Neuronal vulnerability of CLN3 deletion to calcium-induced cytotoxicity is mediated by calsenilin

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Received December 8, 2006; Revised and Accepted December 9, 2006

Calsenilin/DREAM/KChIP3, a neuronal Ca\textsuperscript{2+}-binding protein, has multifunctions in nucleus and cytosol. Here, we identified CLN3 as a calsenilin-binding partner whose mutation or deletion is observed in Batten disease. \textit{In vitro} binding and immunoprecipitation assays show that calsenilin interacts with the C-terminal region of CLN3 and the increase of Ca\textsuperscript{2+} concentration \textit{in vitro} and in cells causes significant dissociation of calsenilin from CLN3. Ectopic expression of CLN3 or its deletion mutant containing only the C-terminus (153–438) and capable of binding to calsenilin suppresses thapsigargin or A23187-induced death of neuronal cells. In contrast, CLN3 deletion mutant containing the N-terminus (1–153) or (1–263), which is frequently found in Batten disease, induces the perturbation of Ca\textsuperscript{2+} transient and fails to inhibit the cell death. In addition, the expression of calsenilin is increased in the brain tissues of \textit{CLN3} knock-out mice and SH-SY5Y/CLN3 knock-down cells. Down-regulation of CLN3 expression sensitizes SH-SY5Y cells to thapsigargin or A23187. However, additional decrease of calsenilin expression rescues the sensitivity of SH-SY5Y/CLN3 knock-down cells to Ca\textsuperscript{2+}-mediated cell death. These results suggest that the vulnerability of \textit{CLN3} knock-out or CLN3 deletion (1–153)-expressing neuronal cells to Ca\textsuperscript{2+}-induced cell death may be mediated by calsenilin.

INTRODUCTION

Calsenilin/DREAM/KChIP3 (hereafter referred to as calsenilin) was identified as a Ca\textsuperscript{2+}-sensor that binds to the C-terminus of presenilin-1 and -2 (1), that serves as a transcription repressor known as downstream regulatory element antagonist modulator (2), and that binds to the A-type voltage-gated potassium channel (3). Calsenilin contains four EF-hands at its C-terminus for calcium-binding and binds to DNA in a calcium-dependent manner (1,2). Thus, calsenilin is believed to transduce and regulate diverse calcium-mediated signals. Calsenilin also exhibits pro-apoptotic activity in neuronal culture system (4). The increased expression of calsenilin was suggested to be required for cell death in neuronal cells following exposure to A\textsubscript{β}42 (5) and sensitizes neuronal cells to serum deprivation or Ca\textsuperscript{2+}-induced cell death (6). γ-secretase inhibitors, DAPT and compound E, suppress calsenilin-mediated death (7). Moreover, the phosphorylation of calsenilin at Ser63 by casein kinase I regulates its cleavage by caspase 3 during apoptosis (8–10). While expression regulation of calsenilin is observed in the brains of Alzheimer’s disease patients (5,10) and of epilepsy patients (11), a role of calsenilin in human diseases is not well understood yet. CLN3 is a transmembrane protein that is localized in lysosome/endosome (12–15). Mutation or deletion of \textit{CLN3} gene results in pathologic intracellular inclusions in many cell types including cortical neurons (16) and at last causes Juvenile Neuronal Ceroid Lipofuscinosis (JNCL), also called Juvenile Batten disease (12). Juvenile form of Batten disease is an autosomal recessive and the age of onset is usually 4–7 years, followed by visual failure. Clinical features of Batten disease are seizures, blindness and cognitive decline, leading to death at the age of 20 years (12,17,18). \textit{CLN3} knock-out mice show JNCL-like phenotype such as degeneration of optic nerve, hypertrophy and loss of specific populations of interneurons.

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and GABAAergic neurons (19–21). \( \text{CLN}3\text{ex}^{7/8} \) knock-in mice exhibit not only recessively inherited degenerative changes in retina, cerebral cortex and cerebellum, but also neurological deficits and premature death (22). Neuronal death, loss of photoreceptor cells and the accumulation of lipopigments in lysosomes are representative pathologies of JNCL (23,24). CLN3 was also shown to regulate cell growth and caspase-dependent or -independent cell death operative in neurodegeneration and tumorigenesis (25–28). Up-regulation of CLN3 expression increases growth rate of NT2 cells and is observed in a number of cell lines and solid human colon cancer (26,28). Down-regulation of CLN3 expression increases apoptosis in some human cancer cells (28). These observations show an important role of CLN3 in cell death, which may be associated with Batten disease. However, molecular mechanism of such anti-apoptotic activity of CLN3 is not well elucidated.

In this study, we isolated CLN3 as a protein interacting with calsenilin using yeast two-hybrid assay. Calsenilin binds to CLN3 that correlates the ability of CLN3 to suppress \( \text{Ca}^{2+} \)-mediated cell death. Increased expression of calsenilin in \( \text{CLN}3 \) knock-down cells mediates \( \text{Ca}^{2+} \)-induced neuronal cell death, providing a molecular basis for the cytoprotective activity of CLN3.

**RESULTS**

Calsenilin binds to the C-terminus of CLN3

In an attempt to identify calsenilin-interacting proteins, we screened the human fetal brain cDNA library using full-length calsenilin as bait for yeast two-hybrid assay. From 10 millions of yeast transformants, we have isolated three putative positive clones including CLN3, calmodulin2 and EST (accession number AC079447). In a yeast two-hybrid assay, calsenilin interacted with the C-terminus (amino acid residue 385–438) of CLN3 (data not shown). \textit{In vitro} GST-pull down assay showed that CLN3 interacts with GST-calsenilin but not with GST (Fig. 1A). The radio-labeled CLN3 pulled down by the GST-calsenilin represents \( \sim 5\% \) of input. The endogenous interaction of CLN3 and calsenilin was then examined in SH-SY5Y human neuroblastoma cells and the brains of wild-type mice and \( \text{CLN}3 \) knock-out mice. Co-immunoprecipitation assay using CLN3 antibody showed that calsenilin was detected in the immunoprecipitates containing CLN3, indicating that CLN3 interacts with calsenilin in cells (Fig. 1B and C). Calsenilin antibody did not cross-react with other KChIPs (Supplementary Material, Fig. S1) and the other lysosomal glycoprotein, Lgp120, did not interact with calsenilin (Supplementary Material Fig. S2). We were unable to demonstrate the interaction by reverse immunoprecipitation analysis because the molecular weight of CLN3 overlaps with the heavy chain of immunoglobulin. In addition, ectopic expression of CLN3 shows aggregated and vesicular structure, whereas calsenilin distributes in cytosol with dots (Fig. 1D, upper panel). However, co-expression of CLN3 with calsenilin induced an apparent change in the subcellular localization of calsenilin, showing the localization pattern of calsenilin is similar to that of CLN3 (Fig. 1D, lower panel).

To determine the calsenilin-binding region of CLN3, we generated several deletion mutants tagged with HA epitope (Fig. 2A). Two of such CLN3 deletion mutants, \( \text{CLN}3\Delta\text{A1-HA} \) (1–153) and \( \text{CLN}3\Delta\text{D2-HA} \) (1–263), are mostly found as truncated forms in Batten disease patients. The \( \text{[}^{35}\text{S}\text{]} \) labeled deletion mutants of CLN3 were evaluated for their abilities to bind to calsenilin \textit{in vitro} (Fig. 2B). \( \text{CLN}3\Delta\text{A1-HA} \) (1–153) and \( \text{CLN}3\Delta\text{D2-HA} \) (1–263) mutants lacking the C-terminus failed to bind to calsenilin, whereas \( \text{CLN}3\Delta\text{A3-HA} \) (153–438) mutant lacking the N-terminus bound to calsenilin (Fig. 2B). \( \text{CLN}3\Delta\text{A4-HA} \) (314–438) was not labeled by \textit{in vitro} translation. Intracellular interaction was examined after transfection with HA-tagged CLN3 deletion and calsenilin (Fig. 2C). Western blot analysis following immunoprecipitation showed that \( \text{CLN}3\Delta\text{A3-HA} \) (153–438), but not \( \text{CLN}3\Delta\text{A1-HA} \) (1–153) and \( \text{CLN}3\Delta\text{D2-HA} \) (1–263), formed protein complexes with calsenilin in cells, consistent with the results of \textit{in vitro} binding assay.
Also, CLN3-D4-HA (314–438) interacted with calsenilin. These results suggest that CLN3 interacts with calsenilin through its C-terminal region spanning residues 314–438.

The C-terminal region of CLN3 inhibits Ca$^{2+}$-mediated cell death

To examine the ability of each CLN3 deletion mutant to affect cell death, we generated SH-SY5Y cells stably expressing wild-type CLN3 (SH-SY5Y/CLN3-#4) or its deletion (SH-SY5Y/CLN3-D1–#4, SH-SY5Y/CLN3-D2–#2, SH-SY5Y/CLN3-D3–#1). We have selected mixed and single clones of CLN3 cells expressing different levels of exogenous CLN3 (Fig. 3). Overexpression of wild-type CLN3, but not other lysosomal membrane glycoprotein (lgp120) (Supplementary Material, Fig. S3), suppressed cell death triggered by thapsigargin, an ER Ca$^{2+}$-ATPase inhibitor or A23187, a Ca$^{2+}$ ionophore (Fig. 3A and B), consistent with the previous reports (25,26). Thapsigargin and A23187 were known to increase cytosolic concentration of free Ca$^{2+}$ and induce cell death (29). The cytoprotective activities of CLN3 stable cells are proportional to the expression level of CLN3. SH-SY5Y/CLN3-D3–#1 cells expressing the C-terminal region of CLN3 were also resistant to cell death triggered by thapsigargin or A23187 (Fig. 3G and H). In contrast, SH-SY5Y/CLN3-D1–#4 and SH-SY5Y/CLN3-D2–#2 cells expressing the N-terminal regions of CLN3 were susceptible to Ca$^{2+}$-induced cell death and rather sensitized to thapsigargin or A23187 (Fig. 3C–F). These results indicate that the cytoprotective activity of CLN3 resides in the C-terminal region spanning residues 153–438.

We then generated SH-SY5Y stable cell lines (SH-SY5Y/CLN3-AS–#3) expressing the reduced amounts of CLN3 using anti-sense cDNA. Reduced expressions of CLN3 were confirmed by western blot analysis (Fig. 4A, upper panel). Interestingly, calsenilin level increased in CLN3 knock-down cells (Fig. 4A, middle panel). Higher level of calsenilin was detected in cells showing lower level of CLN3. Compared to control cells, SH-SY5Y/CLN3-AS-Mix and SH-SY5Y/CLN3-AS–#3 cells expressing less CLN3 were more susceptible to cell death triggered by thapsigargin or A23187 (Fig. 4B and C), adding more evidence that CLN3 is cytoprotective.

CLN3 modulates cytosolic free Ca$^{2+}$ level

As CLN3 suppresses Ca$^{2+}$-mediated cell death, we then examined whether CLN3 affects cytosolic free Ca$^{2+}$ level, [Ca$^{2+}$]$_i$. We monitored an intracellular Ca$^{2+}$ transient after stimulation with extracellular ATP that increases [Ca$^{2+}$]$_i$ in SH-SY5Y cells through the plasma membrane P2X$_7$ purinergic receptor (30) and even induces cell death of sensitive cells (31). Compared to SH-SY5Y/pDNA control cells (Fig. 5A), ATP-induced Ca$^{2+}$ transients significantly decreased in SH-SY5Y/CLN3 cells (Fig. 5B and C). In contrast, ATP-induced Ca$^{2+}$ transients did not decline but maintained higher level until late time points in SH-SY5Y/CLN3-AS cells (Fig. 5D and E). We performed similar experiments with the mixed populations of SH-SY5Y cells expressing CLN3 deletion mutant. Whereas SH-SY5Y/CLN3-D3-Mix (N-terminal deletion mutant) cells exhibited similar Ca$^{2+}$ transient with SH-SY5Y/CLN3 cells (Fig. 5H), expression of CLN3-D1 or CLN3-D2 sustained ATP-induced intracellular calcium level high (Fig. 5F and G), similar to that of CLN3 knock-down cells. In addition, compared to SH-SY5Y/pDNA and SH-SY5Y/CLN3 cells, basal levels of cellular Ca$^{2+}$ increased by 57 ± 10.5% (P < 0.05, n = 3) in SH-SY5Y/CLN3-D1, SH-SY5Y/CLN3-D2 and SH-SY5Y/CLN3-AS cells (Fig. 5D–G). These results show that in contrast to CLN3 and CLN3-D3, CLN3 knock-down and ectopic expression of CLN3 mutant lack in their ability to bind to calsenilin and to suppress cell death sustain [Ca$^{2+}$]$_i$ high, suggesting the presence of a correlation of cell death-regulating and calsenilin-binding abilities of CLN3 with the activity to modulate the intracellular calcium mobilization.

Presence of Ca$^{2+}$ reduces protein–protein interaction between calsenilin and CLN3

As calsenilin is a Ca$^{2+}$-binding protein containing four EF hands, we next examined whether Ca$^{2+}$ affects the binding
of calsenilin to CLN3. In vitro GST-pull down assay showed that the presence of Ca\(^{2+}\) in the reaction buffer decreases the interaction of calsenilin with CLN3; the interaction was significantly reduced by 50 \(\mu M\) Ca\(^{2+}\) and abrogated by higher concentrations of Ca\(^{2+}\) (Fig. 6A). Calcium-dependent intracellular interaction of calsenilin with CLN3 was also evident as examined with immunoprecipitation analysis. Compared to control, treatment of HEK293 cells with thapsigargin for 24 h significantly decreased the amounts of calsenilin detected in the immunocomplexes isolated by HA antibody (Fig. 6B). The effects of intracellular calcium rise on the co-localization of calsenilin and CLN3 were further examined in a single cell level. Treatment with thapsigargin disrupted co-localization of calsenilin and CLN3 by 48 \(\pm\) 6.2\% \((P < 0.001, n = 3)\) at 24 h (Fig. 6C). Subcellular localization of calsenilin was detected in diffused cytosol, which is distinct with that of CLN3 (data not shown). Previous study also showed a distinct subcellular localization of calsenilin by the elevation of intracellular calcium (32). These results suggest that intracellular increase of calcium level may disrupt the protein–protein interaction between calsenilin and CLN3.

Calsenilin contributes to Ca\(^{2+}\)-mediated cell death in CLN3 knock-down cells

We then addressed whether calsenilin plays a role in Ca\(^{2+}\)-mediated death of SH-SY5Y/CLN3-AS-#3 cells. Ectopic expression of calsenilin siRNA in SH-SY5Y/CLN3-AS-#3 cells decreased the expression of calsenilin to the control level (pcDNA) as examined with western blot analysis (Fig. 7C). Down-regulation of calsenilin expression desensitized SH-SY5Y/CLN3-AS-#3 cells to A23187 or thapsigargin-induced death (Fig. 7A and B); SH-SY5Y/CLN3-AS-#3 cells showing similar expression level of calsenilin with control cells are as sensitive to A23187 or thapsigargin-induced cell death as control cells. Also, overexpression of calsenilin further increased death of SH-SY5Y/CLN3-AS-#3 cells (Fig. 7D).

We then examined the expression level of calsenilin in CLN3-overexpressing cells and CLN3 knock-out cells (Fig. 8). Calsenilin level of SH-SY5Y/CLN3-#4 cells expressing more CLN3 was down-regulated by 45 \(\pm\) 8.2\% \((P < 0.05, n = 3)\) compared to control cells (Fig. 8A and B). Also, calsenilin level in the brains of CLN3 knock-out mice increased by 54 \(\pm\) 9.9\% \((P < 0.05, n = 3)\) compared to wild-type mice (Fig. 8C and D). However, KChIP2 and actin did not show any significant changes in CLN3 knock-out mice.

These results suggest that the expression level of calsenilin may be negatively regulated by CLN3.

DISCUSSION

In culture and mice model, calsenilin is believed to contribute to the pathogenesis of human disease, including regulation of A\(^{\beta}_{42}\) production, cell death and pain (33,34). Here, we provide several lines of evidence that susceptibility of CLN3 knock-down cells to Ca\(^{2+}\)-induced cell death is at least mediated by calsenilin. (i) CLN3 binds to calsenilin through its C-terminus; (ii) Like CLN3, the C-terminus of CLN3 protects cells from cell death; (iii) CLN3 regulates intracellular free calcium and ATP-induced Ca\(^{2+}\) transient through its C-terminus; (iv) CLN3 negatively regulates cellular level of calsenilin; (v) Sensitization of CLN3 knock-down cells to death is rescued by additional reduction of calsenilin level; (vi) CLN3 suppresses calsenilin-induced cell death
Our observations that the C-terminus of CLN3 exhibits cytoprotective activity and binds to calsenilin may provide an explanation for the loss-of-function of CLN3 deletion mutants lacking C-terminus found in Batten disease.

Most Batten disease patients have shorter CLN3 protein than normal. (http://www.ucl.ac.uk/ncl/cln3.shtml). The most abundant deletion mutant of CLN3 is a truncated protein generated by the deletion of the C-terminal region spanning residues 154–438 and the introduction of 28 amino acids (12). In contrast to wild-type CLN3 and its deletion mutant lacking N-terminus, the C-terminal deletion mutants of CLN3 lose their cytoprotective activity and rather sensitize SH-SY5Y cells to Ca\(^{2+}\)-mediated cell death. Truncated CLN3 may accumulate in the ER of BHK cells (14) and primary cultured neuronal cells (35), showing defect in trafficking out of ER. Such accumulation of CLN3 mutants in ER may dysregulate calcium signal and induce cellular stress. Thus, the increase of cytosolic free calcium or calcium perturbation triggered by CLN3 deletion mutants, such as CLN3-D\(_1\) and CLN3-D\(_2\), may generate cellular stress, in turn leading to malfunction of the affected neurons and to sensitization of the sensitive cells to Ca\(^{2+}\)-mediated cell death for long-term period.

The functions of CLN3 are believed to be associated with lysosomal event, including pH regulation (36,37) and transporting arginine (38,39), while in human model JNCL fibroblast cells exhibit normal intracellular pH and elevated lysosomal pH (40). There are two dileucine motifs and MX9G motifs essential for targeting lysosome in the C-terminus of CLN3 spanning residues 253–438 (15). In addition, highly conserved amino acids across species are located in 291VYFAE295 and 330VFASRSSL337, which may play a role in the regulation of cell growth and apoptosis (25,26). Recently, CLN3 was also proposed to have a role in autophagy and autophagic cell death (27,41), where lysosomal event is important for the maturation of autophagic vacuole. We also observed autophagic vacuole formation in CLN3 knock-down cells or cells expressing CLN3 mutant lacking the C-terminus, CLN3-\(\Delta\)1 and CLN3-\(\Delta\)2 (data not shown). These results reveal that the ability of CLN3 to regulate cell death seems to overlap with that of autophagic vacuole formation. Thus, in the brain tissues of JNCL patients and CLN3\(_{\text{Dex7/8}}\) knock-in mice, loss of CLN3 function may be associated with the deposits of autofluorescent inclusion bodies and fingerprint structures exhibited within the CA2/CA3 hippocampal pyramidal cell layer and cortex (22,42). However, whether other CLN3 mutants containing amino

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**Figure 4.** Down-regulation of CLN3 sensitizes SH-SY5Y cells to Ca\(^{2+}\)-mediated cell death. (A) SH-SY5Y cells were transfected with control (pcDNA) or antisense cDNA of CLN3 (pCLN3-AS), selected in the presence of G418 for mixed population (CLN3-AS-Mix) or single clone (CLN3-AS-#3) of stable cells, and examined with western blotting using CLN3 and calsenilin antibodies. (B and C) SH-SY5Y/pcDNA, SH-SY5Y/CLN3-AS-Mix and SH-SY5Y/CLN3-AS-#3 stable cells were treated with 5 \(\mu\)M thapsigargin (B) or 3 \(\mu\)M A23187 (C) for the indicated times and cell death (percentage) was examined with Live/Dead cell assay. The results shown are from three independent experiments with mean values ± SD (\(P < 0.005\) for time points at 12 and 24 h, \(n = 3\)).

**Figure 5.** Effects of CLN3 and its deletion mutants on ATP-induced cytosolic-free Ca\(^{2+}\) level. (A–H) SH-SY5Y/pcDNA (A), SH-SY5Y/CLN3-#4 (B), SH-SY5Y/CLN3-Mix (C), SY5Y/CLN-AS-#3 (D), SH-SY5Y/CLN3-AS-Mix (E), SH-SY5Y/CLN3-\(\Delta\)1-Mix (F), SH-SY5Y/CLN3-\(\Delta\)2-Mix (G) and SH-SY5Y/CLN3-\(\Delta\)3-Mix (H) cells were loaded with 2.5 \(\mu\)M Fluo-4 in the presence of 1.25 \(\mu\)M probenecid. After stimulation with 200 \(\mu\)M ATP (arrow), cells (\(n > 33\)) were monitored with the BD\(^{\text{TM}}\) Pathway Bioimager for the indicated times. The plot represents the normalized amplitudes of peak Fluo-4 fluorescence intensity. Bars indicate mean values ± SD (\(n = 3\)).
Figure 6. Calcium-dependent dissociation of calsenilin from CLN3. (A) Calcium-dependent binding of CLN3 to calsenilin in vitro. Total cell extracts were prepared from SH-SY5Y/CLN3-HA stable cells and then incubated with GST or GST-fused calsenilin protein in the presence of 1 mM EGTA or increasing concentrations of CaCl₂. After separation of proteins bound to their affinity resins by SDS-PAGE, CLN3 were detected by western blotting using CLN3 antibody (upper panel) and GST bead-coupled proteins were visualized by Ponceau S staining (lower panel). (B) Effects of thapsigargin on the cellular binding of CLN3 to calsenilin. SH-SY5Y cells were co-transfected with pcalsenilin and pCLN3-HA for 24 h and then treated with thapsigargin (TG, 5 μM) for the indicated times. Cell extracts were immunoprecipitated (IP) with HA antibody and analyzed with western blotting using calsenilin (upper panel) and HA (middle panel) antibodies. Light chains of immunoglobulin (LC) are indicated. (C) Effects of thapsigargin on subcellular co-localization of CLN3 and calsenilin. SH-SY5Y cells were co-transfected with pCLN3-HA and pcalsenilin for 24 h, and then treated with DMSO (Con) or thapsigargin (TG, 5 μM) for another 24 h. Cells were co-immunostained with HA and calsenilin antibodies and observed under a fluorescence microscope. Co-localization level of CLN3-HA and calsenilin in control cells were taken as arbitrary unit 100 and the relative co-localization ratio of CLN3-HA and calsenilin in cells exposed to thapsigargin is represented (200–300 cells/count). Bars indicate mean values ± SD (P < 0.001, n = 3).

Figure 7. Contribution of calsenilin to Ca²⁺-mediated death of SH-SY5Y/CLN3 knock-down cells. (A and B) Reduced expression of calsenilin desensitizes SH-SY5Y/CLN3-AS cells to thapsigargin. SH-SY5Y cells showing reduced expression of CLN3 (CLN3-AS-#3) were transiently co-transfected with pEGFP and either pSUPER or pSUPER-calsenilin (siCalse) for 24 h, and then exposed to 5 μM thapsigargin (A) or 3 μM A23187 (B) for the indicated times. Cell viability was then determined on the basis of the morphology of GFP-positive cells and by staining with trypan blue. (C) Expression level of calsenilin was assessed by western blotting using calsenilin antibody. (D) Overexpression of calsenilin sensitizes SH-SY5Y/CLN3-AS-Mix and SH-SY5Y/CLN3-AS-#3 cells to death. SH-SY5Y/pcDNA, SH-SY5Y/CLN3-AS-Mix and SH-SY5Y/CLN3-AS-#3 cells were transfected with GFP or GFP-fused calsenilin and cell death was assessed on the basis of the morphology of GFP-positive cells under a fluorescence microscope. Bars indicate mean values ± SD [(A and B) P < 0.01 for time points at 12 and 24 h, n = 3. (D) P < 0.05 for time points at 24 and 48 h, n = 3].
acid substitution share similar activity with CLN3 deletion mutant remains to be further characterized.

Although it is not known yet how CLN3 destabilizes or decreases calsenilin, calsenilin is apparently accumulated in the brains of CLN3 knock-out mice and CLN3 knock-down cell lines. Inversely, calsenilin may regulate the cellular function of CLN3, such as cellular trafficking including endocytosis (43). In addition, Hook1 and CLN5 were reported to bind to CLN3 (43,44). Hook1 was recently identified as a microtubule-binding protein and may play a role in membrane-trafficking events (45). CLN5 knock-out mice showed similar phenotype as CLN3-deficient mice, such as loss of vision and neurons (46). Thus, calsenilin may also affect the CLN3-mediated endocytosis and neuronal loss. The current model is that intracellular increase of Ca²⁺ concentration under pathologic conditions may cause the dissociation of calsenilin from CLN3. Our observation that relatively high level of Ca²⁺ is required for such in vitro dissociation implies that additional cellular factor(s) may be needed for more efficient regulation of the intracellular dissociation.

In summary, we found that CLN3 binds to calsenilin and suppresses neuronal cell death mediated by calsenilin and dysregulation of intracellular Ca²⁺. Calsenilin contributes to the increased sensitivity of CLN3 knock-down cells to cell death, providing a new insight into a role of calsenilin and CLN3 in the pathogenic neuronal cell death.

**MATERIALS AND METHODS**

**Yeast two-hybrid assay**

Calsenilin was fused in-frame to the LexA DNA-binding domain of pLexA (pLexA-calsenilin). A human fetal brain cDNA library (3.5 × 10⁶ independent clones) in pB42AD (Clontech) was screened as described in the manufacturer’s protocol. *S. cerevisiae* EGY48 harboring the β-galactosidase reporter plasmid p8op-lacZ was co-transformed with pB42AD-cDNA library and pLexA-calsenilin. Positive clones were identified by growth in a medium lacking leucine, and β-galactosidase activity from 1 × 10⁷ independent co-transformants. The cDNA inserts in the positive
plasmids were sequenced with the pB42AD-forward (5′-ccagcctcttggtatatgata-3′) primer.

Antibody production

GST-calsenilin fusion protein was purified from Escherichia coli BL21(DE3) with Glutathione Sepharose 4B (Amersham Pharmacia Biotech) and injected into a rabbit, following standard immunization procedures. Calsenilin antibody was purified by calsenilin protein affinity chromatography. The rabbit anti-CLN3 antibody (Q-438) was kindly provided by M. Bennett (University of Iowa, USA).

Plasmid construction

Mammalian expression plasmids, pCLN3 and pCLN3-AS, were constructed by subcloning cDNAs into BamHI/EcoRI and EcoRI/BamHI sites of pcDNA3.1 (Invitrogen), respectively. pCLN3-HA, pCLN3-Δ1(1–153)-HA, pCLN3-Δ2(1–263)-HA, pCLN3-Δ3(154–438)-HA and pCLN3-Δ4(314–438)-HA were generated by subcloning the PCR products into EcoRI/BamHI sites of HA-tagged pcDNA3 (pcDNA-HA). For calsenilin (pcCLN3) or calsenilin-green fluorescence protein fusion (pcalsenilin-GFP) plasmids, the entire coding region of calsenilin was subcloned into EcoRI/XhoI sites of pcDNA3.1 or EcoRI/KpnI sites of pEGFP-N1 (Clontech). For pGST-calsenilin, the coding region of calsenilin was inserted into EcoRI/XhoI sites of pGEX4T-3 (Amersham Pharmacia Biotech). For pSUPER-calsenilin, a 64 nucleotide long insert containing a calsenilin 19 nucleotide dsRNA hairpin region and inserted to an inverted repeat, separated by a 5′-ccagcctcttggtatatgata-3′ corresponding to human calsenilin cDNA 514–532) was generated by annealing two complementary oligonucleotides containing the calsenilin sequence as an inverted repeat, separated by a 9-nucleotide-long hairpin region and inserted to BglII/HindIII site of pSUPER (Oligo Engine).

Cell culture and DNA transfection

SH-SY5Y and HEK293 cells were grown in Dulbecco’s Modified Eagles Medium (DMEM) (Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS). Cells were transfected for 24 h with appropriate vector using LipofectAMINE reagent (GIBCO BRL) and selected with G418 (1 mg/ml) for 2 weeks to generate mixed populations (SH-SY5Y/CLN3-Mix, SH-SY5Y/CLN3-AS-Mix, SH-SY5Y/CLN3-Δ1-Mix, SH-SY5Y/CLN3-Δ2-Mix and SH-SY5Y/CLN3-Δ3-Mix). Single cell was then separated by the limited dilution and further cultivated to form stable cell clones (SH-SY5Y/pcDNA, SH-SY5Y/CLN3-#4, SH-SY5Y/CLN3-AS-#3, SH-SY5Y/CLN3-Δ1-#4, SH-SY5Y/CLN3-Δ2-#2 and SH-SY5Y/CLN3-Δ3-#1). Expression levels of CLN3 were examined with western blotting.

Live/Dead cell assay

Cells were loaded with fluorescent dyes calcein-AM (0.5 μM) and ethidium homodimer (0.5 μM) for 15 min at 37°C (Live/Dead assay, Molecular Probes) and then visualized under fluorescence microscope (Olympus). Percentage of viable cells (normalized to control) was calculated as ratios [(numbers of live cells/total cells) × 100]. All data are expressed as mean values ± SD. Significance of differences between groups was assessed by Student’s paired t test. For trypan blue staining, cells were harvested and resuspended in 1:1 mixture of 2% trypan blue dye (Sigma) and 1× phosphate-buffered saline (PBS).

Calcium imaging

Cells were seeded overnight in 96-well plates (Greiner) with density of 7500–10000 cells/well and incubated with the Ca2+-sensitive fluorescent dye Fluo-4 (2.5 μM, Molecular Probes) in DMSO with probenecid (1.25 μM, Sigma) in FBS-free DMEM at 37°C for 45 min. After removing and washing the dye with washing solution [5 mM HEPES (pH 7.0), 1.25 μM probenecid in HBSS], cells were stimulated with 200 μM ATP. Cytosolic Ca2+ fluorescent signals were acquired with time series (1 image/s) using The BD Pathway Bioimager (BD biosciences). Fluorescence intensities were then extracted from each image using Atto Vision software (BD biosciences), which splits the color channel and calculates the ratio for all frames. Individual cells were analyzed as a separated region of interest.

In vitro protein-binding assay

GST-fused proteins were immobilized by incubating with Glutathione Sepharose 4B and then incubated with [35S]-methionine-CLN3 (Promega) in a binding buffer [20 mM Tris-Cl (pH 7.2), 0.15 M NaCl, 0.2% Triton X-100 and protease inhibitors]. SH-SY5Y/CLN3-#3 cells were lysed for 40 min in ice-cold lysis buffer A [50 mM Tris (pH 7.4), 300 mM NaCl, 1% Triton X-100, 0.1% BSA and protease inhibitors], and cell extracts were incubated with GST-fusion protein for in vitro binding assay. Beads were recovered and then washed with the binding buffer or lysis buffer A several times and the bound proteins were subjected to SDS-PAGE.

Whole brain extracts

Flash frozen whole brains of 7-month-old wild-type control mice 129S6/SvEv and homozygous CLN3 knock-out mice on a 129S6/SvEv background were kindly provided by D. Pearce (University of Rochester, USA). Tissue extracts were prepared using an Ultra-Turex T8 homogenizer (IKA Labortechnik) at 8000 r.p.m./stroke for 3×5 strokes in ice-cold buffer containing 0.32 M sucrose, 10 mM KH2PO4, 1 mM EDTA, 20 μM leupeptin, 1 μM pepstatin and 0.5 mM phenylmethylsulphonyl fluoride (pH 7.0). Tissue debris was removed by centrifugation at 800g for 5 min at 4°C and supernatant was analyzed by western blotting or stored at −80°C until further use.

Immunoprecipitation analysis

SH-SY5Y cells were lysed for 40 min in ice-cold lysis buffer A. Mouse brain extracts prepared from age-matched wild-type mice and CLN3 knock-out mice were diluted to 0.5% in lysis buffer.
buffer A. CLN3 antibody (Q-438) or preimmune serum was incubated with cell extracts for 2 h at 4°C on a rotor and protein complexes were immunoprecipitated with Protein A or G Sepharose 4B (Amersham Pharmacia Biotech) and the reaction products were analyzed by western blotting.

**Immunofluorescence staining and confocal microscopy**

SH-SY5Y cells were fixed with fresh 4% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS and permeabilized with 0.1% Triton X-100 (Sigma) for 15 min. Non-specific binding was blocked with 3% BSA for 30 min. Cells were then labeled with primary antibodies, including HA antibody (monoclonal) or calsenilin antibody (affinity purified), for overnight at 4°C and secondary antibodies, rhodamine red (TRITC)-conjugated goat anti-rabbit IgG (Immunootech; 1:200) combined with fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM (Jackson Laboratories; 1:200). The sample was examined with an UltraVIEW confocal imaging system (Perkin-Elmer Life Sciences) and an Eclipse TE 2000-U microscope (Nikon) using a 100× oil immersion objective (Plan Fluor, Nikon).

**Statistical analysis**

Results comparing three or more samples were analyzed using ANOVA and Bonferroni tests, or two samples were analyzed by t-test. Only P-values <0.05 were considered significant. Statistical analysis was performed using SigmaPlot program.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**ACKNOWLEDGEMENTS**

The authors thank D. Pearce (University of Rochester, USA) for providing whole brains of CLN3 knock-out and wild-type mice, and M. Bennett (University of Iowa, USA) for CLN3 antibody. This work was supported by the Brain Research Center of 21C Frontier and Research Center for Functional Cellulomics and Ubiquitomics of the Korea Science and Engineering Foundation and by the Korean Research Foundation (KRF-2005-201-C00039).

**Conflict of Interest statement.** There are no conflicts of interest in this manuscript.

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