의학석사 학위논문

소포체 스트레스가 시상하부 신경세포 일차섬모 형성에 미치는 영향 연구

Study on the effect of endoplasmic reticulum stress on the formation of primary cilia in hypothalamic neurons

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ABSTRACT

Background and hypothesis: Most mammalian cells including hypothalamic neurons have non-motile primary cilia on their surface. Once-forgotten organelle, primary cilia are now regarded to have crucial roles in sensing multiple external cues and transducing internal signals. Inhibition of ciliogenesis in hypothalamic neurons leads to obesity in mice and impaired insulin and leptin signaling, suggesting a critical role of hypothalamic neuron primary cilia in body weight control. On the other hand, primary cilia lengths are shortened in mice with high fat diet (HFD), which may contribute to the progression of obesity. However, underlying mechanism of obesity-associated short cilia phenotype in hypothalamic neurons are unknown. Similarly, it has been reported that endoplasmic reticulum (ER) stress is induced in the hypothalamus of obese mice and contributes to aggravating obesity. Therefore, I hypothesized that ER stress may disrupt primary cilia formation in the hypothalamic neurons, through which it impairs leptin and insulin signaling.

Purpose: During my master's study, I investigated the effects of ER stress on primary cilia formation in hypothalamic neurons by performing in vitro and in vivo

experiments.

Methods: I induced ER stress by treating cells with palmitate and chemical ER stress inducers thapsigargin and tunicamycin in N1 hypothalamic neuronal cells. I stained the cilia using type 3 adenylyl cyclase (AC-3) antibody and analysed the primary cilia length and ciliated cell percentages. I also determined the expression levels and promoter activity of antegrade intraflagella transport (IFT) components such as IFT88, IFT20, and KIF3A, which critically mediate cilia formation, upon treatment with palmitate, thapsigargin and tunicamycin. Finally, I injected palmitate and thapsigargin into the third cerebroventricle of the mouse and examined changes in hypothalamic primary cilia.

Results: The average cilia lengths as well as the ciliated cell percentages were significantly decreased by treatment with palmitates, thapsigargin, and tunicamycin. Moreover, promoter activities and protein expression in IFT88, IFT20 and KIF3A were also downregulated in cells treated with palmitate, and ER stress inducers, in a dose-dependent manner, but the mRNA expressions were increased in those treatment. In consistent with in vitro data, intracerebroventricular injection of palmitate and ER

stress inducers potently decreased the primary cilia lengths and frequency in the hypothalamic arcuate nucleus of mice.

Conclusions: ER stress inhibits ciliogenesis in hypothalamic neurons, which may lead to reduced ability of sensing metabolic signals such as leptin and insulin, and contributes to obesity and obesity-related metabolic complication.

Keywords: primary cilia, ER stress, hypothalamus, arcuate nucleus

ABBREVATIONS

ACTH (Adrenocorticotropic hormone), **AC-3** (type 3 adenylyl cyclase), **AgRP** (Agouti-related peptide), **α-MSH** (α-melanocyte stimulating hormone), **ATF-6** (activating transcription factor 6), **BiP** (binding immunoglobulin protein), **CART** (cocaine and amphetamine-regulated transcript), **DAPI** (4′,6-diamidino-2-phenylindole) **ER** (endoplasmic reticulum), **GRP78** (78 kDa glucose regulated protein), **ICV** (intracerebroventricular), **IFT** (intraflagellar transport), **IFT 20** (intraflagellar transport 20 homolog), **IFT 88** (intraflagellar transport 88 homolog), **IRE1α** (inositol-requiring enzyme 1α), **KIF3A** (kinesin family member 3A), **LV** (lateral ventricle), **MBH** (mediobasal hypothalamus), **MC4R** (melanocortin 4 receptor), **NPY** (neuropeptide Y), **PERK** (pancreatic endoplasmic reticulum kinase), **PFA** (paraformaldehyde), **POMC** (proopiomelanocortin), **PVDF** (polyvinylidene difluoride), **SDS-PAGE** (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), **STAT3** (Signal transducer and activator of transcription 3), **TLR-4** (toll-like receptor-4), **TUDCA** (tauroursodeoxycholic acid), **UPR** (unfolded protein response), **XBP-1** (X-box binding protein 1)

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INTRODUCTION

1. Obesity: Epidemic and undercomprehended pathophysiology

Obesity is a medical condition that negatively affects human health due to excessive accumulation of fat in the body [1]. Obesity is caused by excessive energy intake in the body compared to energy expenditure in genetically susceptible individuals. Food intake is a fundamental and protective mechanism for survival and is one of the greatest pleasures in human [2]. In modern human society, however, people often eat excessively more than necessary. As a result, the prevalence of obesity and overweight is on the sharp rise in both developed and developing countries, those are considered one of the most important health problem worldwide [3]. In addition, hypertension, dyslipidemia, type 2 diabetes and cardiovascular disease, cancer and psychological disorders in obese subjects have been not only an individual medical concern, but also a socioeconomic burden [4].

Although many pharmacologic agents for treating obesity have been developed for a long time, most drugs have been proven to be ineffective or difficult to use due to intolerable adverse effects [5]. These suggest that the pathophysiological

mechanisms leading to obesity are forming an extremely complicated network between various signalling pathways, and our understanding to date is not sufficient for combating obesity [6].

2. Hypothalamus as a key controlling center of body weight

Central nervous system (CNS) plays an indispensable role in regulation of energy homeostasis. The CNS detects and integrates signals related to energy and nutritional status from the periphery and directs physiologic responses of the peripheral organs to maintain the energy homeostasis. Peripheral organs and the CNS are constantly crosstalking to respond appropriately to diverse signals from ever-changing external environment. Therefore, most anti-obesity drugs currently approved by the U.S. Food and Drug Administration act mainly in the CNS, except orlistat [7].

Hypothalamus has been considered as a key master organ in the brain for the integration of humoral and neural cues related to regulation of food intake and energy expenditure [8]. The brain area called the arcuate nucleus (ARC), which is located in the mediobasal hypothalamus (MBH) adjacent to the third ventricle, is critical for

the regulation of energy metabolism [9]. In ARC, there are two distinct neuronal populations; one produces orexigenic neuropeptides Agouti-related peptide (AgRP) and neuropeptide Y (NPY) and the other produces anorexigenic neuropeptides proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) [10]. Activation of POMC/CART neurons induces the release of α-melanocyte stimulating hormone (α-MSH), a final product of POMC, at the axon terminus, and subsequently it binds the melanocortin 4 receptor (MC4R) on the upper order neurons, leading to inhibition of food intake and stimulation of energy expenditure [11]. In contrast, the activation of AgRP/NPY neurons leads to the release of AgRP, which counteracts the effect of α-MSH by competing with α-MSH on the MC4R binding [11]. These neurons are thought to be the first-order neurons that sense and integrate peripheral metabolic signals, and send to the second-order neurons located in the perifornical area adjacent to the paraventricular nucleus (PVN), the fornix and lateral hypothalamus (LH), and the autonomic preganglionic neurons in the brainstem and spinal cord [12]. These first- and second-order neurons integrate and relay the signals to control food intake and energy expenditure.

3. Primary cilia as a signalling hub in hypothalamic regulation for energy metabolism

Primary cilium is also a cellular organelle protruding from the plasma membrane. Primary cilium was first discovered about 150 years ago and has been considered to be non-functional or have a very minor function over the past 100 years. However, recent advances in science technology have provided new insights on the roles of cilia [13]. Thus far, at least 15 signalling pathways are shown to be associated with primary cilia [14] and thus these vestigial organelles are now regarded as a hub in transducing many signalling pathways [15].

Non-motile primary cilia are ubiquitously present in most vertebrate cells except dividing cells [16]. Primary cilia is a structure in which the plasma membrane surrounds a central core of a microtubule-based skeletal structure called as axoneme. In addition, many protein complexes involved in the signal pathway are found in the ciliary membrane. Because there are no machinery for protein synthesis in the cilia, ciliary components are made externally and transported into the cilia through

specialized transport system. This transport system is called intraflagellar transport (IFT). Anterograde IFT transports newly synthesized ciliary proteins from the base to the tip of the cilia whereas retrograde IFT transports ciliary proteins from the tip to the base of the cilia. IFT particles form two complexes: IFT complex A (IFT-A) and IFT complex B (IFT-B) [17]. During antegrade transport, cargo proteins bind to IFT-B with kinesin-2 motor protein that provides the power of motility and travel along the axoneme [18]. Retrograde transport is mediated by IFT-A and dynein-2 motor protein [19]. The critical roles of IFT in the formation and function of the primary cilia have been elucidated by genetic studies. For example, genetic abnormalities in intraflagellar transport 88 homolog (IFT88) (a component of IFT-B) and kinesin family member 3A (KIF3A) (a subunit of kinesin-2) cause the absence or shortening of the cilia and abnormal signal transduction [20-22].

Inherited human diseases with impaired ciliary structures and/or functions are referred as ciliopathies. Interestingly, obesity is a common manifestation in certain type of human ciliopathies such as Bardet-Biedl syndrome and Alström syndrome [23]. Neurons in hypothalamus have particularly long primary cilia [24]. Specific inhibition

of ciliogenesis in the hypothalamic neurons such as POMC neurons induce increased food intake and obesity [25]. Therefore, primary cilia in the hypothalamic neurons have crucial roles for central regulation of energy metabolism. On the other hand, the average cilia lengths are shortened in the hypothalamus of diet-induced obese mice [14, 25]. Similarly, cilia are shortened in the hypothalamic neurons in leptin-deficient mice, which was recovered by leptin treatment [26, 27]. These findings suggest that hypothalamic ciliogenesis can be affected by obesity and the presence or absence of leptin.

4. Inflammation and ER stress in obesity

Endoplasmic reticulum (ER) is an intracellular organelle near nucleus, which plays important roles in synthesis, folding, modification and trafficking of transmembrane or secretory proteins after translation from mRNA [28]. ER stress, a condition of impaired ER functions, is induced by excessive misfolding or unfolding proteins, depletion of calcium ion or ATP, and alteration of redox state in ER lumen [29]. ER stress activate the cellular responses, termed the unfolded protein response

(UPR), that help to recover from ER stress [30]. The UPR is mediated by three signalling pathways: the pancreatic ER kinase (PERK)-Eukaryotic Initiation Factor 2 (eIF2α)-Activating transcription factor 4 (ATF4) pathway, the inositol-requiring 1a (IRE-1a)/X-box binding protein 1 (XBP-1) pathway and the activating transcription factor 6 (ATF6) pathway [31]. The primary goal of UPR is to inhibit the translation to reduce the load of unfolded proteins, and to increase the capacity of protein-folding machineries [32]. If these adaptive mechanisms are insufficient for resolving ER stress, apoptotic pathway is activated [33].

ER stress is found in adipose tissue and liver of obese animals [34, 35]. ER stress observed in obesity is associated with chronic low grade inflammation [36] and shown to interfere insulin signalling through activation of c-Jun kinase (JNK) in peripheral metabolic organs [37]. Likewise, ER stress have been observed in in the hypothalamus of obese animals and contributes to obesity-associated hypothalamic dysfunction [38]. POMC, a precursor protein of anorexigenic α-MSH, is initially synthesized as a larger inactive precursor in the rough ER of ARC neurons. Upon adequate folding in the ER lumen, it undergoes post-translational modifications and cleavage to produce final

peptides such as α-, β-, and γ-MSH and β-endorphin in the secretory granules until they are secreted [38]. Therefore, well-orchestrated ER quality control is crucial for generation of fully functional proteins. Increased ER stress could disturb the synthesis and maturation of POMC final products.

Recent studies have suggested a strong association between hypothalamic ER stress and obesity. Chemical ER stress inducers (i.e., homocysteine, tunicamycin, and thapsigargin) increased the hypothalamic expression of negative regulators of leptin signalling pathway such as SOCS3 and PTP1B [34, 39] and blocked leptin and insulin signalling in the hypothalamus [34], leading to increased food intake and weight gain. Genetic manipulation of ER stress in the hypothalamus also affects energy metabolism and obesity development and progression. For example, ATF4 overexpression in the MBH increased food intake and body weight, whereas MBH expression of dominant-negative ATF4 ameliorates impaired insulin signalling and glucose metabolism in obesity [40]. Lack of ATF4 in AgRP neurons decreased food intake and increased energy expenditure. As a result, it prevented diet-induced obesity (DIO) [41]. Notably, POMC neuron-specific ATF4 deletion also produced a similar lean phenotype [40, 42].

Collectively, these data have demonstrated that ER stress in both AgRP and POMC neurons contributes to the development of DIO.

AIM OF STUDY

Obese mice displayed impaired ciliogenesis in the hypothalamus, which was suggested as However, underlying mechanisms are still unknown. Excessive fat overload causes ER stress in multiple tissues including the hypothalamus in obese animals. Therefore, in my master thesis, I investigated whether ER stress may interfere ciliogenesis in hypothalamic neurons by performing in vitro and in vivo experiments.

MATERIALS AND METHODS

1. Cell culture

N1 murine hypothalamic neuronal cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin (100 units/ml each). The cells were cultured in a CO_2 incubator supplied with air containing 5% CO_2. Prior to the experiments, 1×10^5 N1 cells were plated onto 12-well culture plates. To stimulate ciliogenesis, cells were cultured in serum-derived condition for 48 hours before cilia analysis. For this, culture medium was changed to DMEM without FBS when the confluency of the cells reached 90%.

2. Animals

Eight weeks old male C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea). Mice were fed a standard chow diet (CD; Samyang, Seoul, Korea) and maintained under controlled temperature (22 ± 1℃) and a 12 h light-dark cycle (lights on 8 AM) with free access to food and water. All animal procedures were approved by

the Institutional Animal Care and Use Committee of the Asan Institute for Life Science (Seoul, Korea).

3. Intracerebroventricular cannulation and injection

A stainless steel intracerebroventricular (ICV) cannula were implanted into the lateral ventricle (LV) of mice using a stereotaxic surgery (stereotaxic coordinates: 0.6 mm caudal to bregma, 1 mm right to the sagittal sinus, and 2.0 mm ventral to the sagittal sinus). After 1 week of surgery, animals were daily handled to avoid stress. Angiotensin-2 (50 ng) was injected through the ICV cannula to confirm the correct positioning of each cannula. Mice with a negative drinking response to angiotensin-2 were excluded for further studies.

4. Induction of ER stress

In vitro, ER stress was induced by treating cells with different doses of ER stress inducers such as palmitate (Sigma, 0~60 μM), thapsigargin (Sigma, 0~5 μM) or tunicamycin (Santa Cruz Biotechnology, 0~12 nM) dissolved in serum-free medium for indicated times before fixing for cilia staining. All substances were tested in more

than two wells at a time and repeated three or more independent experiments. In vivo, palmitate (1.6 μg/day) or thapsigargin (10 ng/ day) was daily injected into the LV in the early light phase through the ICV cannula to induce ER stress in the hypothalamus and to evaluate the effects of ER stress on hypothalamic ciliogenesis (n = 5 per group). Mice were sacrificed in the next day of final injection in 5 hour-fast condition.

5. Cilia staining

To analyze ciliogenesis in hypothalamic neuron cells, immunohistochemical staining was performed using primary antibodies against type 3 adenylyl cyclase (AC-3), known as a ciliary marker. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) at room temperature for 15 minutes. After permeabilization with 1% Triton-X, cells were treated with blocking solution (3% normal goat serum) for 60 min and then incubated with AC-3 antibody (1: 1,000 dilution, rabbit, Santa Cruz Biotechnology) at 4 for 48 hours. After incubation with a secondary antibody (1: 1000, Invitrogen) conjugated with Alexa Fluor for 1 hour at room temperature, cells were treated with 4′,6-diamidino-2-phenylindole (DAPI,

1:10,000, Invitrogen) to stain nuclei and then mounted.

For staining cilia in the mouse hypothalamus, mice were anesthetized with intraperitoneal injection of 40 mg/kg Zoletil® and 5 mg/kg Rompun®. Mice were perfused with 50 ml of saline followed by 50 ml of 4% PFA via the left ventricle of heart. Whole brains were collected, post-fixed with 4% PFA for 24 hours, and then dehydrated in 30% sucrose solution until brains sank to the bottom of the container. Coronal brain sections including the hypothalamus were sliced 150 μm thick using a cryostat (Leica, Wetzlar, Germany). Tissue sections were incubated with the primary antibody of AC-3 at 4 for 48 hours. After washing, slides were incubated with Alexa-Fluor-555-conjugated donkey anti-rabbit antibody at room temperature for 1 hour. For nuclear staining, slides were treated with DAPI for 10 minutes before mounting.

6. Cilia analysis

Cilia immunofluorescence imaging was obtained using a laser scanning confocal microscope (Carl Zeiss or Leica). The cilia in neuron cell lines were analysed in randomly selected fields (at least 100 cilia per well and three wells per condition). Cilia

analysis of cultured cells was conducted using the ImagePro Plus program (Media Cybernetics, Silver Spring, MD). The total cell number was determined by DAPI staining of cell nuclei, and data are presented as the percentage of ciliated cells. Cell experiments were repeated at least three times.

The cilia images in the mice hypothalamic ARC were taken in three brain slices per animal (10 fields per slice) using confocal microscopy through a Z-axis stack, and images were merged using maximum intensity projection. Measurements of cilia length and number were performed using the IMARIS program.

7. Western blot

Seventy-five micrograms of cell lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). After incubation in the blocking buffer, the membranes were incubated overnight at 4 with antibodies against KIF3A (1:000; rabbit; Abcam), IFT88 (1:1000; rabbit; Proteintech), GRP78 (1:1000; goat; Santa Cruz Biotechnology) and β-actin (1:1000; mouse; Sigma-Aldrich).

Blots were developed using horseradish peroxidase-linked anti-rabbit secondary antibody (1:5000) and the chemiluminescent detection system (PerkinElmer). Band density was measured with a densitometer (VersaDoc Multi Imaging Analyzer System; Bio-Rad) and corrected to the density of β-actin.

8. Promoter analysis

The promoter regions of the genes encoding human KIF3A (nucleotide positions −1067 to +64), mouse IFT88 (nucleotide positions –913 to +81), and mouse IFT20 (nucleotide positions −1111 to +60) were cloned and ligated into the pGL3 vector (Promega). N1 cells were plated onto 12-well plates and transfected with promoter-reporter constructs (50 ng) and CMV-β-gal (25 ng) using Lipofectamine (Invitrogen). Forty-eight hours after transfection, cells were harvested in order to assay luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity. Data are shown as fold increases over the controls. Transfections were performed in duplicate, and the experiments were repeated ≥ 3 times.

9. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 22.0 (SPSS, Chicago, IL). Statistical significance between groups was tested using one-way or two-way analysis of variance (ANOVA) followed by the *post-hoc* test. Significance was defined as $P < 0.05$.

RESULTS

1. Treatment with ER stress inducers inhibits ciliogenesis in hypothalamic neuronal cells.

First, I investigated the effect of ER stress on ciliogenesis using well-ciliated N1 hypothalamic neuronal cells. Saturated fatty acid palmitate is known to induce ER stress in many cells and tissues [43]. Thapsigargin is an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and causes ER stress by decreasing Ca^{2+} content in the ER lumen [44]. Tunicamycin inhibits the first step in the biosynthesis of N-linked glycans in proteins, and produces unfolded or misfolded proteins, leading to ER stress in cells [45]. Therefore, I used palmitate, thapsigargin and tunicamycin to induce ER stress in N1 cells. N1 hypothalamic neuron cells were treated with palmitate (25 μM), thapsigargin (250 nM) or tunicamycin (3 nM) for 6 hours.

The lengths of primary cilia was significantly reduced in cells treated with ER stress inducers. The average lengths of primary cilia in control, palmitate, thapsigargin, and tunicamycin groups were 3.18 ± 0.07 μm, 2.50 ± 0.06 μm, 2.52 ± 0.05 μm, 2.54

± 0.06 μm, respectively (Fig. 1). In addition, the percentages of cells with primary cilia was also decreased (control: 88.0 ± 0.7 %, palmitate: 70.3 ± 0.8 %, thapsigargin: 72.0 ± 4.1 %, tunicamycin: 78.6 ± 4.2 %) (Fig. 2). Therefore, treatment with ER stress inducers significantly inhibited ciliogenesis in hypothalamic N1 cells.

2. ER stress inducers suppresses the transcription of antegrade IFT proteins

To generate cilia, ciliary proteins are transported to the ciliary tip area via antegrade IFT that is mediated by IFT complex-B and kinasein-2 motor. IFT88 is a component of IFT complex-B. KIF3A is a component of kinesin-2. So, I investigated whether ER stress inducers may affect the expression of IFT88 and KIF3A using western blotting. I also confirmed induction of ER stress by determining the GRP78 expression.

Palmitate treatment at the doses of 3.75, 7.5, 15, 30, and 60 μM significantly increased GRP78 protein, indicating that ER stress was induced appropriately (Fig. 3). In contrast, IFT88 and KIF3A expression levels were reduced in cells treated with palmitate (15, 30, and 60 μM) (Fig. 3). Similarly to palmitate treatment, treatment with

thapsigargin (0.1~5 μM) to N1 cells increased GRP78 expression but reduced the expression of both IFT88 and KIF3A (Fig. 4). On the other hand, tunicamycin treatment decreased KIF3A protein expression but did not reduced IFT88 protein expression (Fig. 5).

I also determined the effect of ER stress on the transcriptional activity of ciliogenesis genes such as IFT20, IFT88 and KIF3A genes. Treatment with palmitate, thapsigargin and tunicamycin significantly suppressed the promoter activities of IFT20, IFT88, and KIF3A genes in a dose-dependent manner (Fig. 6~8). Therefore, treatment with ER stress inducers inhibited the transcriptional activity and protein expression of key components of antegrade IFT machineries in N1 hypothalamic neuronal cells.

3. Treatment of ER stress inducers decrease the number and length of primary cilia in the neurons in ARC of the hypothalamus.

I finally investigated whether induction of ER stress may affect ciliogenesis in the mouse hypothalamus. ER stress was induced by injecting ER tress inducers: palmitate (1.6 μg per day) or thapsigargin (10 ng per day) via the ICV cannulae into

LV for 5 consecutive days. Notably, the lengths and frequency of primary cilia were decreased in the mice injected with palmitate and thapsigargin compared to those injected with saline (Fig. 9). Ciliary changes by ER stress inducers were much pronounced in the 3D reconstruction images of cilia. These in vivo data confirm that ER stress can disrupt cilia formation in the hypothalamus.

DISCUSSION

In my master thesis, I have shown that induction of ER stress by treating hypothalamic neuron cells with palmitate, thapsigargin, and tunicamycin significantly reduced the average cilia lengths as well as the ciliated cell percentages. Consistently, ICV injection of palmitate and thapsigargin in mice led to impaired ciliogenesis in the hypothalamus. Together, these data strongly suggested that ER stress can inhibit ciliogenesis in the hypothalamic neurons.

A previous study has demonstrated reduced cilia lengths in the hypothalamus of mice with HFD-induced obesity [26]. However, the mechanism of DIO-associated short cilia phenotype in the hypothalamus has not been shown so far. Therefore, my study has firstly provided the evidence that ER tress may be a potential mechanism of obesity-induced ciliary changes in the hypothalamus.

Increased ER stress has been reported not only in the peripheral tissues but also in the hypothalamus of obese animals [34, 35, 46]. Obesity-induced ER stress is related to abnormal fat overload in the tissues. Low-grade inflammation found in the multiple tissues of obese animals may also contribute to the development of ER stress as

suggested previously [47, 48]. Consistent with these observations, treatment with saturated fatty acid palmitate increased the GRP78 expression, which is a marker of ER stress.

Notably, ER stress-induced impairment in ciliogenesis appeared to be regardless of the cause of ER stress. Indeed, palmitate may induce ER stress by activating cellular stress response and inflammation. Thapsigargin induces ER stress by depleting intra-ER calcium content. Tunicamycin induces ER stress by inhibiting the glycation of proteins inside the ER. All these ER stress inducers commonly disrupted the cilia formation, suggesting that ER stress generally interfere cilia formation. It will be worth to test in the future that ER stress can inhibit ciliogenesis in the other types of cells and tissues.

As a molecular mechanisms underlying ER stress-induced inhibition of ciliogenesis, I found that ER tress downregulated the transcription of ciliogenic genes such as IFT20, IFT88, and KIF3A that involved antegrade IFT. Supporting it, treatment of palmitate, thapsigargin and tunicamycin reduced the expression levels of KIF3A and IFT88. Furthermore, the transcriptional activities of KIF3A-, IFT20-, and

IFT88-encoding genes was significantly supressed by treatment of ER stress inducers. Under ER stress conditions, transcription and translation of many genes except ER chaperons was generally suppressed [32] while helps ER to recover their protein folding capacity during ER stress. Upon ER stress, three UPR pathways, PERK-eIF2α-ATF4 pathway, IRE1α-XBP1 pathway and ATF6 pathways are activated [31]. All three pathways may be involved in the transcriptional regulation of UPR target genes. Therefore, I am currently investigating which UPR pathways may mediate ER stress-caused transcriptional inhibition of ciliogenic genes.

To make cilia, protein components of ciliary plasma membrane and axoneme are synthesized in the ribosome in the cytosol, matured and properly folded in the ER and Golgi, and then transported to the periciliary area around the basal body via Golgi-derived vesicles. Therefore, in addition to transcriptional regulation, ER stress may potentially disrupt the protein maturation and folding of ciliogenic genes in the ER at the post-transcriptional stage.

Induction of ER stress has been shown to disrupt insulin and leptin signalling in the hypothalamus [49]. Reduced insulin and leptin signalling in the hypothalamic

neurons has been suggested to be a potential mechanism of obesity and energy imbalance. Notably, artificial inhibition of ciliogenesis in the bilateral MBH by stereotaxic injection of small inhibitory RNAs specific to KIF3A and IFT88 reduced anorexigenic responses to leptin, insulin and glucose [14]. In addition, artificial inhibition of hypothalamic ciliogenesis accelerated weight gain and disrupted energy balance. Taken these data together, ER stress-induced impairment in hypothalamic ciliogenesis may contribute to the development of central leptin and insulin resistance in obese condition and thus the progression of obesity and related metabolic disorders. It will be interesting to test whether resolving ER stress in obese mice by treating chemical ER chaperons such as tauroursodeoxycholic acid (TUDCA) can reverse obesity associated short-cilia phenotypes in the hypothalamus.

On the other hand, primary cilia are transiently expressed in preadipocytes and mediate Hedgehog and Wnt signaling pathways [14, 50]. Inhibition of ciliogenesis as well as of Hedgehog and Wnt signaling in preadipocytes promoted adipogenesis [14, 50]. These data may suggest that ER stress in adipocyte tissues possibly promotes obesity by inhibiting ciliogenesis in developing adipocytes. This possibility will be an

interesting topic in the future study.

In conclusion, my study has convincingly demonstrated that ER stress interferes cilia formation and lengthening in the hypothalamic neurons. ER stress-induced disruption of cilia formation in the hypothalamus could decrease the neuronal ability to sense metabolic signals from the periphery and thus to maintain energy homeostasis.

Table 1. Primer sets used in real time PCR analysis

Gene		Primer (5' to 3')
Kif3a	Forward	AGCTGCGATAATGTGAAGGTG
	Reverse	GTTCCCCTCATTTCATCCACG
Ift88	Forward	GCAGTGACAGTGGCCAGAAC
	Reverse	AAGGTTCATCTGTCCCAGGC
Xbp-1	Forward	TGCTGAGTCCGCAGCAGGTG
	Reverse	GCTGGCAGGCTCTGGGGAAG
Gapdh	Forward	ACTCTTCCACCTTCGATGC
	Reverse	CCTGTTGCTGTAGCCGTAT

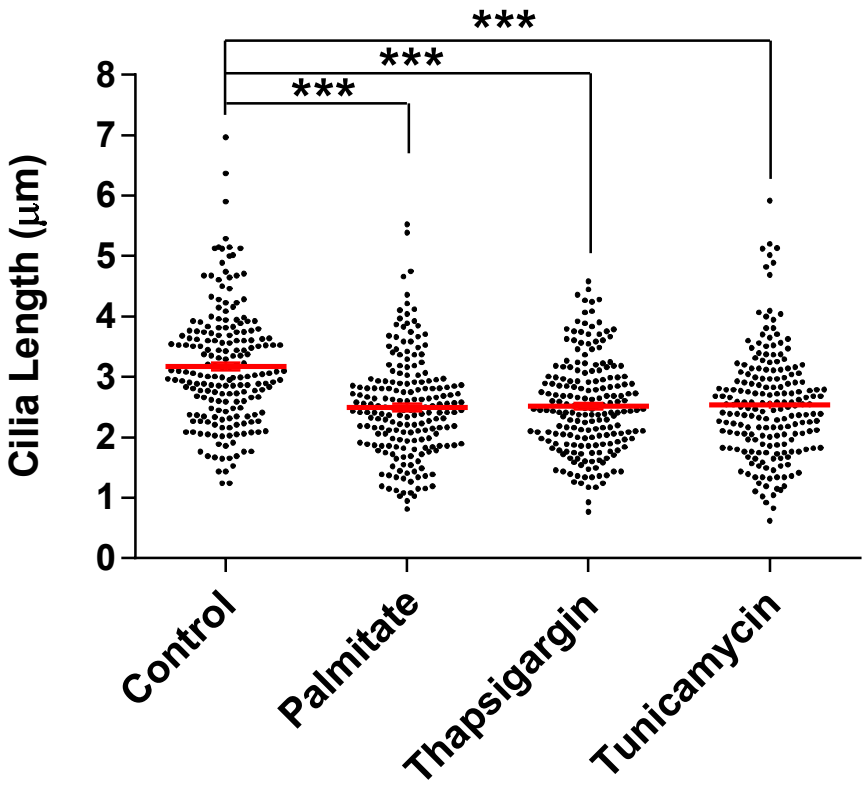

Figure 1. Effects of palmitate and chemical ER stress inducers (thapsigargin and tunicamycin) treatment on primary cilia lengths in N1 hypothalamic neuronal cells.

Cells are treated with 25 μM palmitate, 250 nM thapsigargin, or 3 nM tunicamycin for 6 hours. Results are presented as mean ± standard error of mean (SEM). $^{***}P < 0.001$ vs. untreated control.

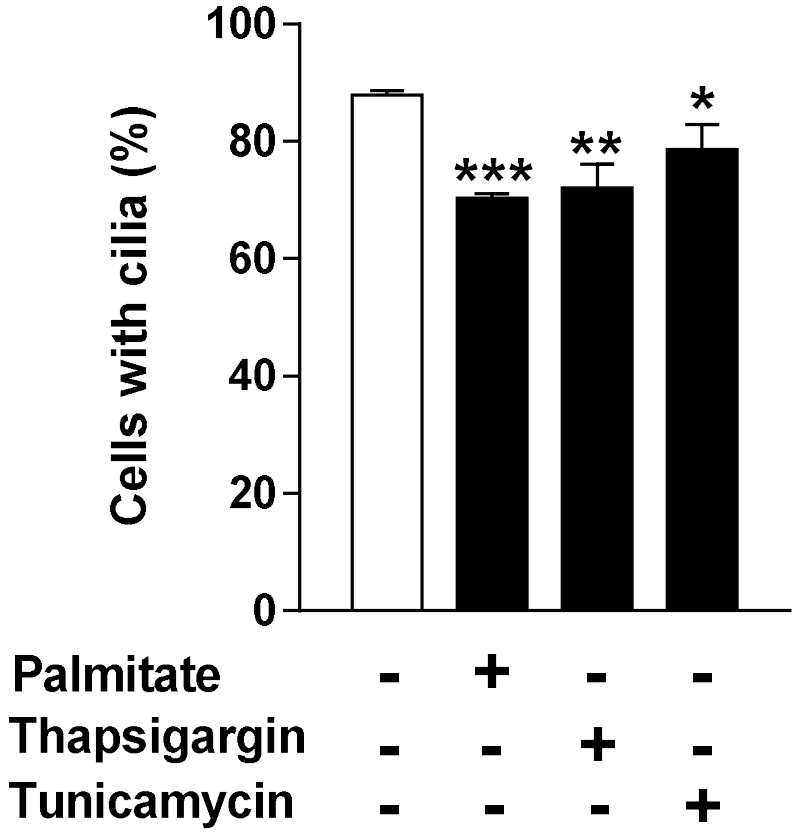

Figure 2. Effects of ER stress inducers (thapsigargin and tunicamycin) treatment on ciliated cell percentages in N1 hypothalamic neuronal cells.

Cells are treated with 25 μM palmitate, 250 nM thapsigargin, or 3 nM tunicamycin for 6 hours. Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$ vs. untreated control.

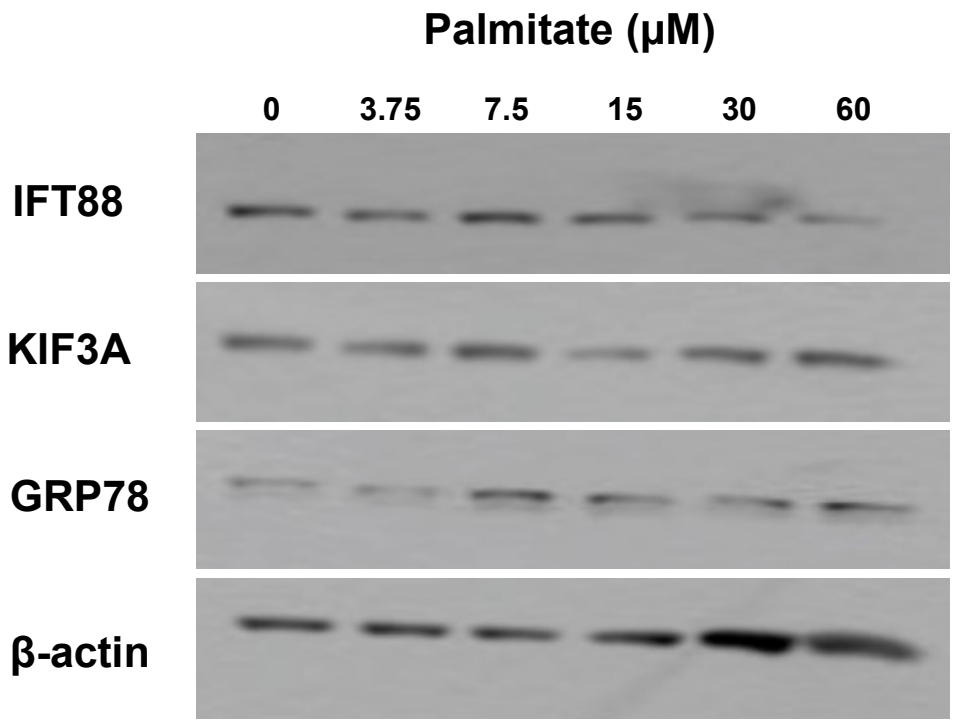

Figure 3. Effects of palmitate treatment on protein expression of antegrade IFT-related proteins (IFT88 and KIF3A) and ER stress protein (GRP78) in N1 hypothalamic neuronal cells.

Cells were treated with various concentrations of palmitate (0 ~ 60 μM) for 6 hours.

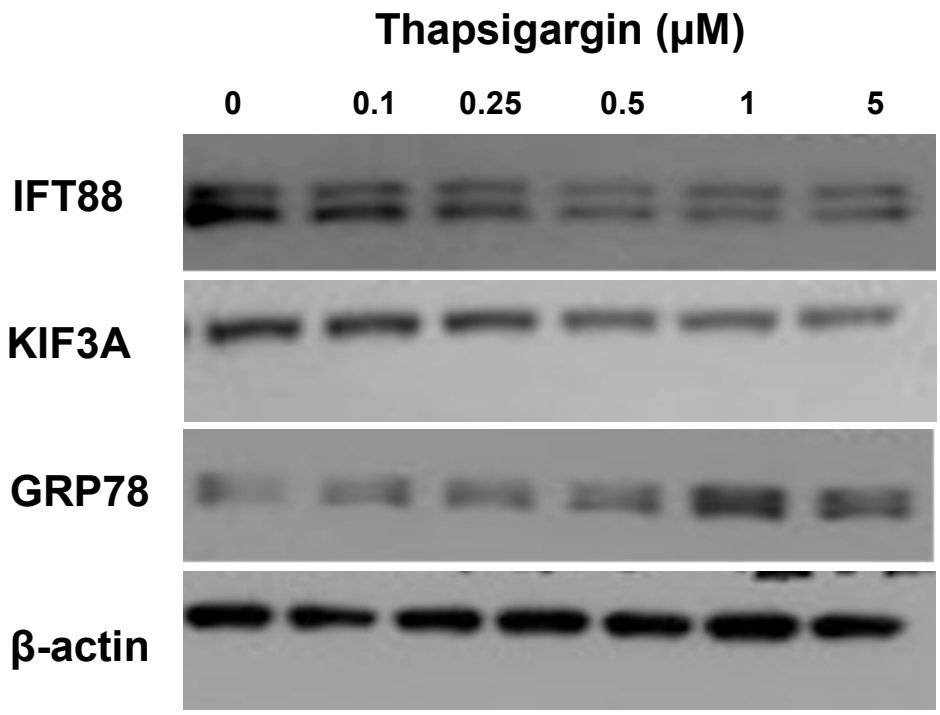

Figure 4. Effects of thapsigargin treatment on protein expression of antegrade IFT-related proteins (IFT88 and KIF3A) and ER stress protein (GRP78) in N1 hypothalamic neuronal cells.

Cells were treated with various concentrations of thapsigargin (0 ~ 5 μM) for 6 hours.

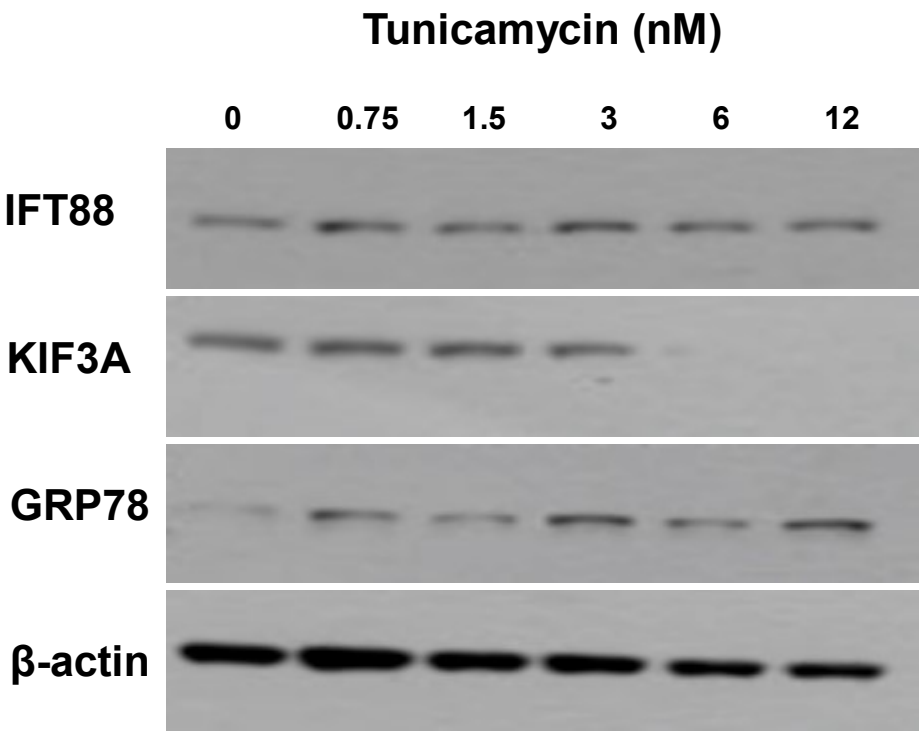

Figure 5. Effects of tunicamycin treatment on protein expression of antegrade IFT-related proteins (IFT88 and KIF3A) and ER stress protein (GRP78) in N1 hypothalamic neuronal cells.

Cells were treated with various concentrations of tunicamycin (0 ~ 12 nM) for 6 hours.

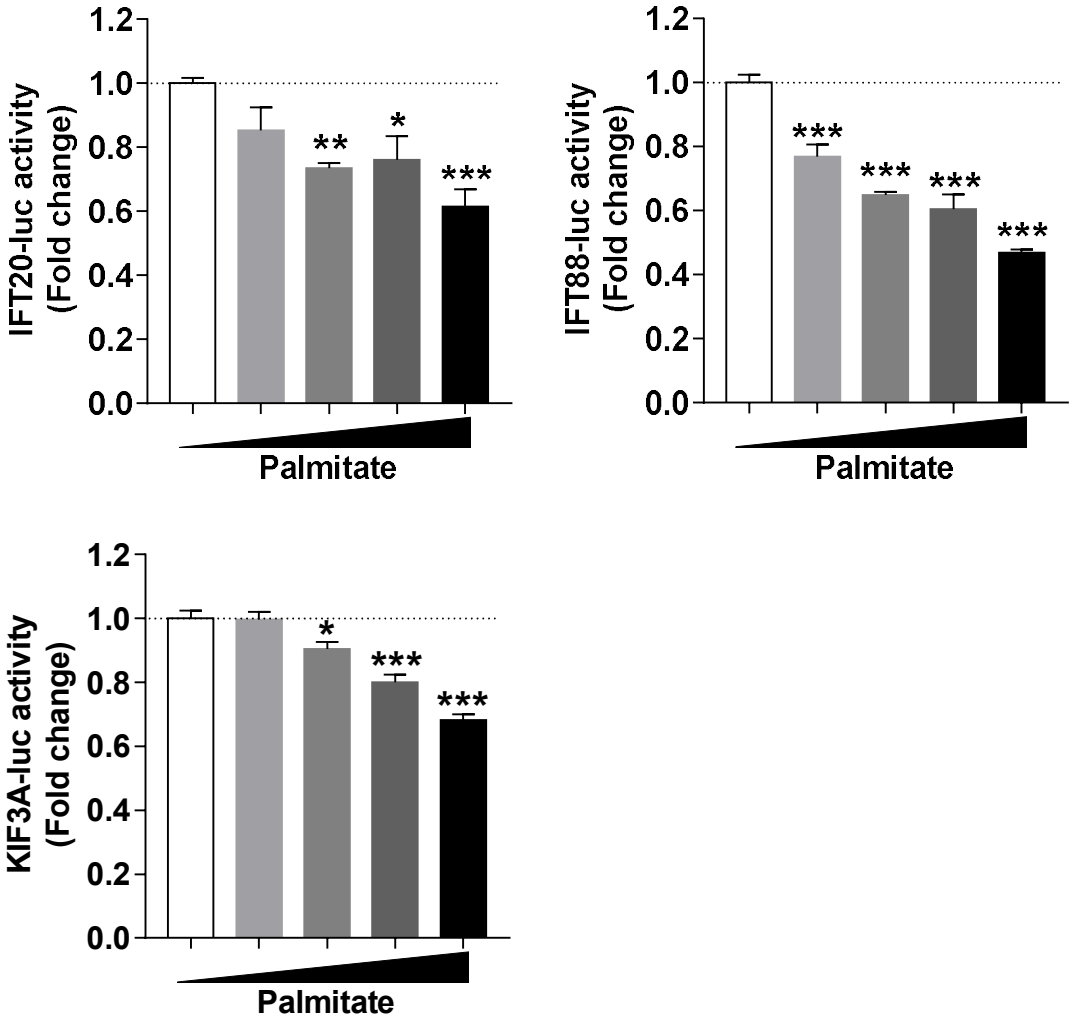

Figure 6. Palmitate treatment downregulated the promoter activities of IFT20, IFT88, and KIF3A in N1 hypothalamic neuron cells.

Cells were treated with palmitate (mock, 7.5, 15, 30, 60 μM) for 6 hours before promotor analysis. Results are presented as mean ± SEM. **P* < 0.05, ***P* < 0.05, ****P* < 0.001 vs. control.

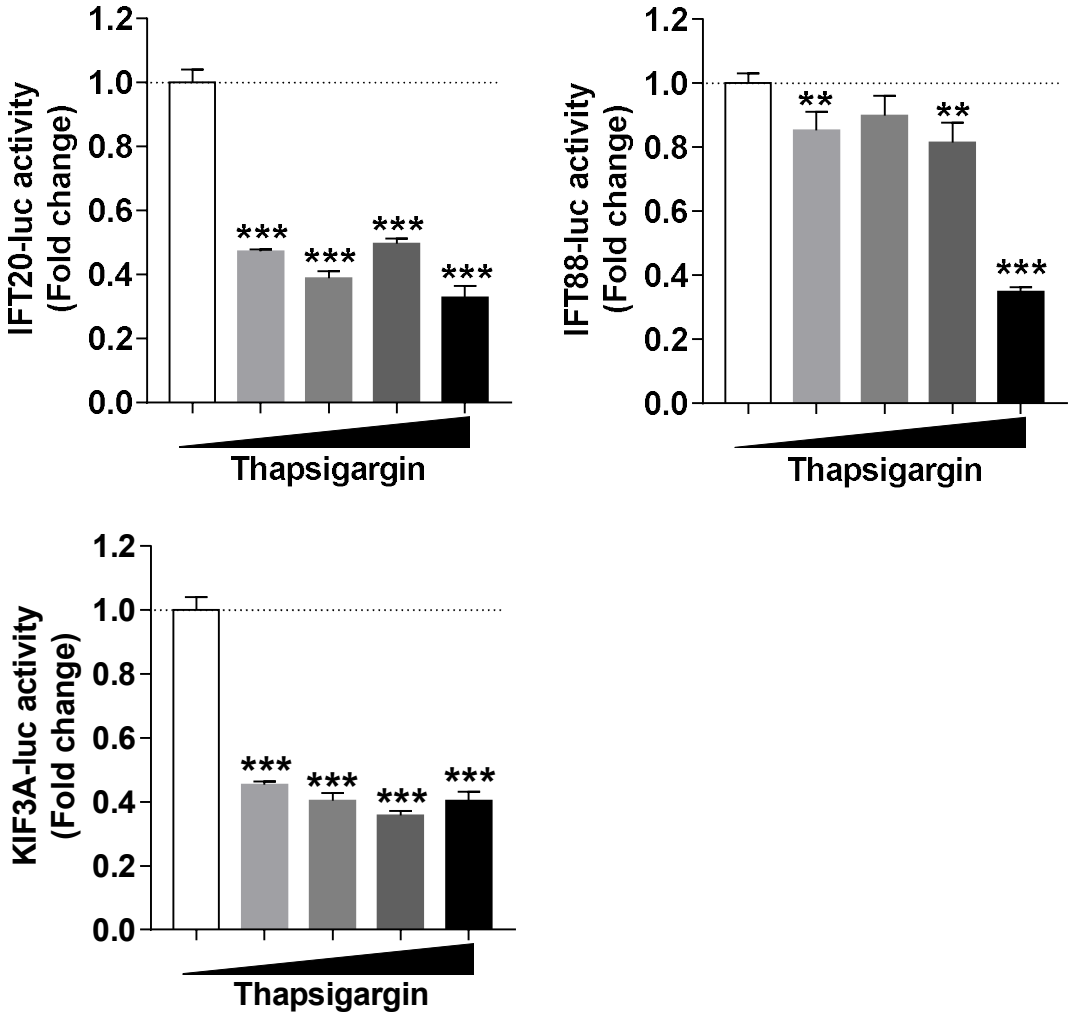

Figure 7. Thapsigargin treatment reduced the promoter activities of IFT20, IFT88, and KIF3A in N1 hypothalamic neuron cells.

Cells were treated with thapsigargin (mock, 0.25, 0.5, 1, 5 μM) for 6 hours before promotor analysis. Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$ vs. control.

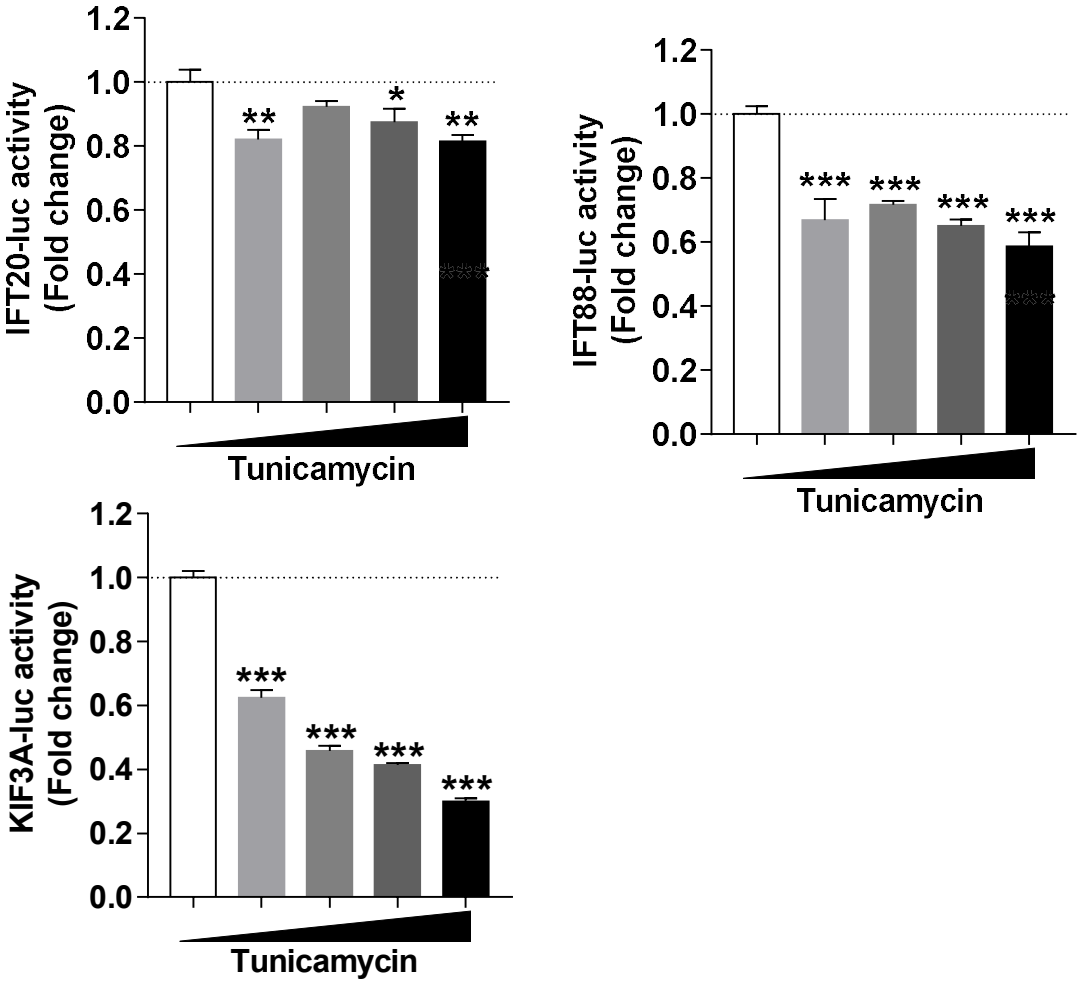

Figure 8. Tunicamycin treatment decreased the promoter activities of IFT20, IFT88, and KIF3A in N1 hypothalamic neuron cells.

Cells were treated with tunicamycin (mock, 1.5, 3, 6, 12 nM) for 6 hours before promotor analysis. Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$ vs. control.

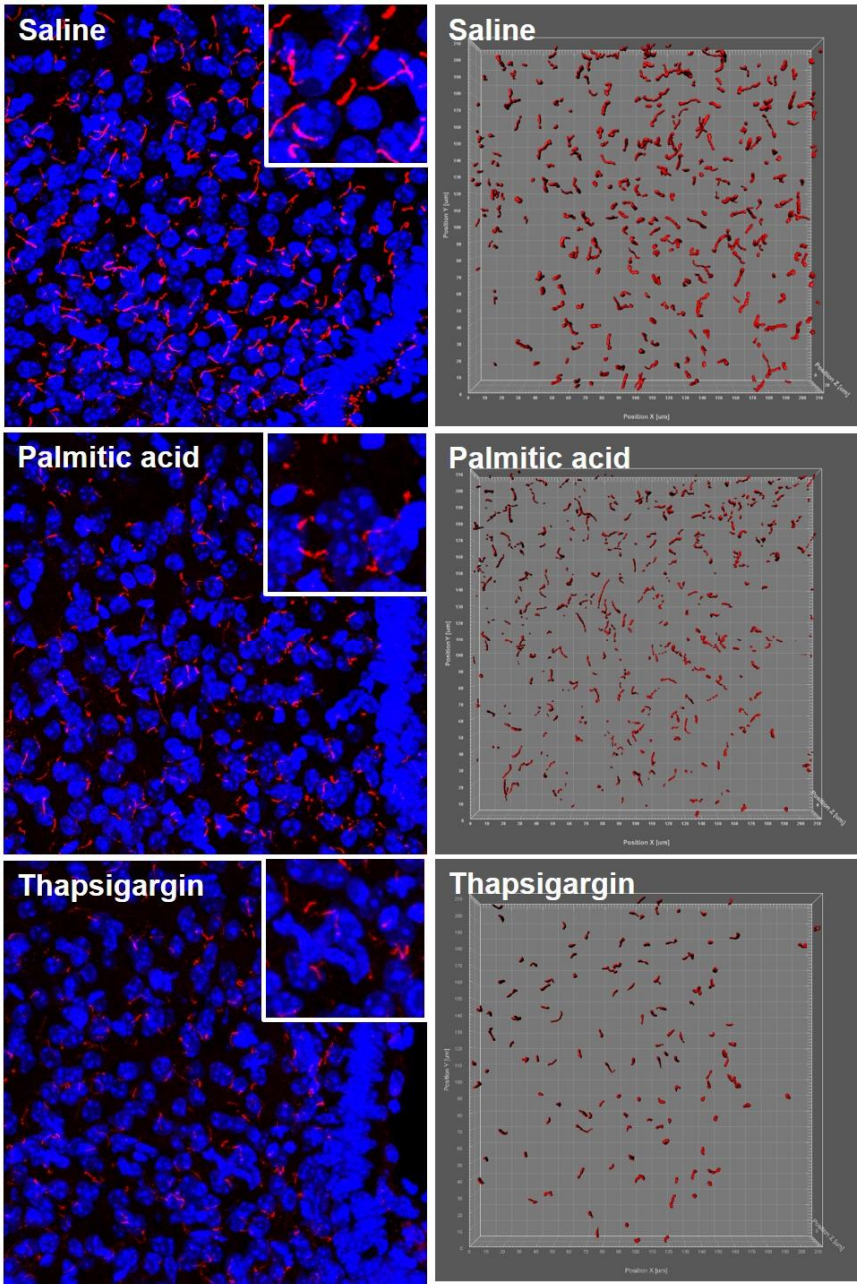

Figure 9. Effects of intracerebroventricular administration of palmitate and thapsigargin on the primacy cilia in the mouse hypothalamic arcuate nucleus.

Primary cilia stained with cilia marker adenylyl cyclase 3. Left: immunofluorescence image. Right: 3D-reconstructed cilia images, red: primary cilia, blue: DAPI.

REFFERENCES

1. Hebebrand, J., et al., *A Proposal of the European Association for the Study of Obesity to Improve the ICD-11 Diagnostic Criteria for Obesity Based on the Three Dimensions Etiology, Degree of Adiposity and Health Risk.* Obes Facts, 2017. **10**(4): p. 284-307.
2. Zheng, H. and H.R. Berthoud, *Eating for pleasure or calories.* Curr Opin Pharmacol, 2007. **7**(6): p. 607-12.
3. World Health Organization, *Obesity and Overweight Fact Sheet.* 2018.
4. Gonzalez-Muniesa, P., et al., *Obesity.* Nat Rev Dis Primers, 2017. **3**: p. 17034.
5. Kim, G.W., et al., *Antiobesity pharmacotherapy: new drugs and emerging targets.* Clin Pharmacol Ther, 2014. **95**(1): p. 53-66.
6. Levian, C., E. Ruiz, and X. Yang, *The pathogenesis of obesity from a genomic and systems biology perspective.* Yale J Biol Med, 2014. **87**(2): p. 113-26.
7. Pilitsi, E., et al., *Pharmacotherapy of obesity: Available medications and drugs under investigation.* Metabolism, 2019. **92**: p. 170-192.
8. Roh, E., D.K. Song, and M.S. Kim, *Emerging role of the brain in the homeostatic regulation of energy and glucose metabolism.* Exp Mol Med, 2016. **48**: p. e216.
9. Elias, C.F., et al., *Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area.* J Comp Neurol, 1998. **402**(4): p. 442-59.
10. Dietrich, M.O. and T.L. Horvath, *Hypothalamic control of energy balance: insights into the role of synaptic plasticity.* Trends Neurosci, 2013. **36**(2): p. 65-

73.

11. Timper, K. and J.C. Bruning, *Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity.* Dis Model Mech, 2017. **10**(6): p. 679-689.

12. Roh, E. and M.S. Kim, *Brain Regulation of Energy Metabolism.* Endocrinol Metab (Seoul), 2016. **31**(4): p. 519-524.

13. Bloodgood, R.A., *From central to rudimentary to primary: the history of an underappreciated organelle whose time has come. The primary cilium.* Methods Cell Biol, 2009. **94**: p. 3-52.

14. Song, D.K., J.H. Choi, and M.S. Kim, *Primary Cilia as a Signaling Platform for Control of Energy Metabolism.* Diabetes Metab J, 2018. **42**(2): p. 117-127.

15. Berbari, N.F., et al., *The primary cilium as a complex signaling center.* Curr Biol, 2009. **19**(13): p. R526-35.

16. Goto, H., H. Inaba, and M. Inagaki, *Mechanisms of ciliogenesis suppression in dividing cells.* Cell Mol Life Sci, 2017. **74**(5): p. 881-890.

17. Louvi, A. and E.A. Grove, *Cilia in the CNS: the quiet organelle claims center stage.* Neuron, 2011. **69**(6): p. 1046-60.

18. Cole, D.G., et al., *Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons.* J Cell Biol, 1998. **141**(4): p. 993-1008.

19. Pazour, G.J., C.G. Wilkerson, and G.B. Witman, *A dynein light chain is essential for the retrograde particle movement of intraflagellar transport (IFT).* J Cell Biol, 1998. **141**(4): p. 979-92.

20. Pazour, G.J., et al., *Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella.* J Cell Biol, 2000. **151**(3): p. 709-18.

21. Cortellino, S., et al., *Defective ciliogenesis, embryonic lethality and severe impairment of the Sonic Hedgehog pathway caused by inactivation of the mouse complex A intraflagellar transport gene Ift122/Wdr10, partially overlapping with the DNA repair gene Med1/Mbd4.* Dev Biol, 2009. **325**(1): p. 225-37.

22. Takeda, S., et al., *Left-right asymmetry and kinesin superfamily protein KIF3A: new insights in determination of laterality and mesoderm induction by kif3A-/- mice analysis.* J Cell Biol, 1999. **145**(4): p. 825-36.

23. Ferkol, T.W. and M.W. Leigh, *Ciliopathies: the central role of cilia in a spectrum of pediatric disorders.* J Pediatr, 2012. **160**(3): p. 366-71.

24. Fuchs, J.L. and H.D. Schwark, *Neuronal primary cilia: a review.* Cell Biol Int, 2004. **28**(2): p. 111-8.

25. Davenport, J.R., et al., *Disruption of intraflagellar transport in adult mice leads to obesity and slow-onset cystic kidney disease.* Curr Biol, 2007. **17**(18): p. 1586-94.

26. Han, Y.M., et al., *Leptin-promoted cilia assembly is critical for normal energy balance.* J Clin Invest, 2014. **124**(5): p. 2193-7.

27. Kang, G.M., et al., *Leptin Elongates Hypothalamic Neuronal Cilia via Transcriptional Regulation and Actin Destabilization.* J Biol Chem, 2015. **290**(29): p. 18146-55.

28. Hetz, C., *The unfolded protein response: controlling cell fate decisions under*

ER stress and beyond. Nat Rev Mol Cell Biol, 2012. **13**(2): p. 89-102.

29. Mekahli, D., et al., *Endoplasmic-reticulum calcium depletion and disease.* Cold Spring Harb Perspect Biol, 2011. **3**(6).

30. Mori, K., *The unfolded protein response: the dawn of a new field.* Proc Jpn Acad Ser B Phys Biol Sci, 2015. **91**(9): p. 469-80.

31. Kadowaki, H. and H. Nishitoh, *Signaling pathways from the endoplasmic reticulum and their roles in disease.* Genes (Basel), 2013. **4**(3): p. 306-33.

32. Ron, D. and P. Walter, *Signal integration in the endoplasmic reticulum unfolded protein response.* Nat Rev Mol Cell Biol, 2007. **8**(7): p. 519-29.

33. Sano, R. and J.C. Reed, *ER stress-induced cell death mechanisms.* Biochim Biophys Acta, 2013. **1833**(12): p. 3460-3470.

34. Cakir, I., et al., *Obesity induces hypothalamic endoplasmic reticulum stress and impairs proopiomelanocortin (POMC) post-translational processing.* J Biol Chem, 2013. **288**(24): p. 17675-88.

35. Ozcan, U., et al., *Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes.* Science, 2004. **306**(5695): p. 457-61.

36. Ghemrawi, R., S.F. Battaglia-Hsu, and C. Arnold, *Endoplasmic Reticulum Stress in Metabolic Disorders.* Cells, 2018. **7**(6).

37. Zhang, X., et al., *Endoplasmic reticulum stress mediates JNK-dependent IRS-1 serine phosphorylation and results in Tau hyperphosphorylation in amyloid beta oligomer-treated PC12 cells and primary neurons.* Gene, 2016. **587**(2): p. 183-93.

38. Cakir, I. and E.A. Nillni, *Endoplasmic Reticulum Stress, the Hypothalamus, and Energy Balance.* Trends Endocrinol Metab, 2019. **30**(3): p. 163-176.

39. Hosoi, T., et al., *Endoplasmic reticulum stress induces leptin resistance.* Mol Pharmacol, 2008. **74**(6): p. 1610-9.

40. Zhang, Q., et al., *Central activating transcription factor 4 (ATF4) regulates hepatic insulin resistance in mice via S6K1 signaling and the vagus nerve.* Diabetes, 2013. **62**(7): p. 2230-9.

41. Deng, J., et al., *Deletion of ATF4 in AgRP Neurons Promotes Fat Loss Mainly via Increasing Energy Expenditure.* Diabetes, 2017. **66**(3): p. 640-650.

42. Xiao, Y., et al., *An ATF4-ATG5 signaling in hypothalamic POMC neurons regulates obesity.* Autophagy, 2017. **13**(6): p. 1088-1089.

43. Zhang, Y., et al., *Palmitic and linoleic acids induce ER stress and apoptosis in hepatoma cells.* Lipids Health Dis, 2012. **11**: p. 1.

44. Garcia-Casas, P., et al., *Inhibition of Sarco-Endoplasmic Reticulum Ca(2+) ATPase Extends the Lifespan in C. elegans Worms.* Front Pharmacol, 2018. **9**: p. 669.

45. Diehl, J.A., S.Y. Fuchs, and C. Koumenis, *The cell biology of the unfolded protein response.* Gastroenterology, 2011. **141**(1): p. 38-41, 41 e1-2.

46. Zhang, X., et al., *Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity.* Cell, 2008. **135**(1): p. 61-73.

47. Lumeng, C.N. and A.R. Saltiel, *Inflammatory links between obesity and metabolic disease.* J Clin Invest, 2011. **121**(6): p. 2111-7.

48. Tripathi, Y.B. and V. Pandey, *Obesity and endoplasmic reticulum (ER) stresses.* Front Immunol, 2012. **3**: p. 240.

49. Hummasti, S. and G.S. Hotamisligil, *Endoplasmic reticulum stress and inflammation in obesity and diabetes.* Circ Res, 2010. **107**(5): p. 579-91.

50. Oh, E.C., S. Vasanth, and N. Katsanis, *Metabolic regulation and energy homeostasis through the primary Cilium.* Cell Metab, 2015. **21**(1): p. 21-31.

ABSTRACT IN KOREAN

배경 및 가설: 시상하부 뉴런을 포함하여 대부분의 포유류 세포들은 세포 표면에 운동성이 없는 일차섬모를 가지고 있다. 예전에 일차섬모는 특별한 기능이 없거나 진화의 흔적기관 정도로만 여겨졌으나, 최근에는 일차섬모가 다양한 외부 신호들을 감지하고, 새포내 신호 전달에 중요한 역할을 함이 밝혀져 주목을 받고 있다. 시상하부 뉴런의 일차섬모 형성을 억제시키는 것은 비만을 유발하고 인슐린과 렙틴의 신호 전달에 이상을 일으키는데, 이는 시상하부 뉴런의 일차섬모가 체중과 에너지 대사 조절에 있어 중요한 역할을 하고 있음을 암시한다. 흥미롭게도 고지방식으로 유도한 비만 생쥐에서 일차섬모의 길이가 짧아져 있는데, 비만증에서 시상하부 뉴런의 일차섬모 길이의 감소가 어떤 기전을 통해 발생하는지는 아직 알려져 있지 않다. 한편 비만을 동반한 생쥐의 시상하부에서 소포체 스트레스가 증가되어 있고, 증가한 소포제 스트레스가 비만의 악화에 기여함이 보고된 바 있다. 따라서 본 학위 논문에서는 비만증에서 소포체 스트레스가 시상하부 뉴런 일차섬모 형성을 방해하여 시상하부 뉴론에 렙틴과 인슐린의 신호전달을 방해할 것이다는 가설을 세웠다.

연구 목적: 소포체 스트레스가 시상하부 뉴런에서 일차섬모의 형성에 미치는 영향을 in vitro 와 in vivo 실험을 통하여 연구하였다.

연구 방법: 마우스 시상하부 뉴런의 세포주인 N1 세포를 포화지방산의 일종인 팔미트산과 화학적 소포체 스트레스 유도물질인 탑시가진과 튜니카마이신을 처리하여 소포체 스트레스를 유도하였다. Adenylyl cyclase (AC3)를 사용하여 일차섬모를 염색하였고, 일차섬모의 길이와 일차섬모를 가진 뉴런의 분율을 분석하였다. 또한 일차섬모의 anterograde intraflagellar transport (IFT)의 구성요소인 IFT88, IFT20, KIF3A 의 단백 발현량과 프로모터 활성도를 측정하였다. 마지막으로 마우스 뇌의 뇌실 안에 삽입한 캐뉼라를 통해 팔미트산과 탑시가진을 주입하고 시상하부 일차섬모의 변화를 조사하였다.

연구 결과: 포화지방산인 팔미트산과 소포체 스트레스 유도물질인 탑시가진, 튜니카마이신을 N1 세포에 처리하였을 때, 일차섬모의 길이와 일차섬모를 가진 세포 분율이 모두 감소하였다. 또한 상기 치료는 일차섬모 형성에 필요한 유전자들의 프로모터 활성과 단백 발현을 용량 의존적으로 감소시켰다. 마우스의 뇌실로 팔미트산과 탑시가진을 주입하였을 때, 세포 실험에서의 결과와 유사하게 시상하부의 궁상핵에서 현저한 일차섬모의 길이와 수가 감소하였다.

결론: 이상의 결과로부터 시상하부 뉴런에서 에너지 과잉상태에 따른 ER stress 는 일차섬모의 형성을 억제함으로써 시상하부 뉴론의 대사신호 감지 능력을 감소시켜 비만의 진행에 기여할 수 있음을 알 수 있었다.