Endoplasmic Reticulum Stress Contributes To The Decline in Doublecortin Expression in Immature Neurons in Mice with Long-Term Obesity and Memory Impairment

Kiyomi Nakagawa  
Gifu University

Saiful Islam  
Bangladesh Council of Scientific and Industrial Research Laboratories

Masashi Ueda  
National Center of Neurology and Psychiatry

Toshiyuki Nakagawa  
Gifu University

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Abstract

Adult hippocampal neurogenesis (AHN) plays an important role in hippocampus-dependent function. The number of doublecortin (Dcx)-positive immature neurons in the dentate gyrus (DG) decreases during aging and especially in the early stages of Alzheimer’s disease (AD) and is further reduced in later stages of AD. Obesity in midlife is associated with dementia later in life; however, the underlying mechanisms by which obesity results in the development of dementia later in life remain unknown. Here, we show that endoplasmic reticulum (ER) stress was activated in the hippocampus, and processes of Dcx-expressing immature neurons were shortened, coexpressing CHOP in APP23 AD model mice with high-fat diet (HFD)-induced long-term obesity as well as aged Lepr\textsuperscript{db/db} (\textit{db/db}) mice. Moreover, in differentiating cells from hippocampal neurospheres, Dcx mRNA was rapidly degraded via a microRNA (miRNA) pathway after thapsigargin treatment in vitro. These results indicate that loss of Dcx mRNA induced by ER stress during AHN may be an underlying mechanism of memory impairment in obese individuals later in life.

Introduction

New neurons are generated in distinct regions of the adult human brain, i.e., the hippocampal subgranular zone (SGZ) and subventricular zone (SVZ). A total of 700 new neurons are generated daily in the hippocampus, but this number declines during aging. Under physiological conditions, adult hippocampal neurogenesis (AHN) is involved in hippocampus-dependent functions such as pattern separation and stress resilience, and AHN is impaired in Alzheimer’s disease (AD) and Depression. The number of cells positive for doublecortin (Dcx), which is specifically expressed in immature neurons, in the dentate gyrus (DG) is decreased in mild cognitive impairment (MCI) and early stages of AD and further reduced in later stages. Staining with markers of different differentiation stages has revealed substantial impairment of Dcx-positive cell maturation in the DG in AD. Dcx, which stabilizes and maintains microtubules in maturing neurons, has been identified as a causal gene of X-linked lissencephaly and double cortex syndrome, resulting in arrest of neuronal migration. The Dcx transcript has a long 3’ untranslated region (UTR), indicating that it is involved in regulation of mRNA stability and suggesting that Dcx mRNA is a target of microRNA (miRNA)-128. Consistently, the Dcx transcript level is increased in Dicer\textsuperscript{-/-} neural stem cells (NSCs). Female mice heterozygous for a mutation in Dcx show abnormalities in hippocampal lamination, defects in contextual and conditioned fear memory, and mild deficits in the Morris water maze (MWM) performance, indicating that Dcx expression in the hippocampus is important for learning.

The number of people living with dementia is increasing; however, age-specific incidence rates of dementia have recently decreased in several countries because of decreases in the prevalence of some risk factors due to lifestyle changes. It has been indicated that there are twelve modifiable risk factors. Eliminating these risk factors could prevent or delay the onset of dementia. Obesity is one of these modifiable risk factors. Consistently, memory is impaired in AD model mice with high-fat diet (HFD)-
induced obesity\textsuperscript{15} and the offspring of AD model mice crossed with diabetic \textit{ob/ob} mice\textsuperscript{16} or diabetic \textit{db/db} mice\textsuperscript{17}, compared with AD model mice. A 28-year study on changes in body mass index (BMI) revealed that obesity at age 50 is associated with dementia\textsuperscript{18}, indicating that obesity in midlife is related to dementia later in life. However, the underlying mechanisms by which obesity results in the development of dementia later in life remain unknown. Interestingly, the survival and proliferation of hypothalamic NSCs are disrupted by IкB kinase \(\beta\) (IKK\(\beta\))/nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) activation in the context of long-term HFD-induced obesity\textsuperscript{19} and a decline in release of exosomal miRNA by the loss of hypothalamic NSCs regulates aging\textsuperscript{20}. Several studies have suggested that endoplasmic reticulum (ER) stress is critical for obesity-induced inflammation\textsuperscript{21,22}. Analysis of the brain transcriptomes of mice with different \textit{APOE} genotypes and previously published RNA-seq data from the brains of human patients with AD and control subjects has shown that the expression of activating transcription factor 4 (ATF4), which is increased during the unfolded protein response (UPR), is significantly upregulated in the brains of AD patients\textsuperscript{23}. The UPR is induced by ER stress through three different pathways, which are initiated by inositol-requiring protein-1 (IRE1), protein kinase RNA-activated (PKR)-like ER-localized translation initiation factor 2\(\alpha\) (eIF2\(\alpha\)) kinase (PERK), and ATF6\textsuperscript{24}. IRE1 mediates nonconventional \textit{X-box binding protein-1 (XBP-1)} mRNA splicing and ER-localized mRNA degradation through a process called regulated IRE1-dependent decay (RIDD). PERK phosphorylates eIF2\(\alpha\) to suppress general translation, and ATF4 is specifically translated. Prolonged ER stress induces cell death\textsuperscript{25} in several diseases, including neurodegenerative diseases and diabetes, possibly through IRE1 activation and C/EBP-homologous protein (CHOP) expression\textsuperscript{26}. eIF2\(\alpha\) is phosphorylated at residue Ser51 by four protein kinases, including PERK, general control nonderepressible-2 (GCN2) kinase, double-stranded PKR, and heme-regulated inhibitor kinase (HRI), under several conditions, such as ER stress, amino acid starvation, viral infection, and heme deficiency through a process known as the integrated stress response (ISR). Several studies have indicated that eIF2\(\alpha\) phosphorylation and ATF4 expression are important for memory formation\textsuperscript{27–29}. We have shown that ER stress and autophagy impairment enhance \(\gamma\)-secretase activity to increase amyloid-\(\beta\) (A\(\beta\)) production\textsuperscript{30} through the binding of ATF4 to the regulatory region of the presenilin-1 (PS1) gene\textsuperscript{31}. Downregulation of ATF4 expression by quercetin in mouse models of AD improves memory impairment\textsuperscript{17}.

Diabetes affects AHN by increasing the levels of glucocorticoids and the hyperglycemia\textsuperscript{32}. However, whether long-lasting obesity affects AHN remains unclear. Therefore, we asked whether ER stress is activated in the brain of aged mice with long-term obesity and memory impairment and affects neurogenesis. In this study, we found that ER stress was activated and that the processes of Dcx expressing immature neurons were shortened in the hippocampi of mice with long-term obesity. Furthermore, \textit{Dcx} mRNA expression rapidly decreased in differentiating NSCs after thapsigargin treatment. These results indicate that loss of \textit{Dcx} mRNA induced by ER stress during AHN may be an underlying mechanism of memory impairment in obese individuals later in life.

\textbf{Results}
Spatial memory was impaired in wild-type and AD model mice with long-term obesity

We previously showed that the expression levels of ATF4 are increased in the cortex, hippocampus, and amygdala in the offspring of obese and diabetic APP23 AD model mice crossed with db/db mice through a process known as the ISR. ATF4 expression in the brain is decreased by quercetin, and this change is accompanied by an improvement in memory. It has been indicated that glucocorticoids reduce the number of BrdU-labeled cells in the DG in 60-day-old mice. To examine the effect of the ER stress on AHN in the brains of mice with long-term obesity, we first examined whether memory was impaired in HFD-induced obese mice. Obesity was induced by HFD feeding for 41 weeks in 66-week-old APP23 AD model mice (Fig. 1a-1c) and for 64 weeks in 74-week-old wild-type C57BL6 mice (Fig. 1d-1f). Fasting blood sugar (FBS) and insulin levels in serum were increased in both groups of mice subjected to long-term HFD feeding compared with mice fed a standard diet (Fig. 1b and 1e). To examine whether long-term HFD-induced obesity affects memory, we performed the novel object location (NOL) test. Analysis of the ratio of the time spent exploring the target object to the time spent exploring all three objects revealed that the time spent exploring the object in the novel location was significantly decreased in APP23 mice with long-term obesity (Fig. 1c). Although the exploration index for the object in the novel location was not significantly increased in mice fed a standard diet (Fig. 1c), it was likely that their memory was slightly impaired because it has been reported that the number of CA1 pyramidal neurons is significantly decreased in 14- to 18-month-old APP23 mice compared with controls. In aged wild-type mice, the index for the object in the novel location was significantly increased in mice fed a standard diet but not mice with long-term obesity (Fig. 1f). Next, we examined whether spatial memory was affected in eighteen-week-old Lepr/db/db (db/db) mice, which had a higher body weight than wild-type and heterozygous littermates (control), even at 6 weeks of age (Fig. 1g). We found that in the MWM test, the latency to find the invisible platform during the acquisition phase was not changed and the number of platform crossing was significantly decreased for db/db mice (Fig. 1h). These results suggested that spatial memory was impaired in mice with long-term obesity fed a HFD and further worsened in APP23 mice and in leptin receptor-deficient mice fed a standard diet.

ER stress was activated in the brains of mice with long-term obesity

To investigate the role of ER stress in the brains of mice with long-term obesity, we first used 59- to 61-week-old APP23 mice. After eight weeks of HFD feeding beginning at 21 weeks of age, the body weights of the mice fed a HFD were significantly increased. The body weights of the mice fed a HFD and the mice fed the standard diet were 42.5 ± 0.9 g and 28.3 ± 3.6 g, respectively, when the mice were approximately 60 weeks of age. Western blot analysis showed that the expression levels of CHOP in the hippocampi of mice with long-term obesity were increased compared with those in the hippocampi of mice fed the standard diet (Fig. 2a). Consistently, obesity was detected in wild-type C57BL/6 mice fed a HFD for 4 weeks at the age of 16 weeks, and the body weights of mice fed a HFD and the standard diet were 55.8 ±
9.7 g and 36.0 ± 3.9 g, respectively, at the age of 86 weeks. Western blot analysis showed that the expression levels of CHOP in the hippocampi of mice with long-term obesity were significantly increased compared with those in the hippocampi of mice fed the standard diet (Fig. 2b). Furthermore, the expression levels of CHOP in the hippocampi of db/db mice with long-term obesity were significantly higher than those in the hippocampi of control mice at 46 weeks of age (Fig. 2c). We also observed an increase in ATF4 expression in wild-type and db/db mice with long-term obesity. Since three signaling pathways, the PERK, ATF6, and IRE1 pathways, are activated during ER stress, we examined the expression of Xbp-1s and ATF6 fragment in the hippocampi of 60-week-old db/db mice. We found that the N-terminal cleaved product of ATF6 and Xbp-1s were not detected in the hippocampi of aged db/db mice but were detected in mouse ES cells treated with 2 mM dithiothreitol (DTT) (Fig. 2d). These results suggested that long-term obesity induced ER stress, particularly the ISR, in the hippocampus.

**CHOP and Dcx were coexpressed in immature neurons in mice with long-term obesity**

To investigate the impact of the ER stress in AHN, we performed immunostaining for Ki67, which is continuously produced from S phase until exit of the cell cycle35, in 10-week-old (Fig. 3a) and 45-week-old (Fig. 3b) db/db mice. The number of Ki67-positive cells in the DG of the hippocampus of db/db mice was similar to control mice in both young and aged mice (Fig. 3a and 3b). Additionally, the levels of mature BDNF in the hippocampi of 46-week-old control and db/db mice were similarly measured (Fig. 3c). Next, since Dcx is sensitive to postmortem breakdown36, the mice were anesthetized and quickly perfused with 4% paraformaldehyde for fixation, and then the expression of Dcx in the hippocampi of control and db/db mice was examined by immunohistochemistry. The number of Dcx-positive cells in the DG was not different between 45-week-old control and db/db mice (Fig. 3d and 3f) or between 10-week-old control and db/db mice (data not shown). However, confocal microscopy showed that the processes on Dcx-positive cells in the DG were significantly shortened in 45-week-old db/db mice compared with 45-week-old control mice (Fig. 3e and 3f). These Dcx-positive cells with a short process expressed CHOP in 45-week-old db/db mice (Fig. 3g). Consistently, we observed that the Dcx-positive cells with a short process expressed CHOP in APP23 mice with long-term obesity (Fig. 3h). These results suggested that the ER stress was activated in Dcx-positive immature neurons with a short process.

**Thapsigargin reduced Dcx expression in immature neurons through Dcx mRNA degradation**

To investigate the role of the ER stress in Dcx expression in immature neurons, we cultured neurospheres isolated from the mouse hippocampus according to a previously established protocol37. After neurospheres were cultured on laminin-coated dishes for 5 days in vitro (DIV) (Fig. 4a), several marker proteins, i.e., Dcx, Calreticulin, Nestin, and βIII Tubulin, were expressed (Fig. 4c). Therefore, we challenged these cells with thapsigargin, a sarcoplasmic reticulum/ER Ca^{2+}-ATPase inhibitor38, for 6 h and then cultured them in new medium to induce ER stress according to a previously published method25. The expression levels of Dcx were decreased after thapsigargin treatment, as confirmed by
immunohistochemistry and western blotting. Consistently, immunostaining revealed that the processes on cells expressing both Dcx and CHOP was shortened (Fig. 4b) like in vivo (Fig. 3g and 3h). Interestingly, loss of Dcx protein was observed in thapsigargin-treated cells, but the levels of other marker proteins were not decreased (Fig. 4c). Proneural basic helix-loop-helix neurogenin 1 and 2 directly activate $Dcx$ expression by binding to the promoter and upregulate $p35$ expression$^{39}$. $p35$ protein expression was also not decreased by thapsigargin treatment (Fig. 4c). Since the protein levels of Dcx were not rescued by 25 µM MG132, 10 µg/mL E-64d and 10 µg/mL pepstatin A (data not shown), or 40 µM z-VAD-FMK (Fig. 4d), as determined by western blotting, we measured the levels of $Dcx$ mRNA by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) (Fig. 4e). We found that the mRNA levels of $Dcx$, but not $Nestin$ or $p35$, quickly decreased during incubation in new medium after thapsigargin treatment (lower panel in Fig. 4e). The levels of $Dcx$ mRNA were not rescued by the IRE1 inhibitor 4µ8C, indicating that $Dcx$ mRNA is not a substrate for RIDD (Fig. 4f). Since the $Dcx$ transcript has a long 3’ UTR, which binds Musashi1 RNA-binding protein, mRNA stability and translation are possibly regulated by the binding proteins$^9$. We found that the levels of $Dcx$ mRNA were not rescued by zVAD (Fig. 4g), suggesting that caspase substrates might not be involved in $Dcx$ mRNA stability and that apoptosis might not decrease $Dcx$ mRNA expression. Next, we knocked down $Dicer$ in differentiating NSCs using a small hairpin RNA (shRNA) and small interfering RNA (siRNA) and then treated the cells with thapsigargin to examine whether the ER stress-induced reduction in $Dcx$ mRNA expression was regulated by Dicer, as it has been reported that $Dcx$ mRNA expression is upregulated in $Dicer$-deficient NSCs$^{12}$. Neurospheres cultured on laminin were infected with lentiviral particles carrying shRNA targeting $Dicer$ at 1 DIV, transfected with siRNA targeting $Dicer$ at 4 DIV, and then treated with thapsigargin at 5 DIV. Real-time RT-PCR showed that thapsigargin-induced downregulation of $Dcx$ mRNA expression was significantly prevented by knockdown of $Dicer$ in differentiating NSCs (Fig. 4h).

**Discussion**

ER stress is activated by several chronic diseases, including obesity, diabetes$^{40}$, and depression$^{41}$, which may cause dementia later in life$^{14}$. However, it is unknown why it takes a long period of time for dementia to develop after body weight increases. In the present study, we demonstrated that long-term obesity induced ER stress, mainly the ATF4-CHOP axis, in the hippocampal DG, leading to a decrease in the number of processes on Dcx-expressing immature neurons due specifically to loss of $Dcx$ mRNA stability.

The ATF4-CHOP axis is initiated by eIF2α phosphorylation, which is mediated by four protein kinases, through a process called the ISR, which contributes to the pathogenesis of diseases, including cognitive disorders$^{27}$. For example, PKR-induced ISR signaling is activated in a Down syndrome (DS) mouse model and individuals with DS$^{29}$. PERK phosphorylation$^{42}$ and eIF2α phosphorylation$^{43}$ are observed in AD patients. Consistently, the gene expression of the ISR-related signaling molecule ATF4 is upregulated in the AD brain$^{23}$. Since ATF4 directly binds to the promoter of the CHOP (also known as growth arrest and DNA damage-inducible protein, Gadd153) gene$^{44}$ and CHOP induction is dependent on ATF4 expression$^{45}$, CHOP is expressed when the ISR is activated. CHOP was detected in the hippocampi of
mice with long-term obesity by western blotting (Fig. 2), and immunohistochemistry and confocal microscopy confirmed that CHOP was localized in Dcx-expressing immature neurons of the hippocampal DG (Fig. 3g, 3h). We observed significant short processes on Dcx-positive immature neurons in the hippocampal DG in mice with long-term obesity (Fig. 3). It has been demonstrated that Dcx-positive immature neurons of the hippocampal DG are selectively vulnerable to mild controlled cortical impact (CCI) of the mouse brain, which is used to model traumatic brain injury (TBI); this selective vulnerability may contribute to memory impairment following TBI\(^4\). These vulnerable immature neurons have short processes that express phosphorylated PERK, phosphorylated eIF2\(\alpha\), and CHOP\(^4\). Furthermore, immature neurons that are vulnerable to CCI are not observed in Chop knockout mice, suggesting that local caspase activation without cell death results in dendritic loss\(^4\). Our experiment showed that the pan-caspase inhibitor zVAD had no effect on the protein expression of Dcx or on Dcx mRNA stability during ER stress (Fig. 4d, 4g). Further experiments are needed to explore the mechanisms of dendritic loss in Dcx-expressing immature neurons. ER stress is involved in the pathogenesis of obesity and diabetes\(^4\), which exacerbate cognitive dysfunction in \(db/db\) with mouse model of AD\(^5\). The results presented here demonstrated that ER stress/ISR might be activated in immature neurons in mice with long-term obesity. However, the mechanisms of ER stress/ISR activation in immature neurons remain unclear. Reactive oxygen species (ROS) are produced as byproducts of protein oxidation in the ER through ER oxidoreductin-1 (ERO1) and protein disulfide isomerase (PDI)\(^5\). ERO1\(\alpha\) transcription is increased by CHOP\(^5\). Overexpression of ERO1\(\beta\) causes ER stress in pancreatic \(\beta\) cells\(^5\). ER stress is also elicited by ROS\(^5\) and by inactivation of PDI through nitrosylation\(^5\). In the C17.2 NSC line, oxidative stress induced by high glucose medium activates ER stress and prevents Tuj1 and glial fibrillary acidic protein (GFAP) expressions, which are rescued by the superoxide dismutase mimetic tempol and ER stress inhibitor 4-phenylbutyrate\(^5\). Thus, reduction-oxidation (redox) reactions and ER stress stimulate each other\(^5\).

Although we do not know which occurs first in the brains of obese mice, we hypothesize that oxidative stress might induce ER stress/ISR in Dcx-expressing immature neurons \(in vivo\). To confirm this hypothesis, further studies are needed; for example, \(in vivo\) dynamic nuclear polarization magnetic resonance imaging (DNP-MRI)\(^5\) may allow detection of redox conditions in the brains of young and aged obese mice.

Differentiating NSCs were treated with thapsigargin for 6 h, washed, and cultured in medium without thapsigargin. ER stress was activated in these cells, as indicated by the induction of 78-kDa glucose-regulated protein (Grp78)/immunoglobulin heavy chain-binding protein (BiP) and CHOP expression. The Dcx protein was absent in thapsigargin-treated cell lysates, although the proteins Nestin and \(\beta\) III Tubulin were present (Fig. 4c). Interestingly, we found that loss of the Dcx protein induced by ER stress was mainly caused by rapid elimination of Dcx mRNA (Fig. 4e) because the protein expression of Dcx was not rescued by inhibitors of the proteasome, autophagy, and caspase. Dcx is a microtubule-associated protein that leads to microtubule polymerization\(^5\), and its mRNA is not a substrate for RIDD (Fig. 4e), probably because Dcx mRNA is not an ER-localized mRNA. The Dcx transcript has a long 3' UTR\(^5\), which binds Musashi1\(^6\) and miRNAs\(^1,2,6,2,6\) to repress Dcx mRNA translation or to regulate Dcx mRNA
stability. The mRNA levels of *Dcx* were decreased in differentiating NSCs 6 h after thapsigargin treatment for 6 h (Fig. 4e), indicating ER stress induced loss of *Dcx* mRNA. The mRNA expression of *Dcx* in differentiating NSCs was significantly rescued by *Dicer* knockdown after thapsigargin treatment (Fig. 4h). Consistently, the *Dcx* transcript level is increased in unstressed adult NSCs from *Dicer* knockout mice	extsuperscript{12}. The *Dcx* 3' UTR is targeted by miR-128, resulting in downregulation of Dcx protein expression in SH-SY5Y neuroblastoma cells	extsuperscript{11} and in Neuro2A cells	extsuperscript{12}, but the mechanism by which *Dcx* mRNA is regulated is controversial	extsuperscript{11,12}. Moreover, it may interesting to investigate the levels of miR-128 in the serum of individuals with preclinical AD or MCI and long-term obesity since circulatory levels of miR-128 are significantly increased in patients with type 2 diabetes and depression compared to those with type 2 diabetes	extsuperscript{66}. This evidence and our results demonstrate that ER stress may stimulate a miRNA pathway to regulate *Dcx* mRNA stability, resulting in the loss of Dcx in immature neurons of the hippocampal DG (Fig. 4i).

In conclusion, ER stress/the ISR are activated in immature neurons in the hippocampal DG of mice with long-term obesity. Loss of *Dcx* mRNA by ER stress/the ISR during AHN may be an underlying mechanism of memory impairment in obesity later in life.

**Methods**

**Western blot analysis and chemicals**

Western blotting was performed as described previously	extsuperscript{31}. Mouse tissue and cells were lysed in RIPA [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate, and 1% Nonidet P-40] with Roche cOmplete™ mini tablets (Roche, Mannheim, Germany), 25 µM MG132 (EMD Chemicals, Inc; San Diego, CA), and phosphatase inhibitors, i.e., 20 mM β-glycerophosphate (Sigma-Aldrich Co., LLC; St. Louis, MO) and 20 mM sodium orthovanadate (Fujifilm, Osaka, Japan). After centrifugation at 13,100 x g, the supernatants were used for western blotting. The antibodies used for western blotting were as follows: anti-CHOP antibody (Thermo Fisher Scientific, Waltham, MA; Cell Signaling Technology, Inc; Danvers, MA); anti-ATF4 antibody (Santa Cruz Biotechnology, Inc; Santa Cruz, CA; Thermo Fisher Scientific); anti-p35 (C-19) antibody; anti-GAPDH antibody (Santa Cruz Biotechnology, Inc); anti-α-tubulin antibody (Sigma-Aldrich Co., LLC; St. Louis, MO); anti-Dcx antibody (Abcam, Cambridge, UK; Novus Biologicals, Centennial, CO; Santa Cruz Biotechnology, Inc); anti-Calreticulin antibody (StressGen Biotechnologies Corp., Victoria, Canada); anti-Nestin antibody (EMD Millipore Corp., Temecula, CA); anti-βIII tubulin antibody (Covance, Inc; Princeton, NJ); and anti-BiP (Grp78, KDEL) antibody (ENZO Life Sciences International, Inc., Plymouth Meeting, PA). HRP-conjugated anti-rat, anti-mouse, and anti-rabbit IgG (H+L) antibodies (SouthernBiotech; Birmingham, AL) and thapsigargin (Santa Cruz Biotechnology, Inc.) were used. FBS levels were measured with a FreeStyle Freedom Lite device (Abbott Diabetes Care Inc., Alameda, CA). Insulin levels were measured by a mouse insulin enzyme-linked immunosorbent assay (ELISA) Kit (RTU) (Shibayagi, Gunma, Japan). BDNF levels were measured with a Mature BDNF Rapid™ ELISA Kit (Bisensis Pty Ltd., Thebarton, Australia). All other chemicals were
purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Kanto Chemical Co., Inc., (Tokyo, Japan), and Sigma-Aldrich Co., LLC.

**Animals and the HFD**

C57BL/6J mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). APP23 mice, which express human \( APP_{751} \) cDNA and a Swedish double mutation on the C57BL/6 genetic background \(^{34}\), were kindly provided by Dr. M. Staufenbiel (Novartis Pharma Ltd; Basel, Switzerland). Obese and diabetic \( db/db \) (Lepr\(^{db/db}\)) mice on the C57BL/6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in a temperature- and light-controlled room (24°C; 12-h light/dark cycle). The mice were fed on AIN93G (standard diet) or HFD-60 (Oriental Yeast Co., Ltd; Tokyo, Japan). All animal studies were approved by the Gifu University Graduate School of Medicine Animal Care and Use Committee under the guidelines for experiments on animals provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All animal experiments were performed according to the ARRIVE guidelines.

**Behavioral procedures**

The NOL test was performed according to the method described by Roy et al.\(^{67}\). Briefly, each mouse was habituated to a cage without objects for 15 min on day 1. The mice were exposed to three different objects, i.e., conical (diameter x height: 5 x 11.5 cm), cylindrical (6.5 x 10.5 cm), and reagent (5 x 13.2 cm) bottles, for 5 min three times per day at 2-min intervals. The mice were exposed to three objects on day 3 and placed in their home cages for a retention interval (two minutes). Then, one of the objects was moved to the opposite corner. The behavior of each mouse was monitored using video recording software and an automated tracking system (SMART v3.0 software; Panlab, Barcelona, Spain) and a video camera (HDC-HS350; Panasonic; Osaka, Japan). When the distance between the nose of the mouse and an object less than 2 cm or when the mouse sniffed or touched the object with its snout, the mouse was considered to be exploring the object, as previously described\(^{68}\). The cages and objects were cleaned with 70% ethanol and 1% acetic acid solution before each trial to eliminate dominant odors.

The MWM test was performed according to a previously described protocol\(^{69}\). Briefly, the apparatus was a 100-cm diameter tank containing water at a temperature of approximately 22°C water, skim milk and a submerged platform. Four acquisition trials from each of the five starting positions were performed each day. The time limit for each trial was 60 sec. Mice that did not reach the platform were guided to the platform and left on the platform for 30 sec. On day 5, the platform was removed for the probe test. Each trial was recorded and analyzed with the SMART v3.0 automated tracking system.

**Immunostaining**

Immunostaining was performed as described previously\(^{25}\) and analyzed by confocal microscopy (LSM710, Carl Zeiss; Göttingen, Germany). Briefly, mice were anesthetized and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The mouse brains were postfixied for 2 h in the same fixative, which was then replaced with 15% sucrose in 0.1 M PB.
Sections (14 µm) were incubated in PBS containing 10% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 0.1% Triton X-100 at room temperature for 1 h. Sections were incubated with an anti-Dcx antibody and anti-CHOP antibody or an anti-Ki67 antibody (Thermo Fisher Scientific) in PBS containing 1% normal goat serum and 0.1% Triton X-100 at 4°C for 12 h. For detection of Ki67, sections were incubated in 10 mM sodium citrate (pH 6) at 80°C for 30 min and cooled to room temperature before adding antibody. The sections were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (H + L) and Alexa Fluor 546-conjugated anti-mouse IgG (H + L) antibodies (Thermo Fisher Scientific) and Hoechst [1 µg/mL bis-benzimide (Sigma-Aldrich Co. LLC)] to detect fluorescence signals and nuclei. The fluorescence intensity profiles were analyzed with Zen software (Carl Zeiss).

**Neurosphere culture, knockdown of Dicer, and thapsigargin treatment**

Neurospheres were cultured and isolated from the hippocampi of 10-day-old C57BL/6J mice according to a previously described protocol. Neurospheres were grown in neurobasal medium supplemented with B-27 without vitamin A, 20 ng/mL basic FGF, 20 ng/mL EGF, GlutaMAX, and gentamicin (Thermo Fisher Scientific). For differentiation, the cells were plated on dishes coated with natural mouse laminin (Thermo Fisher Scientific), and the medium was exchanged with medium A, which consisted of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12, Wako Pure Chemical Industries, Ltd.), MACS® NeuroBrew®-21 (Miltenyi Biotec, Bergisch Gladbach, Germany), 0.5 x N-2 supplement (Thermo Fisher Scientific), 20 ng/mL basic FGF, and gentamicin, the next day. On the following days, the medium A was changed in the morning replaced with medium B, which consisted of DMEM/F12, MACS® NeuroBrew®-21, 0.5 x N-2 supplement, and gentamicin, in the afternoon. Then, the medium was changed every two days. The cells were maintained at 37°C in an atmosphere containing 5% CO₂. Thapsigargin was added to the medium at a final concentration of 0.23–0.69 µM, the cells were incubated for 6 h, the cells were washed with medium, and the medium was replaced with new medium, and the cells were incubated for the indicated times. Knockdown of Dicer was induced by an shRNA-expressing lentivirus (sc-4090-V, Santa Cruz) and siRNA (s101206, Thermo Fisher Scientific). Differentiating cells were infected with a lentivirus expressing shRNA targeting Dicer or control shRNA (sc-108080, Santa Cruz) with 5 µg/mL polybrene at 1 DIV and then transfected with siRNA targeting Dicer or control siRNAs at 4 DIV using TransIT-X2 (Takara Bio Inc., Shiga, Japan). The following day, the cells were treated with thapsigargin. The sequences of the siRNAs targeting Dicer were as follows: 5'-GCCGAUCUCUAUUACGUAtt-3' and 5'-UACGUAAUAGAGAGAUCGCgc-3'. For immunostaining of cells, cells were fixed with 4% paraformaldehyde in 0.1 M PB 4°C for 15 min. Cells were incubated in PBS containing 10% normal goat serum and 0.3% Triton X-100 at room temperature for 1 h. Cells were incubated with an anti-Dcx antibody and anti-CHOP antibody and an anti-GFAP antibody (Thermo Fisher Scientific) in PBS containing 1% normal goat serum and 0.1% Triton X-100 at 4°C for 12 h. Cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (H + L) and Alexa Fluor 546-conjugated anti-
mouse IgG (H + L) and Alexa Fluor 647-conjugated anti-rat IgG (H + L) antibodies (Thermo Fisher Scientific).

**Semiquantitative and quantitative RT-PCR**

RNA was isolated from cells with TRIzol (Thermo Fisher Scientific) as described previously\(^{31}\). Reverse transcription was performed using M-MLV reverse transcriptase (Thermo Fisher Scientific) with random primers (Toyobo Co., Ltd., Osaka, Japan). RT-PCR analysis was performed using an S1000 Thermal Cycler (Bio-Rad, Hercules, CA) with TaKaRa Ex Taq (TaKaRa, Shiga, Japan). Semiquantitative polymerase chain reaction (PCR) was performed for 25 cycles. Quantitative RT-PCR was performed using a TP870 Thermal Cycler Dice (TaKaRa) with Thunderbird SYBR qPCR Mix (TOYOBO CO., LTD. Osaka, Japan). The PCR primer pairs were as follows: mouse *Dcx*: 5′-GCTACATTATACCATTGACGGATCCAG-3′ and 5′-TCATCACCATAAATCATGAGACAG-3′; mouse *Nestin*: 5′-GAGTCAGATCGCTCAGATCC-3′ and 5′-GGAGGACCACAGTAGAAGTGG-3′; mouse *p35*: 5′-ctgcagcccatcctcacatc-3′ and 5′-gaacacttaagcttagcgtgcgg-3′; mouse *Xbp-1*: 5′-GAATGCCCAAAAGGATATCAGACTC-3′ and 5′-GGCCTTGTGTAGAGAAACCAGGAG-3′; mouse *Dicer*: 5′-AGACCAACCTGCTCATTGCAAC-3′ and 5′-CACCATGCCGTCATTGCAAC-3′; and mouse *β-Actin*: 5′-CCTAAGGCCAACCACGG-3′ and 5′-CACGCAGATTCTCCCTC-3′.

**Statistics**

Statistical analyses were performed using SPSS Statistics 27 (IBM, Armonk, NY). Statistical significance \((p < 0.05)\) was determined using Student’s t test (two-tailed).

**Abbreviations**

ATF4, activating transcription factor 4; Aβ, amyloid-β; eIF2a, eukaryotic translation initiation factor 2a; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; siRNA, small interfering RNA; UTR, untranslated region; PKR, protein kinase RNA-activated; PERK, PKR-like ER-localized eIF2a kinase; GCN2, general control nonderepressible 2; IRE1, inositol-requiring protein-1; CHOP, C/EBP-homologous protein

**Declarations**

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**Author contributions**
K.N. and T.N. contributed to the experimental design and data interpretation; K.N., S.I., M.U., and T.N. contributed to the data analysis. All authors critically read and approved the manuscript.

Additional Information

All authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Memory was impaired in aged mice with long-term obesity (a-f) APP23 mice (a-c) (25 to 27 weeks old) and C57BL/6 mice (d-f) (10 weeks old) were fed a standard chow diet (st.) or a HFD (60% fat) for 43 weeks or 67 weeks, respectively. The body weights (BW) of APP23 (n=7 standard diet-fed mice; n=9 HFD-fed mice) (a) and C57BL/6 (n=12 standard diet-fed mice; n=12 HFD-fed mice) (d) mice. The error bars represent the standard errors of the mean (SEMs). *: p<0.05. (b, e) FBS and insulin levels in serum.
(c, f) Performance of mice with long-term obesity and control mice in the NOL test. One of three objects was moved from the previous location (P) to a novel location (N). Index (left panels). Representative trajectories of mice exploring the objects (right panels). (c) Total distance traveled (arbitrary units): standard diet-fed mice (n=5): 22.5 ± 2.2 (P), 23.5 ± 3.7 (N); HFD-fed mice (n=9): 22.6 ± 4.5 (P), 21.2 ± 4.4 (N). (f) Total distance traveled (arbitrary units): standard diet-fed mice (n=12): 36.2 ± 2.3 (P), 38.8 ± 2.2 (N); HFD-fed mice (n=12): 31.0 ± 2.6 (P), 32.9 ± 2.8 (N). (g) BW of db/db mice. control mice (n=5-7); db/db mice (n=5-7). (h) Performance of db/db mice in the MWM test. n=8 control mice; n=8 db/db mice. Latency to reach the invisible platform over four days (left panel). Number of platform crossings during the probe test on day 5 (right panel). The error bars represent the SEMs. *p < 0.05. n.s.: not significant.
Figure 2

The ER stress was activated in the brains of mice with long-term obesity (a, b, c) CHOP and ATF4 expression in the hippocampi of APP23 (a) and C57BL/6 (b) with long-term HFD-induced obesity, and db/db mice (c). st.: standard diet; H: high-fat diet. The arrow in each panel indicates ATF4. (d) ATF6 and Xbp-1 expression in the hippocampi of 60-week-old db/db mice. The arrowheads in each panel indicate
the ATF6 fragment or Xbp-1s. ES: mouse embryonic stem cell line. ES cells were treated with 2 mM DTT for 1 min or 30 min. The error bars represent the SEMs. n.s.: not significant.

**Figure 3**

CHOP and Dcx were coexpressed in immature neurons in mice with long-term obesity (a, b) Ki67-positive cells in the SGZ of the DG in 10-week-old (c: control mice, n=2; db/db mice, n=2) (a) and 45-week-old (control mice, n=2; db/db mice, n=2) (b) db/db mice. Scale bars: 100 µm. (c) Mature BDNF expression in
hippocampi of 46-week-old mice. control mice (n=5); db/db mice (n=5). (d-f) Dcx-positive cells in the DG in 45-week-old control and db/db mice. Serial sections of the DG from zero to 6.30 µm imaged by confocal microscopy. Scale bars: 40 µm (d); 20 µm (e). (f) The average number of Dcx-positive cells in the DG (left panel). The average number of Dcx-positive cells with processes in the DG (right panel). control mice (n=2); db/db mice (n=2). (g, h) CHOP was expressed in Dcx-expressing neurons in 45-week-old db/db mice (g) and 69- to 71-week-old APP23 mice with long-term HFD-induced obesity (h). The arrowhead in each panel indicates Dcx and CHOP-positive cell. Scale bars: 20 µm. The fluorescence intensity profiles are shown along the arrow (bottom panels).
Thapsigargin reduced Dcx expression in immature neurons by decreasing Dcx mRNA levels (a) Experimental schedule and representative images. Hippocampal neurospheres from mice were differentiated on laminin for 5 days. The cells were treated with thapsigargin (Tg) for 6 h, washed once with medium, and then cultured in medium without Tg for the indicated times. (b) Immunostaining for Dcx, CHOP, and GFAP in differentiating cells cultured on laminin after Tg treatment for 6 h and in the
absence of Tg for 18 h. Arrows: Dcx-expressing cells; arrowheads: Dcx- and CHOP-expressing cells. Scale bars: 100 µm (a, b). (c) Several neuronal lineage marker proteins, i.e., Dcx, Calreticulin, Nestin, βIII Tubulin, were expressed in differentiating NSCs cultured on laminin, and the expression of the ER stress markers Grp78 and CHOP was induced by Tg. The arrow indicates Dcx. Cells were lysed 42 h after Tg treatment for 6 h (Tg 6 h + 42 h). (d) The cells were pretreated with 40 μM zVAD for 30 min, Tg (0.23 μM) was added and the cells were incubated for 6 h. After the medium was changed, the cells were incubated with 40 μM zVAD without Tg for 42 h. (e) Semiquantitative RT-PCR 42 h after treatment with Tg (0.23 μM) for 6 h (upper panels). The mRNA levels of Dcx in cells cultured in medium without Tg were decreased at the indicated times after treatment with 0.23 μM Tg for 6 h (lower panel). The error bars represent the SEMs of 3 independent experiments. (f, g) The levels of Dcx mRNA were not changed after treatment with Tg (0.23 μM) for 6 h in the presence of the IRE1 inhibitor 4μ8C (f) and at 6 h after 0.23 μM of Tg treatment for 6 h in the presence of 40 μM zVAD (g). (h) Semiquantitative RT-PCR analysis of differentiating cells 6 h after 0.23 μM Tg treatment for 6 h after Dicer was knocked down using shRNA and siRNA (upper panels). Semiquantitative RT-PCR analysis of Dicer (lower left panel, p<0.05, Student’s t test). shRNA and siRNA for Control (C) and for Dicer (D). Real-time RT-PCR analysis of Dcx (lower right panel, p<0.05, Student’s t test). The error bars represent the SEMs. (i) Model of the effect of long-term obesity on neural maturation in AHN.

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