Formation of Endoplasmic Reticulum-Associated Compartment in Vasopressin Neurons: A Mechanism by Which Endoplasmic Reticulum Stress is Reduced

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Analyses of a mouse model for familial neurohypophysial diabetes insipidus (FNDI), a disease characterized by progressive polyuria due to progressive decreases in arginine vasopressin (AVP) release, revealed that mutant proteins are accumulated in a sub-compartment of the endoplasmic reticulum (ER) of AVP neurons. By forming such a structure called ER-associated compartment (ERAC), AVP neurons are likely to reduce ER stress. However, the formation of ERAC is hampered in FNDI mice which are relatively old or subjected to chronic dehydration. Failure of ERAC formation induces autophagy in AVP neurons, which are finally lost through autophagy-associated cell death. It is also worthwhile that enlargement of a sub-compartment of ER, a structure similar to ERAC, was observed in the AVP neurons in wild-type mice subjected to dehydration. Activating transcription factor 6α (ATF6α), one of three ER stress sensors, contributes to the formation of ERAC, as the ER was dilated diffusely in AVP neurons of dehydrated ATF6α knockout mice. Thus, our data suggest that misfolded proteins are sensed via ER stress sensors including ATF6α, and confined to the ERAC in AVP neurons. This mechanism seems to apply to the AVP neurons of not only FNDI but also wild-type mice.

KEYWORDS: arginine vasopressin, familial neurohypophysial diabetes insipidus, endoplasmic reticulum stress, autophagy, cell death

1. Introduction

Arginine vasopressin (AVP) is an antidiuretic hormone which promotes reabsorption of free water through its V2 receptor in the kidney. AVP is synthesized in magnocellular neurons of the supraoptic (SON) and paraventricular nuclei (PVN) in the hypothalamus. The AVP gene encodes the signal peptide, AVP, neurophysin II (NPII), which is the carrier protein of AVP, and glycoprotein. After translation of mRNA, the preproAVP is converted to proAVP by removal of its signal peptide in the ER. The folded proAVP is packaged into the granules via the Golgi apparatus, and AVP as well as NPII and glycoprotein is cleaved during the axonal transport to the posterior pituitary. The release and synthesis of AVP are precisely regulated by serum Na levels, and only 1–2% increases in serum Na levels significantly increase AVP release and the transcription of AVP gene [1]. The serum Na levels are thus kept almost constant (around 140 mEq/L) by the antidiuretic action of AVP in normal subjects.

The deficiency of AVP leads to polyuria accompanied by polydipsia, a disorder called neurohypophysial diabetes insipidus. Serum Na levels in patients with neurohypophysial diabetes insipidus are around 145 mEq/l unless treated, a level which causes thirst. As a result, patients with neurohypophysial diabetes insipidus drink water until the thirst ceases, and the amount of water intake could be up to 10 L/day. The causes of neurohypophysial diabetes insipidus include tumors (such as craniopharyngioma and germinoma), pituitary surgery, and inflammation (such as lymphocytic infundibulo-neurohypophysitis and IgG4-related disease) [2]. Although rare, neurohypophysial diabetes insipidus also occurs on a familial basis, i.e., familial neurohypophysial diabetes insipidus (FNDI), which is inherited mostly in an autosomal dominant mode [3]. The mutations that cause FNDI are mainly located in the NPII-coding region of the AVP gene locus [3]. FNDI is characterized by progressive polyuria due to progressive deficiency of AVP, and the most plausible explanation for the phenotype had been the progressive loss of AVP neurons due to the accumulation of mutant proteins [4]. In order to examine whether or not this hypothesis is true, we made FNDI model mice in which a mutation (Cys98stop) causing FNDI was introduced in the NPII-coding region of the mouse AVP gene locus. The heterozygous knockin mice manifested progressive polyuria as do patients with FNDI [5]. Thus, we succeeded in making a mouse model for FNDI with which we could analyze how progressive polyuria occurs, how the AVP neurons degrade the misfolded proteins, and how AVP neurons eventually die in FNDI.

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2. Water Balance in FNDI Mice

Urine volumes in FNDI mice increased progressively at least until 12 months after birth, while pituitary AVP content was significantly reduced in FNDI mice compared to wild-type (WT) mice (Fig. 1A, B). Water intake was also increased and urine osmolality was significantly reduced in FNDI mice [5]. These data indicated that progressive polyuria and polydipsia in FNDI mice are due to progressive decreases in AVP synthesis and release. Increases in urine volumes and decreases in pituitary AVP contents were greater in females than in male FNDI mice (Fig. 1A, B). As ovariectomy decreased urine volumes in female FNDI mice and estrogen replacement cancelled the effects of ovariectomy on urine volumes [5], it was suggested that estrogen contributes to the prominent phenotype in females, although the detailed mechanisms still need to be clarified.

3. Morphological Analyses of AVP Neurons in FNDI Mice

Immunohistochemical analyses of 1-month-old mice with specific antibodies for mutant NPII, which react with the C terminal sequence of mutated NPII, revealed that mutant proteins are expressed in AVP neurons of the SON and PVN in FNDI, but not in WT mice [5]. Analyses with antibodies for normal NPII also revealed that while normal NPII is expressed in both cell bodies and axons of AVP neurons in WT mice, it is mainly expressed in cell bodies in FNDI mice (Fig. 2). It is thus suggested that normal AVP precursors were trapped inside the cell bodies of AVP neurons in the presence of mutant proteins, and that the transport of AVP from cell bodies to axonal terminals was hampered in FNDI mice. The results are interpreted as dominant negative effects of mutant NPII, which could at least partially explain the polyuria due to AVP deficiency in FNDI.

Immunohistochemical analyses also revealed that there are round inclusion bodies which are less immunoreactive for NPII in the AVP neurons of 12-month-old, but not 1-month-old FNDI mice (Fig. 3). As the size and number of the inclusion bodies detectable at an age as early as 2 months increased, so did urine volumes over time in FNDI mice. It was therefore suggested that inclusion bodies were made of mutant proteins trapped in cell bodies [5]. The presence of less immunoreactive inclusion bodies in AVP neurons made it difficult to count cell numbers with immunohistochemistry; we therefore performed in situ hybridization to examine whether progressive polyuria was accompanied by progressive loss of AVP neurons. Our data revealed that loss of AVP neurons is minimal, if any, in FNDI mice until the age of 12 months (Fig. 1C, D). These data indicate that loss of AVP neurons, which would finally occur, cannot account for the progressive polyuria in the early phase of FNDI. A possible explanation for there being less staining of...
the inclusion bodies with antibodies for normal or even mutant specific NPII could be that the epitopes of NPII are masked due to its misfolded structure, but this hypothesis remains to be proven.

4. Formation of Endoplasmic Reticulum-Associated Compartment in the AVP Neurons in FNDI Mice

In order to characterize the inclusion bodies in AVP neurons detected with immunohistochemistry in FNDI mice, we performed an electron microscopy analysis. Aggregates were already present in endoplasmic reticulum (ER) lumen in the SON of 1-month-old FNDI mice (Fig. 4A, B), suggesting that misfolded AVP precursors are trapped in the ER. In 3-month-old FNDI mice, there existed round-shaped electron-dense aggregates (Fig. 4C), which may well correspond to inclusion bodies detected with immunohistochemistry in the AVP neurons in FNDI mice (Fig. 3). Of note, the lumen of the ER adjacent to massive aggregates seemed intact (Fig. 3D). These data suggest that aggregates are confined to a sub-compartment of ER in the AVP neurons of FNDI mice [6]. It has been reported that misfolded proteins are segregated into a specialized sub-compartment of ER [7–11], called ER-associated compartment (ERAC). Consistent with a previous study showing that ERAC formation did not induce unfolded protein response (UPR) [7], our data also demonstrated that in spite of the presence of aggregates in the ER of AVP neurons in FNDI mice, expression levels of mRNA of immunoglobulin heavy chain binding protein (BiP) in the SON were similar between FNDI and WT mice [1]. These data suggest that ER stress that may have been caused by the accumulation of mutant proteins was reduced by ERAC formation in the AVP neurons of FNDI mice.

![Fig. 2. Immunohistochemical analyses of AVP neurons in SON stained with antibodies for normal NPII. While normal NPII is expressed in both the cell bodies and axons of AVP neurons in WT mice, it is mainly expressed in cell bodies in FNDI mice. Scale bar, 100 μm. Figures in ref. 5 are modified.](image)

![Fig. 3. Immunohistochemical analyses of AVP neurons in SON of 1-month-old and 12-month-old FNDI mice. Antibodies for mutant or normal NPII were used. Round-shaped inclusions (arrows) which are less immunostained with either antibody are observed in SON of 12-month-old, but not 1-month-old FNDI mice. Scale bar, 20 μm. Figures in ref. 5 are modified.](image)
In 12-month-old FNDI mice, there were massive aggregates which occupied the cytoplasm of AVP neurons (Fig. 4E). There were also some cells in which aggregates were scattered in ER lumens (Fig. 4F), suggesting that ERAC formation failed in some AVP neurons at this age when AVP neurons started to die in FNDI mice. Taken all together, the results suggested that (1) misfolded proteins are confined to the ERAC to maintain the function of ER, (2) increases in the size and number of inclusion bodies detected with immunohistochemistry indicate that misfolded proteins are accumulated in the ERAC over time, and (3) AVP neurons start to die when accumulation of mutant proteins overwhelms the capacity of AVP neurons to confine misfolded proteins into ERAC. It is also reasonable to assume that not only mutant but also normal AVP precursors are accumulated in the ERAC, given that the phenotype of polyuria progresses when there is no loss of AVP neurons in FNDI mice.

Fig. 4. Electron microscopic analyses of AVP neurons in the SON of FNDI mice. Representative photographs of male FNDI mice at 1 month (A, B), 3 months (C, D), and 12 months (E, F) are shown. Aggregates were present in ER (indicated by arrowheads) of 1-month-old mice (A, B). In 3-month-old FNDI mice, there existed round-shaped electron-dense aggregates (C), while lumen of ER adjacent to the massive aggregates seemed intact (D). In 12-month-old FNDI mice, there were massive aggregates occupying the cytoplasm (E). In some cells, aggregates were scattered in ER lumens (F). Aggregates were scattered throughout the dilated ER lumen in AVP neurons in 3-month-old FNDI mice subjected to intermittent WD for 4 weeks (G), and phagophores (indicated by arrowheads) surrounded the ER which contained scattered aggregates (H). There were vacuoles in the cytoplasm while the nuclear structure was relatively preserved in FNDI mice subjected to WD for 12 weeks (I, J). Higher magnification images of boxed areas in (A, C, G, and I) are shown in (B, D, H, and J), respectively. Nu: nucleus. Ag: aggregate. Scale bar: 0.2\,\mu m in B, 1\,\mu m in A, E, F, and 2\,\mu m in C, G, I. Figures in ref. 5 and 6 are modified.

5. **Animal Model to Study Loss of AVP Neurons in FNDI**

Based on morphology, cell death is classified into 3 groups: apoptosis, necrosis, and autophagy-associated cell death [12]. The FNDI mice we generated are potentially a good model for studying the mechanisms by which ER stress...
causes cell death. However, it is difficult to observe morphological changes in cells which are dying gradually over time. In order to elucidate the mechanisms by which AVP neurons are lost in FNDI, we synchronized the AVP neurons toward cell death by subjecting FNDI mice to dehydration, which promotes the synthesis of AVP precursors including the mutant one. Two-month-old FNDI mice were divided into two groups: one with water access *ad libitum* and the other subjected to intermittent water deprivation (WD), consisting of repeated cycles of 2-day-WD followed by a 5-day-recovery period with free access to water [6]. Urine volumes were significantly increased in the WD group compared to the *ad libitum* group at 5–12 weeks after starting WD (Fig. 5A), indicating that WD accelerated the phenotype of polyuria in FNDI mice. The number of AVP neurons in the SON of FNDI mice subjected to intermittent WD for 12 weeks was decreased by 30–40% compared to that of FNDI mice with water access *ad libitum* (Fig. 5B, C). Thus, we succeeded in making an animal model for analyzing how ER stress leads to loss of AVP neurons in FNDI.

6. Mechanisms Underlying Loss of AVP Neurons

Immunohistochemical analyses demonstrated that the sizes and numbers of inclusion bodies in the SON were decreased 4 weeks after starting WD [6], suggesting that the formation is hampered under WD. Electron microscopic analyses showed that aggregates were scattered throughout the dilated ER lumen in AVP neurons in FNDI mice subjected to intermittent WD for 4 weeks (Fig. 4G, H), as observed in some neurons of 12-month-old FNDI mice (Fig. 4F). It was also demonstrated that phagophores, the autophagosome precursors, surrounded the ER which contained scattered aggregates (Fig. 4H), suggesting that autophagy was induced in AVP neurons. To confirm this finding, FNDI mice were mated with green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (LC3) mice. While GFP signals were barely detected in AVP neurons of the SON in FNDI/GFP-LC3 mice with water access *ad libitum*, signals were elicited in AVP neurons in the mice subjected to intermittent WD for 4 weeks (Fig. 6), suggesting that WD induced autophagy in FNDI mice.
Electron microscopic analyses revealed vacuoles in the cytoplasm, and the nuclear structure was relatively preserved in FNDI mice subjected to intermittent WD for 12 weeks (Fig. 4I, J). These data suggest that AVP neurons in FNDI are lost by autophagy-associated cell death.

7. Role of ATF6α in the Formation of ERAC

Activating transcription factor 6 (ATF6) is one of the ER transmembrane proteins [13]. ATF6 is cleaved when it senses ER stress, and the active form of ATF6 translocates to the nucleus where it upregulates transcription of ER chaperones and ER-associated degradation components [14]. There are two subtypes, ATF6α and ATF6β, and the former has been shown to be the main mediator of the UPR [15]. In order to examine the possibility that ATF6α is required for ERAC formation in the AVP neurons, we analyzed water balance in ATF6α knockout (ATF6α−/−) mice [16] as well as FNDI/ATF6α−/− mice. While immunoglobulin heavy chain-binding protein (BiP) mRNA levels were significantly increased in WT mice subjected to intermittent WD for 12 weeks, there were no significant differences in the levels in ATF6α−/− mice between ad libitum and WD groups (Fig. 7A), suggesting that upregulation of BiP expression in AVP neurons in response to chronic dehydration is absent in ATF6α−/− mice [17].

Immunoelectron microscopic analyses demonstrated no apparent differences in the structure of ER in the AVP neurons between WT and ATF6α−/− mice with water access ad libitum [17]. While intermittent WD for 12 weeks induced dilation of ER lumen in AVP neurons in both WT and ATF6α−/− mice, the dilation was more evident in ATF6α−/− mice (Fig. 7B), suggesting that ATF6α and possibly ER chaperones such as BiP are prerequisites for the maintenance of ER morphology of AVP neurons under dehydration. It is also noteworthy that only a small part of ER lumen was dilated in WT mice under dehydration (Fig. 7B). This could mean that ER function might be maintained by the enlargement of a small part of the ER lumen, as is the case with AVP neurons of FNDI mice in which ERAC is formed.

Polyuria as well as loss of AVP neurons in FNDI mice was accelerated in the absence of ATF6α under dehydration [17]. Electron microscopic analyses showed that AVP neurons were lost through autophagy-associated cell death in both FNDI and FNDI/ATF6α−/− mice [17]. Thus, it is suggested that ATF6α is required for the maintenance of ER morphology and function in AVP neurons, both in WT and FNDI mice under dehydration. In case of FNDI mice, ATF6α seems to be required to maintain cell viability in AVP neurons.

8. Conclusion and Perspective

ERAC is formed in the AVP neurons in FNDI mice so as to reduce ER stress and maintain cell viability. The failure of ERAC to form leads to autophagy, which ultimately induces loss of AVP neurons in FNDI mice. The finding that a small part of ER lumen was dilated while the rest seemed intact in WT mice under dehydration suggests that misfolded proteins, which could be induced even in WT mice, are degraded through a mechanism similar to ERAC formation in the AVP neurons of FNDI. Thus, ERAC formation may not be a specific mechanism for FNDI but rather a universal one by which unfolded proteins are segregated (Fig. 8). Furthermore, our data suggest that ATF6α is required for the ERAC formation. On the other hand, as the expression levels of BiP mRNA were similar between ATF6α−/− and WT
mice with access to water *ad libitum*, it was not possible to fully explore the role of ER chaperones in the formation of ERAC. Investigating the AVP neuron-specific knockout of ER chaperones might provide us with more information on how the ERAC is formed in the AVP neurons.

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REFERENCES

[1] Arima H, Kondo K, Kakiya S, Nagasaki H, Yokoi H, Yambe Y, Murase T, Iwasaki Y, Oiso Y (1999) Rapid and sensitive vasopressin heteronuclear RNA responses to changes in plasma osmolality. J Neuroendocrinol 11:337-341.

[2] Arima H, Wakabayashi T, Nagatani T, Fuji M, Hirakawa A, Murase T, Yambe Y, Yamada T, Yamakawa F, Yamamori I, Yamashita M, Oiso Y (2014) Adipsia increases risk of death in patients with central diabetes insipidus. Endocr J 61:143-149.

[3] Babey M, Kopp P, Robertson GL (2011) Familial forms of diabetes insipidus: clinical and molecular characteristics. Nat Rev Endocrinol 7:701-714.

[4] Russell TA, Ito M, Ito M, Yu RN, Martinson FA, Weiss J, Jameson JL (2003) A murine model of autosomal dominant neurohypophyseal diabetes insipidus reveals progressive loss of vasopressin-producing neurons. J Clin Invest 112:1697-1706.

[5] Hayashi M, Arima H, Ozaki N, Morishita Y, Hiroi M, Nagasaki H, Kinoshita N, Ueda M, Shiota A, Oiso Y (2009) Progressive polyuria without vasopressin neuron loss in a mouse model for familial neurohypophysial diabetes insipidus. Am J Physiol Regul Integr Comp Physiol 296:R1641-1649.

[6] Hagiwara D, Arima H, Morishita Y, Wenjun L, Azuma Y, Ito Y, Suga H, Goto M, Banno R, Sugimura Y, Shiota A, Asai N, Takahashi M, Oiso Y (2014) Arginine vasopressin neuronal loss results from autophagy-associated cell death in a mouse model for familial neurohypophysial diabetes insipidus. Cell Death Dis 5:e1148.

[7] Huyer G, Longsworth GL, Mason DL, Mallampalli MP, McCaffery JM, Wright RL, Michaelis S (2004) A striking quality control subcompartment in Saccharomyces cerevisiae: the endoplasmic reticulum-associated compartment. Mol Biol Cell 15:908-921.

[8] Valetti C, Grossi CE, Milstein C, Sitia R (1991) Russell bodies: a general response of secretory cells to synthesis of a mutant immunoglobulin which can neither exit from, nor be degraded in, the endoplasmic reticulum. J Cell Biol 115:983-994.

[9] Kamhi-Nesher S, Shenkman M, Tolchinsky S, Fromm SV, Ehrlich R, Lederkremer GZ (2001) A novel quality control compartment derived from the endoplasmic reticulum. Mol Biol Cell 12:1711-1723.

[10] Granell S, Baldini G, Mohammad S, Nicolin V, Narducci P, Storrie B (2008) Sequestration of mutated alpha1-antitrypsin into inclusion bodies is a cell-protective mechanism to maintain endoplasmic reticulum function. Mol Biol Cell 19:572-586.

[11] Ito D, Yagi T, Ikawa M, Suzuki N (2012) Characterization of inclusion bodies with cytotoxic properties formed by sepinopathy-linked mutant seipin. Hum Mol Genet 21:635-646.

[12] Hotchkiss RS, Strasser A, McDunn JE, Swanson PE (2009) Cell death. N Engl J Med 361:1570-1583.

[13] Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8:519-529.

[14] Chen X, Shen J, Prywes R (2002) The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. J Biol Chem 277:13045-13052.

[15] Thurber DJ, Morrison L, Glombotski CC (2004) Opposing roles for ATF6alpha and ATF6beta in endoplasmic reticulum stress response gene induction. J Biol Chem 279:21078-21084.

[16] Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshiha H, Harada A, Mori K (2007) Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. Dev Cell 13:365-376.

[17] Azuma Y, Hagiwara D, Lu W, Suga H, Goto M, Banno R, Sugimura Y, Oyadomari S, Mori K, Shiota A, Asai N, Takahashi M, Oiso Y, Arima H (2014) Activating transcription factor 6 is required for the vasopressin neuron system to maintain water balance under dehydration in male mice. Endocrinology 155:4905-4914.