura and Usuki 2017b). Therefore, we predict that other factors, such as the levels of antioxidant proteins, are involved in the differences between the ERAI signal peaks.

Because MeHg poisoning is characterized by neuronal cell death, we investigated the cell types affected by MeHg-induced ER stress. As expected, the ERAI signal was observed predominantly in NeuN-positive neurons (Fig. 4). MeHg neurotoxicity has historically been thought to likely occur in small cells such as granule cells (Berlin et al. 2015). Indeed, increases in the ERAI signal were observed near the fourth layer (deep layer) of the cerebral cortex, in which many excitatory granule cells are located. Previous reports suggest that MeHg causes excitatory toxicity to neurons

(Fujimura and Usuki 2017a), and hyperactivated neurons produce oxidative stress (Bondy and Lebel 1993). Therefore, neurons might respond selectively to ER stress caused by MeHg-induced oxidative stress. Additionally, as the ERAI system utilizes the cleavage activity of IRE1α, the selective response may depend on the expression level of IRE1α in each cell. In fact, high expression of IRE1α has been reported in the neurons of the cerebral cortex, the pyramidal cells of the cornu ammonis (CA) 1-3, and the granule cells of the dentate gyrus (Miyoshi et al. 2000).

Compared to neurons, glial cells highly express the antioxidant protein GSH (Sagara et al. 1993). Hence, glial cells may show resistance to MeHg-induced oxidative stress and exhibit low level of ER stress. Moreover, research indicates that apoptotic neurons are phagocytosed by microglia (Sierra et al. 2010). Based on this finding, the ERAI signal in microglia may be derived from the neurons phagocytosed by microglia (Fig. 4c). Alternatively, the cell specificity of ER stress may arise because each cell type may have a different ability to take up MeHg.

Next, we focused on the transient upregulation of ERAI signaling by MeHg in the somatosensory cortex. We previously showed that *XBP1* mRNA splicing is attenuated by exposure to high-dose MeHg, suggesting that IRE1α is a target of *S*-mercuration (Hiraoka et al. 2017). Thus, a decrease in the ERAI signal indicates a decline in *XBP1* mRNA splicing or degradation of the ERAI molecule. In the somatosensory cortex, the expression of HRD1, a downstream factor in the IRE1α-XBP1 axis, peaked at 3 weeks after MeHg exposure, similar to the ERAI signal (Figs. 2c and 5b). Since MeHg accumulates in the cerebral cortex in a time-dependent manner (Fig. S4b), persistent exposure to MeHg might lead to inhibition of *XBP1* mRNA splicing, resulting in attenuation of the ERAI signal.

MeHg is thought to induce ER stress *via* several mechanisms, such as causing *S*-mercuration of PDI (Makino et al. 2014), inducing ER membrane injury (Mori et al. 2007), or inhibiting the

formation of the disulfide bond in nascent proteins. Thus, short-term exposure to MeHg may induce ER stress but not enough to suppress the IRE1α-XBP1 axis. On the other hand, long-term exposure to MeHg may cause ER stress to reach the threshold for suppression of the IRE1α-XBP1 axis. Additionally, the IRE1α branch has been thought to be associated with the switch in UPR signaling from cell survival to cell death (Han et al. 2009; Upton et al. 2012). Therefore, these findings strengthen the possibility that the decline in *XBP1* mRNA splicing by MeHg triggers neuronal cell death.

To ameliorate ER stress, spliced XBP1 functions as a transcription factor inducing the expression of molecular chaperones and ERAD-related proteins (Yoshida et al. 2001, 2003). Hence, persistent ER stress mediated by reducing spliced XBP1 may lead to expression of the apoptosis inducer CHOP *via* the PERK and ATF6 branches. Indeed, CHOP expression increased in a MeHg exposure time-dependent manner (Figs. 5c and d). In a previous study, pharmacologic inhibition of the PERK branch ameliorated MeHg cytotoxicity (Hiraoka et al. 2017). These lines of evidence suggest that MeHg-induced neurotoxicity arises from attenuation of the IRE1α-XBP1 axis and activation of the PERK and ATF6 branches.

In conclusion, we demonstrated the utility of ERAI mice in evaluating the site- and cell type-specific toxicity of MeHg at high resolution. Additionally, since the time-course study showed differential sensitivity to ER stress in each region of the brain, this model will be useful for future studies focused on the central nervous system. Furthermore, we elucidated the temporal change in UPR pathways induced by MeHg exposure. These findings suggest that UPR modulation could be a therapeutic target for treating neuropathy, such as that induced by MeHg poisoning.

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Compliance with ethical standards

Competing interests

The authors declare no conflicts of interest.

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Figure legends

Fig. 1 Visualization of ER stress using ERAI-LUC mice. **a** Whole-body imaging of the ERAI luminescence signal in ERAI-LUC mice after exposure to each agent for 24 h. Left panel: physiological saline, center panel: MeHg (25 mg/kg, S.C.), right panel: tunicamycin (Tm) (0.5 mg/kg, S.C.). **b** *Ex vivo* imaging showing increases in the ERAI luminescence signal in the brain, heart, liver, and kidney of ERAI-LUC mice after whole-body imaging

Fig. 2 Time course analysis of MeHg-induced ER stress using ERAI-Venus mice. **a** Experimental timeline of subchronic exposure to MeHg. ERAI-Venus mice were exposed to 30 ppm MeHg *via* drinking water. At the indicated times, the mice were sacrificed, and the brain tissues were analyzed by immunostaining. **b** ERAI fluorescence signal in the somatosensory cortex of ERAI-Venus mice exposed to MeHg for the indicated time. The scale bar represents 100 μm. **c** Quantification of ERAI-positive cells shown in **b**. Data are expressed as the mean ± S.E.M. values (n=5-6, *p<0.05: significant difference compared with mice without MeHg exposure (0 weeks) by one-way ANOVA with Dunnett's *post hoc* test)

Fig. 3 MeHg-induced ERAI signals in several brain areas. **a-d** Quantification of ERAI-positive cells in the auditory cortex (**a**), visual cortex (**b**), motor cortex (**c**), and striatum (**d**) of mice exposed to MeHg for the indicated times. **e** Merged graph of the data in Fig. 2c and Fig. 4a-c. Data are expressed as the mean ± S.E.M. values (n=5-6, *p<0.05: significant difference compared with mice without MeHg exposure by one-way ANOVA with Dunnett's *post hoc* test)

Fig. 4 Identification of ERAI-active cells induced by MeHg exposure in the cerebral cortex. **a** Detection of the ERAI signal (green) in cells positive for the neuron marker NeuN (red) in the

somatosensory cortex of ERAI-Venus mice exposed to MeHg for three weeks. **b, c** Detection of the ERAI signal (green) in cells positive for the astrocyte marker GFAP (red) (**b**) or the microglia marker Iba1 (red) (**c**) in the somatosensory cortex of ERAI-Venus mice exposed to MeHg for six weeks. Each scale bar represents 20 μm. The arrows indicate the cells coexpressing ERAI and each cell marker. **d** Quantification of cells coexpressing each cell marker and ERAI in **a-c**. Data are expressed as the mean ± S.E.M. values (n=3-6, *p<0.05, ***p<0.001 by one-way ANOVA with Tukey's *post hoc* test)

Fig. 5 Effect of MeHg exposure on each UPR branch in the cerebral cortex. **a, c** Expression of HRD1 (**a**) or CHOP (**c**) in the somatosensory cortex of ERAI-Venus mice exposed to MeHg for the indicated times. Each scale bar represents 100 μm. **b, d** Quantification of HRD1- and CHOP-positive cells shown in **a** and **c**, respectively. Data are expressed as the mean ± S.E.M. values (n=5-6, *p<0.05, **p<0.01: significant difference compared with mice without MeHg exposure by one-way ANOVA with Dunnett's *post hoc* test)

Table 1 Relative quantification of ERAI-driven bioluminescence signals in the brain, heart, liver, and kidney in ERAI-LUC mice exposed to MeHg for 24 h (25 mg/kg, S.C.)