Multiple pathogenic proteins implicated in neuronopathic Gaucher disease mice

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Gaucher disease, a prevalent lysosomal storage disease (LSD), is caused by insufficient activity of acid β-glucosidase (GCase) and the resultant glucosylceramide (GC)/glucosylsphingosine (GS) accumulation in visceral organs (Type 1) and the central nervous system (Types 2 and 3). Recent clinical and genetic studies implicate a pathogenic link between Gaucher and neurodegenerative diseases. The aggregation and inclusion bodies of α-synuclein with ubiquitin are present in the brains of Gaucher disease patients and mouse models. Indirect evidence of β-amyloid pathology promoting α-synuclein fibrillation supports these pathogenic proteins as a common feature in neurodegenerative diseases. Here, multiple proteins are implicated in the pathogenesis of chronic neuronopathic Gaucher disease (nGD). Immunohistochemical and biochemical analyses showed significant amounts of β-amyloid and amyloid precursor protein (APP) aggregates in the cortex, hippocampus, stratum and substantia nigra of the nGD mice. APP aggregates were in neuronal cells and colocalized with α-synuclein signals. A majority of APP co-localized with the mitochondrial markers TOM40 and Cox IV; a small portion co-localized with the autophagy proteins, P62/LC3, and the lysosomal marker, LAMP1. In cultured wild-type brain cortical neural cells, the GCase-irreversible inhibitor, conduritol B epoxide (CBE), reproduced the APP/α-synuclein aggregation and the accumulation of GC/GS. Ultrastructural studies showed numerous larger-sized and electron-dense mitochondria in nGD cerebral cortical neural cells. Significant reductions of mitochondrial adenosine triphosphate production and oxygen consumption (28–40%) were detected in nGD brains and in CBE-treated neural cells. These studies implicate defective GCase function and GC/GS accumulation as risk factors for mitochondrial dysfunction and the multi-proteinopathies (α-synuclein-, APP- and Aβ-aggregates) in nGD.

INTRODUCTION

Gaucher disease, an autosomal recessive disorder, is a common lysosomal storage disease (LSD) (1) that results from defective function of acid β-glucosidase (GCase, encoded by GBA1). The accumulations of its substrates glucosylceramide (GC) and glucosylsphingosine (GS) lead to various visceral and central nervous system (CNS) clinical manifestations in Gaucher disease Type 1 (non-neuronopathic) and Types 2 and 3 (neuronopathic) variants (1–3). GC engorged visceral macrophages or ‘Gaucher cells’ are the pathologic hallmark of Gaucher disease. In the neuronopathic variants, the CNS pathology is also characterized by neuronal cell degeneration and death.

Neuronopathic Gaucher disease (nGD) has histopathological features that include CNS pro-inflammatory responses and are associated with astrogliosis, microgliosis and neuronal degeneration in affected humans and mice (4–9). Neurodegenerative lesions are often complex with progressive accumulation of protein aggregates (proteinopathies), e.g. α-synuclein, ubiquitin, amyloid precursor protein (APP), β-amyloid (Aβ), tau and others in Parkinson disease (10), Alzheimer disease (11) and

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Huntington disease (12). In addition to Gaucher disease, other LSDs also exhibit some accumulations of these proteins, e.g. Tay–Sachs and Sandhoff diseases (13) and Niemann–Pick disease Type C (14). Recently, clinical and genetic studies demonstrated α-synuclein and ubiquitin protein aggregates in the brains of Gaucher disease patients (8,15,16) and mouse models (9,17–20), which typically are present in many non-LSD neurodegenerative diseases. Taken together, these studies suggest that multiple proteinopathies are pathophysiologic features of nGD.

α-Synucleinopathies are characterized by the presence of aggregated, insoluble α-synuclein within Lewy bodies/neurites of the CNS and α-synuclein inclusion-associated neurodegenerative lesions (21). α-Synucleinopathies are typical of Parkinson disease patients, but clinical and genetic studies indicate pathogenic links between Gaucher disease and Parkinson disease (22–24), or Lewy body disease (LBD) (25). In Gaucher patients with Parkinsonism, neuropathological analyses revealed the presence of α-synuclein positive Lewy bodies in hippocampal region (8). In the nGD mouse models, V394L/PS-NA and D409H/PS-NA mice (26), progressive α-synuclein and ubiquitin aggregates were observed in the brains that were coincident with progressive neurological manifestations and brain GC/GS accumulation (9). In addition, α-synuclein aggregation was present in older (>46 weeks) Gaucher disease mice with Gbαl point mutations, D409H and D409V homozygotes (9,17–19). The D409V mice showed hippocampal memory impairments by 52 weeks (19). These findings implicate a common cytotoxic mechanism linking aberrant GCase activity, GC/GS accumulation, neuronal cytotoxicity and α-synucleinopathies in Gaucher disease brains (24,27).

In addition to α-synuclein aggregation, clinical data showed significant Aβ deposits with extensive α-synuclein lesions in cerebral cortex of Parkinson disease patients (28,29), supporting a pathogenic link between α-synucleinopathies and Aβ/APP deposition. Histopathological studies showed significant accumulation of full-length APP species in brain mitochondria from Alzheimer disease patients (30,31), APP transgenic mice (32–34) and neural cell cultures (34). The aggregated APP was closely associated with the outer-membrane channel-forming TOM40 and the inner membrane 23 (TIM23), which led to the incomplete or arrest of APP translocation (30,31,35). Also, Aβ and α-synuclein may act synergistically by promoting each other’s aggregation (36–38). Aβ could drive α-synuclein pathology by impairing protein clearance, activating inflammation, enhancing phosphorylation or directly promoting aggregation (36–38), thereby providing a link to the accompanying neurodegeneration. To date, APP and Aβ have not been reported in Gaucher disease patients or mice, except an in vitro study showing amyloid in GC/α-synuclein tubules (17). Taken together, available studies suggest the possibility of APP/Aβ aggregation in the nGD.

Here, mouse nGD analogs, similar to human Type 3 disease (32) were used to explore brain proteinopathies. Previously, an α-synucleinopathy was characterized in these nGD mice. The current study focused on the cellular compartments and the pathophysiological significance of amyloidopathy and α-synucleinopathy in their brain regional lesions.

RESULTS

Neurological and histopathological phenotypes of nGD mice

9H/PS-NA and 4L/PS-NA mice had high levels of GC/GS in their brains and significant neurological phenotypes by >10 weeks and progressed in severity until death at ~20–22 weeks (26). Starting from 12 weeks, neuronal degeneration with large amounts of α-synuclein/ubiquitin aggregates were observed (9,17,18). To determine additional pathogenic-prone proteins as seen in other neurodegenerative diseases, e.g. Parkinson and Alzheimer diseases, immunohistochemistry studies using anti-APP, -Aβ, -PS-1 antibodies were conducted with brain sections from 12-week nGD mice. Large APP particles (5–13 μm) were present in all brain regions, but more numerous in the cerebral cortex (Cor), caudate putamen (CPu), hippocampus (Hp) and substantia nigra (SN) (Fig. 1A, arrows), where α-synuclein/ubiquitin had been previously observed (9). The rabbit polyclonal anti-APP corresponds to peptide sequence of amino acids 653–662 of APP and can detect the full-length APP. APP signals were around or on the edge of the nuclei in a cap shape. The amount of APP accumulation in the brains was estimated by immunoblotting using the same rabbit polyclonal anti-APP; quantitative analysis showed a 1.65-fold of APP in the cortices of 9H/PS-NA and 4L/PS-NA mice compared with that in wild-type (WT) mice (Fig. 1B and C). The brain sections from their parental lines PS-NA mice (39) and Gbαl point-mutated homozygous 4L/4L and 9H/9H mice (40) were examined. There were smaller sized and a few of APP signals in age-matched PS-NA brains, but not in 4L/4L and 9H/9H brains. However, sporadic APP signals were observed in the cortex of aged (52 weeks) Gbαl point-mutated homozygous mice 4L/4L and 9H/9H mice (40) were examined. These smaller sized and a few of APP signals in age-matched PS-NA brains, but not in 4L/4L and 9H/9H brains. However, sporadic APP signals were observed in the cortex of aged (52 weeks) Gbαl point-mutated homozygous mice 4L/4L and 9H/9H mice. In addition to APP, Aβ and PS-1 signals were seen in the same regions using anti-Aβ and PS-1 specific antibodies (Supplementary Material, S1 and S2). Therefore, at least five different protein aggregates, i.e. α-synuclein, ubiquitin, APP, Aβ and PS-1 coexisted in the brains of nGD mice, 9H/PS-NA and 4L/PS-NA.

APP aggregates in various types of neural cells

The type of neural cells containing APP was evaluated by immunofluorescence analysis using dual-antibody immunostaining. Anti-NeuN, -GFAP and -CNPase were used as the markers for neurons, astrocytes and oligodendrocytes, respectively (41,42). Dual-antibody stained brain sections showed APP signals associated with NeuN-positive neurons, GFAP-positive astrocytes and CNPase-positive oligodendrocytes (Fig. 2A, arrows). These results indicate APP aggregates in three major types of neural cells in the brains of nGD mice. Interestingly, many overlapping signals for α-synuclein and APP were seen in the above regions of both 9H/PS-NA (Fig. 2B, arrows) and the related mouse 4L/PS-NA brains, indicating the co-existing of APP with α-synuclein, and their potential interaction in the neural cells.

Cellular compartment localization of APP/α-synuclein in brains of 9H/PS-NA mice

Since the affected cellular functions and the pathophysiological mechanism potentially involved in the progression of the
proteinopathies, the cellular organelles were evaluated for accumulated APP/α-synuclein-prone protein aggregates. The large amounts of APP/α-synuclein aggregates within neural cells suggested impairment of their degradation pathway and altered autophagy machinery (43). P62 and LC3 (Atg8) were chosen as autophagy reporters because of their importance in the recognition, transport and sequestration of aggregated-prone protein oligomers in fused autophagolysosomes (44). Dual-antibody immunostaining showed no significant association of APP signals with P62 or LC3 signals in the brain sections from 9H/PS-NA mice, although both P62 and LC3 signals were greatly increased compared with the WT controls (Fig. 3A, arrows). In contrast, many APP signals co-localized with the mitochondrial marker TOM40 (Fig. 3A, arrows). The pixel scatter diagrams support the co-localizations of APP/TOM40 signals (Fig. 3A). The same distribution patterns were also observed in the

Figure 1. APP accumulation in the brains of 4L/ and 9H/PS-NA mice. (A) Serial brain sections from 12-week WT, 4L/PS-NA and 9H/PS-NA mice were processed for immunohistochemistry analysis using rabbit polyclonal anti-APP antibodies. Multiple APP aggregates (brown particles) were seen in cerebral cortex (Cor), caudate putamen (Cpu), hippocampus (Hp) and substantia nigra (SN) regions of 4L/PS-NA and 9H/PS-NA brains. The inset in each image is the enlarged region pointed by arrows. The nuclei of neural cells were counterstained with hematoxylin (blue). The images were captured with 40 × objective lens. The scale bars are 40 μm and only show on the first image (Cor, WT). (B) Immunoblots of APP in WT (lanes 1–3), 4L/PS-NA (lanes 4–6) and 9H/PS-NA (lanes 7–9) cerebral cortex (50 μg lysate protein) from 12-week mice (n = 3) were developed with rabbit polyclonal anti-APP antibody. The migrated APP size was ~112 kDa according to the protein molecular weight markers at the left. The blot was stripped and re-probed with β-actin antibody as the loading control (bottom panel). (C) Quantification of immunoblots was conducted using ImageQuant 5.2 software. The scanned density values relative to WT (open bar, 100%), 4L/PS-NA (gray bar) and 9H/PS-NA (black bar) were plotted at Y-axis after normalization to the density of β-actin at each loading.
4L/PS-NA mice. Similar results were observed using anti-\(\alpha\)-synuclein antibodies: \(\alpha\)-synuclein had co-localization with the mitochondrial surface receptor TOM20, but not with P62 or LC3 (Fig. 3B, arrows). The attached pixel scatter diagrams support the co-localizations of \(\alpha\)Syn/TOM20 signals (Fig. 3B). These results indicated the preferential mitochondrial localization of APP/\(\alpha\)-synuclein aggregates, altered autophagy machinery, and impaired APP/\(\alpha\)-synuclein degradation in the brains of nGD mice.

**Association of APP/\(\alpha\)-synuclein with mitochondria in cortical neural cells**

To further confirm the APP/\(\alpha\)-synuclein mitochondrial aggregation in nGD mice, cerebral cortical neural cells were isolated from 12-week 9H/PS-NA mice for cellular compartment studies following short term of culture. Dual-antibody immunostaining was applied using anti-APP and cellular component specific antibodies for mitochondria (TOM20, TOM40 and Cox IV), autophagosomes (P62 and LC3), and lysosomes (LAMP1). Significantly larger-sized (>5 \(\mu\)m) APP aggregates were observed in the cerebral cortical neural cells of nGD, but not in those from WT (Fig. 4A). Most APP signals were co-localized with mitochondrial markers TOM40 and Cox IV, but scant APP signals merged with autophagosome marker P62 (Fig. 4A) and LC3 (Supplementary Material, Fig. S3), or the lysosomal marker LAMP1 (Fig. 4A). Co-localization of APP in each compartment was evaluated by Pearson correlation coefficients from 14 to 33 images and was 0.76 ± 0.045 with TOM40, 0.76 ± 0.029 with Cox IV, 0.51 ± 0.033 with P62 and 0.23 ± 0.029 with LAMP1 (Fig. 4B). These coefficients for APP/LC3 were 0.11 ± 0.045 (Supplementary Material, Fig. S3). The pixel scatter diagrams for two fluorescent signals in the each image are shown in Supplementary Material, Figure S4. Pearson correlation coefficients here show the estimated possible preference of APP signals associated with the organelles. Notably, the merged APP/mitochondrial signals were in clustered pattern, which was different from the granule pattern in WT neural cells. These results supported the *in vivo* observations from brain sections and further confirmed the altered autophagy pathway and APP aggregates preferentially in mitochondria of nGD brain cells.

The distribution pattern of \(\alpha\)-synuclein aggregation was also observed in the isolated cerebral neural cells from 12-week 9H/PS-NA mice (Fig. 5A). Pearson correlation coefficients with cellular organelle markers were TOM20 (0.76 ± 0.040), TOM40 (0.35 ± 0.056), Cox IV (0.28 ± 0.061), P62 (0.33 ± 0.046) and LAMP1 (0.14 ± 0.012) (Fig. 5B). The pixel scatter diagrams for two fluorescent signals in the each image are shown in Supplementary Material, Figure S5. Interestingly, \(\alpha\)-synuclein showed greater association with TOM20 (mitochondrial surface receptor) rather than with TOM40 or Cox IV. These results suggest a differential distribution of \(\alpha\)-synuclein aggregates in the mitochondria compared with those of APP.

**GC/GS accumulation, APP/\(\alpha\)-synuclein aggregates, and mitochondrial arrest in CBE-treated primary cortical neural cell cultures**

CBE-treated WT primary neural cell model was developed since CBE is a covalent active site-directed inhibitor of GCase. CBE treatment (2 \(\mu\)M, 7 days) nearly completely inhibited GCase activities of cultured neural cells and greatly increased the average GC and GS levels. The GC level in CBE (+) neural
cells was 15-fold ($^*P < 0.0001$) higher than the CBE (-) controls (Fig. 6A). The average GS level in CBE-treated (+) neural cells was 28-fold ($^*P = 0.0005$) higher than the CBE (-) controls (Fig. 6A). Among the GCs with different fatty acid acyl chains, GC18-0 increased from 52.37 to 65.43% of total GC (Fig. 6B). GC18-0 is the predominant species in neuronal cells indicating that the majority of cultured cells were neuronal cells. The results of GC/GS accumulation in CBE-treated cells are similar to reported tissue GC/GS levels in the brains of 9H/PS-NA and 4L/PS-NA mice (9).

The cell types of isolated WT cerebral cortical neural cells were identified using specific antibodies. Among 10–20 random images (20 $\times$), three major types of neural cells: neurons, astrocytes and oligodendrocytes were present in the ratio of $\approx$ 3:2:1 (87:58:29 cells per image) in the culture media (Fig. 6C). CBE treatment led to $\approx$ 36–46% neural cell loss, i.e. $\approx$ 56% neurons, 63% astrocytes and 54% oligodendrocytes survived in the treatment. Their population in CBE (+) media was $\approx$ 3:2.3:1 (49:37:16 cells per image) for neuron: astrocytes: oligodendrocytes (Fig. 6C). The results indicated that the cell loss due to direct CBE effects was equal in all three types of neuronal cells. APP aggregates ($>2 \mu m$) in CBE (+) neural cells were compared with CBE (-) control WT cells (Fig. 7A). APP aggregates in each neural cell types were evaluated by dual-antibody immunostaining. Among these neural cells $\approx$ 59% (SEM $\pm$ 4.2%) was Map2-positive neurons, 45% (SEM $\pm$ 3.2%) GFAP-positive astrocytes and 52% (SEM $\pm$ 3.8%) CNPase-positive oligodendrocytes after background subtraction ($\approx$ 5–9%) (Fig. 7B, $n = 8–16$ random 20 $\times$ images). The results here show the estimated preference of APP aggregation in each neural cell types resulting from CBE treatments. The cellular distribution of APP aggregation in CBE-treated neural cells was analyzed by dual-antibody immunofluorescence analysis. Pearson correlation coefficients for co-localization of APP aggregates ($>2 \mu m$) with cellular organelle markers were: 0.64 $\pm$ 0.048 with TOM40, 0.30 $\pm$ 0.035 with Cox IV, 0.45 $\pm$ 0.077 with P62 and 0.27 $\pm$ 0.046 with LAMP1 (Fig. 7C and D). This coefficient for APP with LC3 was 0.24 $\pm$ 0.041 (Supplementary Material, Fig. S3). The pixel scatter diagrams for two fluorescent signals in the each image are shown in Supplementary Material, Figure S6. CBE-treatment also led to $\alpha$-synuclein accumulation in these WT neural cells: $\alpha$-synuclein aggregations were found in 38% (SEM $\pm$ 3.7%) of Map2-positive neuronal cells, 42% (SEM $\pm$ 3.1%) of GFAP-positive astrocytes, and 30% (SEM $\pm$ 3.8%) of CNPase-positive oligodendrocytes after subtracting the background (5–13%) (Fig. 8A and B, $n = 10$, random 20 $\times$ images). The co-localization of $\alpha$-synuclein aggregates ($>2 \mu m$) was 0.42 for TOM20, 0.35 for TOM40, 0.35 for Cox IV, 0.15 for

Figure 3. APP/$\alpha$-synuclein cellular compartments in the brains of 9H/PS-NA mice. (A) Brain sections from 12-week 9H/PS-NA (top) and WT (bottom) mice were processed for dual-antibody immunostaining using anti-APP with anti-P62 (left), anti-LC3 (center) and anti-TOM40 (right) as indicated. APP signals are pointed by arrows. The pixel scatter diagrams for APP/TOM40 signals are shown. (B) Brain sections were from 12-week 9H/PS-NA (top) and WT (bottom) mice and processed for dual-antibody immunostaining using anti-$\alpha$-synuclein with anti-P62 (left column), anti-LC3 (center column) and anti-TOM20 (right column). $\alpha$-synuclein signals are pointed by arrows. The pixel scatter diagrams for $\alpha$-synuclein/TOM20 signals are shown. The images were captured with 40 $\times$ objective lens. The scale bars are 20 $\mu m$. Human Molecular Genetics, 2014, Vol. 23, No. 15 3947
P62 and 0.12 for LAMP1 (Fig. 8C and D). This coefficient for α-synuclein with LC3 was 0.50 (Supplementary Material, Fig. S3). The distribution of α-synuclein aggregates in the cellular compartments was in the order: mitochondrial > autophagosome > lysosomes (Fig. 8D). The pixel scatter diagrams for two fluorescent signals in each image are shown in Supplementary Material, Figure S7. Taken together, APP/α-synuclein accumulation in neural cells correlated with increased cellular GC/GS levels. APP/α-synuclein accumulation in CBE-treated WT cultured neural cells reproduced the finding of APP/α-synuclein aggregations in the in vivo genetic mouse models and their preferential mitochondria compartmentation.

Altered mitochondrial morphology and function in the affected cerebral cortical neural cells

The preferential distribution and cluster pattern of APP/α-synuclein aggregates in mitochondria indicated morphological and potential functional abnormalities in nGD mouse brains and CBE-treated cortical neural cells. The representative ultrastructural images of cortical neural cells isolated from 12-week 9H/PS-NA brains showed degenerating neural cells with irregular cytoplasmic and nuclear membranes, condensed chromatin and electronic dense and size large (up to 1.2 μm) mitochondria (Fig. 9A). To evaluate the effects on mitochondrial functions by APP/α-synuclein accumulations, brain cortices and CBE-treated cortical neural cells were subjected to mitochondrial adenosine triphosphate (ATP) production and oxygen consumption analyses. In comparison with WT controls, ATP production was reduced by 24% (n = 2, *P = 0.0102) in CBE-treated WT cortical neural cells. For mitochondria isolated from cerebral cortical cells of 4L/PS-NA or 9H/PS-NA mice, 32% (n = 2, ***P = 0.0004) to 38% (n = 2, ***P = 0.0001) decreases in ATP production were obtained with 23% decrease in the parental mice (PS-NA, 9H/9H and 4L/4L) (Fig. 9B). Oxygen consumption rates were decreased by 37% (n = 2, **P = 0.0048) in CBE-treated WT cortical neural cells. Those from cerebral cortical cells of 4L/PS-NA or 9H/PS-NA mice were 28% (n = 2, *P = 0.0054) to 42% (n = 2, **P = 0.0001) decreased with 34% in PS-NA, 25% in 9H9H and 19% in 4L4L mice (Fig. 9C). These results show the compartmentation of APP/α-synuclein accumulation in mitochondria leading to the abnormal mitochondrial morphology and function in the brains of nGD mice, which could contribute to neurodegeneration and neuron loss in the affected brains.

DISCUSSION

Multi-protein aggregation in the brain of nGD mice

Here, various sizes of APP, Aβ and PS-1 aggregates and the mitochondrial involvement were observed in the brains of the nGD mice, 9H/PS-NA and 4L/PS-NA. Together with previously observed α-synuclein/ubiquitin aggregates (9), at least five aggregated-prone proteins were present in these brains. Also,
the regional and cellular distribution of these protein aggregates was similar in both studies. Such histological features and synucleinopathies and amyloidopathies have also been reported in more common neurodegenerative diseases, e.g. Parkinson disease (28,29,45) and Alzheimer disease (46,47), as well as in Down syndrome (48). These similar pathologic findings suggest a common cellular and molecular impairment/alteration in their pathophysiologic processes of protein degradation.

Figure 5. α-Synuclein distribution in cellular compartments of cortical neural cells from 9H/PS-NA mouse brains. (A) Co-localization of α-synuclein with cellular organelles. Cortical neural cells isolated from 12-week of 9H/PS-NA (top panels) and WT (bottom panels) mice were applied to dual-antibody immunostaining using rhodamine-conjugated anti-α-synuclein with organelle markers: FITC (green)-conjugated TOM20, TOM40, Cox IV, P62 and LAMP1 as in Figure 4. The merged images show various degree of co-localization of α-synuclein with each marker (yellow). Neural cell nuclei were stained by DAPI (blue). The images were captured with 63 × objective lens. The scale bars are 20 μm. (B) PCC analysis was conducted from co-localized Rhodamine (α-synuclein) and FITC (each organelle marker) signals in (A). The bar graphs show mean PCC (r) at Y-axis plotted to each dual-antibody set (X-axis). The PCC for αSyn-TOM20 was 0.76 (SEM ± 0.040, n = 21); αSyn-TOM40 was 0.35 (SEM ± 0.056, n = 30); αSyn-Cox IV, 0.28 (SEM ± 0.061, n = 13); αSyn-P62, 0.33 (SEM ± 0.046, n = 40); αSyn-LAMP1, 0.14 (SEM ± 0.028, n = 41). Pixel scatter diagrams for each image are shown in Supplementary Material, Figure S5. The results of Pearson analysis demonstrate preferential mitochondrial association of α-synuclein in 9H/PS-NA cortical neural cells.

Figure 6. CBE-induced GC/GS increase in newborn cerebral cortical neural cells. The neural cells isolated from mouse newborn cerebral cortices were cultured in the medium with 2 mM GCase-irreversible inhibitor CBE (+) or without CBE (−) for 7 days. Neural cells were harvest for GC/GS (A–B) and immunofluorescence (A–B, C) analyses. (A) Electrospray ionization-liquid chromatography-tandem mass spectrometry analysis shows significantly increased total cellular GC (> 15-fold, P = 0.0397) and GS (> 28-fold, P = 0.0085) levels in CBE-treated (+) compared with un-treated CBE (−) cells. (B) The proportion of GC species. GC18-0 was dominant species indicating majority of neurons in the neural cells. (C) Population of neural cells. Bar graphs present percentage of each neural cell types in CBE (+) and CBE (−) cultures. Cell type specific marker for neurons, astrocytes and oligodendrocytes were Map2, GFAP and CNPase, respectively. CBE treatment had equal effect on each cell type survival.
However, the initiation of APP, Aβ and α-synuclein aggregation in nGD derives from a totally different pathogenic mechanism, i.e. lipid accumulation.

The pathogenesis of the protein aggregates and neurodegeneration in these models is complex and has contributions from the Gba1 and Psap mutations as well as the resulting biochemical pathology. Importantly, no significant APP aggregates were observed in the brains of age-matched parental lines of the 12-week hypomorphic prosaposin (PS-NA) mice (with WT Gba1) or the isolated Gba1 point-mutated homozygous mice, 4L/4L and 9H/9H. Previously, no significant α-synuclein aggregates were observed in these same parental lines (9). However, α-synuclein aggregates were observed in 9H/9H and 9V/9V brains after a year of age (9). These observations indicate a significant interaction effect, due to the mixed genotypes, on cellular/molecular impairments, rather than a simple phenotype mixture. Biochemically, the GC/GS levels in the brain of those same parental lines (4L.9H, and PN-NA mice) were relatively lower than that in these back-crossed nGD (4L/9H-PS-NA) mice (26). In comparison, moderate to high level accumulation of GC (4–8-fold) and GS (28–108-fold) were in the brains of 4L/PS-NA and 9H/PS-NA mice, thereby implicating high levels of GC/GS as a risk factor for the proteinopathies in nGD (9). The ex vivo CBE-neural cell model was developed to address the potential for involvement of the unfolded protein response in these pathogenic processes. In these studies, CBE was added to neural cells from strain-matched mice with WT Gba1 and the synthesized GCase would fold normally. The addition of CBE would destroy the activity, but not the structure of GCase (49). The similar findings with CBE also provide additional evidence that the pathology is related to the compromised GCase activity rather than the effects of the hypomorphic prosaposin deficiency. These studies showed APP and α-synuclein aggregation was linked to neural cell accumulation of GC and GS, which may be the initiating factors for significant APP/α-synuclein aggregation. The sporadic presence of APP and α-synuclein (9) aggregates in the cortex of aged 4L/4L and 9H/9H (52 weeks) brains, suggests a chronic age-related CNS effect in Gba1 mutant mice and as seen in some human Gaucher disease Type 1 patients (8).

Synergetic interaction between APP and α-synuclein accumulation?

Immunohistochemistry showed APP and α-synuclein co-localized significantly with NeuN, indicating their co-existence in the neuronal cells and suggesting their potential interaction in these cells. Similar cellular co-localization of pathogenic proteins APP and α-synuclein occurs in the CNS of patients with Parkinson and Alzheimer diseases and even more commonly in dementia LBD (28,29,38,47,50,51). Studies from Alzheimer disease brains suggested that the alteration of APP expression increased
neuronal vulnerability to Lewy body formation and to degeneration (52). APP and/or Aβ were also found in the brains of some Parkinson disease patients and mouse model (28,29,51). Taken together, there could be a synergistic effect between Aβ and α-synuclein as described in transgenic animals and in vitro experiments (17,36). Although the cellular and molecular mechanisms are not fully determined in aggregates, these studies raised the possibility that APP and/or Aβ could influence α-synuclein fibril formation and pathophysiology progression of CNS degeneration. Thus, the multi-proteinopathies could develop in parallel as directly linked by common factors or as synergy among these degenerating processes (53,54). The consequences of multiple protein aggregates and inclusions in neurodegenerative diseases could lead to severe impairment of cellular function in the affected brains.

Cellular compartmentation of APP/α-synuclein aggregates and mitochondrial dysfunction

Abnormal levels and aggregation of APP/α-synuclein and other aggregated-prone proteins (i.e. ubiquitin, Aβ and PS-1) were widely distributed in the brains of 9H/PS-NA and 4L/PS-NA mice. Aggregated-prone proteins and inclusions represent an end-stage of pathophysiolologic process as seen in other neurodegenerative diseases. However, their cellular and molecular pathway could be very different during the disease development and progression. In particular, the subcellular localization of APP/α-synuclein aggregates provides an insight into impairment/alteration of cellular organelles. Here, preferential co-localization of APP/α-synuclein was shown to mitochondrial proteins (TOM20, TOM40 and Cox IV). The sizes of co-localized APP/αSyn and mitochondrial signals were larger than the size of normal mitochondria, which suggests that multiple mitochondria and possibly other cellular components (e.g. proteasomes) were trapped in the APP/αSyn aggregates. These mitochondria were abnormal in structure and function. APP and α-synuclein contain mitochondrial targeting sequences indicating that this organelle is part of the normal pathway for these proteins (55,56). Massive influx of APP/α-synuclein and/or their aggregates into mitochondria could block the mitochondrial protein import channel, and lead to translocational arrest, of other proteins essential to organelle function, which could occur by APP within the inner membrane smaller TIM23 channel after first passing through the wide TOM40 channel (35). The reduced (28–40%) mitochondrial ATP production and oxygen consumption in 4L/PS-NA and 9H/PS-NA brain cortices and CBE-treated cerebral cortical neural cells are a hallmark of mitochondrial dysfunction, which led to insufficient supply of metabolic needs for normal neural cell functions. Here, at the first time we show APP aggregation in the mitochondria of nGD mouse brains and the resultant mitochondrial dysfunction. Mitochondrial abnormalities could lead to oxidative stress and promote protein covalent cross-linking (35,57–61), which was also evidenced by immunoblots showing α-synuclein oligomers in our and other studies.
In addition to protein aggregates, the abnormal levels of GS, a lysosphingolipid, is a neuronal toxic agent and a potent inhibitor of mitochondrial cytochrome c oxidase, potentiates oxidative stress and protein/lipid peroxidation. Taken together, all of these observations suggest that mitochondrial dysfunction plays a pivotal role in neurodegenerative disorders including Alzheimer and Parkinson diseases and Gaucher disease.

Unlinked autophagy mechanism in APP/α-synuclein accumulated neural cells

Central to the pathogenesis of prone-protein metabolism, e.g. APP and α-synuclein aggregates, in neural cells is the impairment/alteration of their degradation pathway. The studies here demonstrated significant response of cellular autophagy system to the abnormal levels of cellular APP/α-synuclein-prone...
protein particles, but less corresponding contact or interaction between cellular autophagy machinery and pathogenic APP/α-synuclein aggregates. The involved mechanism is not clear; it could be because of the changes in surface recognition configuration due to APP/α-synuclein aggregation, which led to recognition/interaction failure. In this case, the mitochondrial targeting signal sequences on APP/α-synuclein favored the massive autophagy-unlinked APP/α-synuclein to mitochondrial influx. Consequently, the autophagy machinery unlinked protein aggregates in affected neural cells accelerates the pathophysiology and neurodegenerative process. Similar autophagy deregulation, which showed autophagy vacuoles accumulate in degenerating neurons and impaired processing, has been reported in Parkinson and Alzheimer diseases (70–73), in affected SH-SY5Y cells (74) and B103 rat neuroblastoma cells (75), in wobbler mouse neurons (76), and in Gaucher disease K14-Ind/Inl Gba1−/− mice (20). The less co-localization of lysosomal marker LAMP1 with APP/α-synuclein aggregates also supported impaired/altred autophagosome-lysosomal degradational pathway of these prone proteins in the brains of nGD mice. In addition, lysosomal cathepsin D could be involved in α-synuclein levels. It was shown, the enhanced neuronal ubiquitin signals and axonal spheroid formation in 4L/PS-NA brains were similar to those seen in cathepsin D-deficient mice (18). Significant changes in cathepsin D distribution in the brain and neurons of Gaucher disease mice were detected, particularly in a manner consistent with release of cathepsin D from the lysosome to the cytosol (77). In this study, cathepsin D was less associated with lysosomal signal LAMP1 in 9H/PS-NA cortical neural cells (data not shown) suggesting its lysosomal release.

Pathogenic implications of glycosphingolipids

CBE-induced neural cell model tightly linked the endogenous cellular GC/GS levels to the increased APP/α-synuclein aggregation, although the underlying mechanism is not clear. In addition to Gaucher disease, α-synucleinopathies and amyloidopathies were also observed in the brains of several other GSL-involved human and mouse LSDs, such as Niemann–Pick disease, type C1 and Mucopolysaccharidoses I mice (9), GM1 gangliosidosis, Sandhoff mice (13,78–80), and Niemann–Pick disease, type C1 patients (81). These phenotypic similarities suggest the presence of common pathophysiologic mechanisms for LSDs with neurodegeneration (82), although their initial factors in various GSL-related neurodegenerative diseases are different and dependent on the accumulated substrates. These observations supported the contention that GSLs (including GC/GS) and gangliosides contribute to the pathogenesis of APP/α-synuclein accumulations. To date, some ex vivo studies have been conducted for understanding of the biological pathways from lysosomal enzyme deficiency to APP/α-synuclein accumulation, neuron dysfunction and death. GSL loading on cultured patient fibroblasts (Niemann–Pick disease, type A and B, Tay–Sachs, and Sandhoff) and human cells (SHSY-5Y, and H4 neuroglioma cells) led to increased autophagy vesicles and decreased lysosome-dependent degradation of APP (83). In contrast, inhibition of GSL biosynthesis reduced APP and Aβ peptide in SH-SY5Y (83). Interestingly, in vitro study showed that Aβ selectively bond to GSLs containing a C2-OH group in ceramide backbone (HFA-GSLs), but not to a non-OH fatty acid (NFA)-GSLs (84). The current findings in nGD mouse models provided a biological system and potential clues to the pathogenesis of nGD including the APP/α-synuclein cellular distribution in Gba1 mutant mice and cultured cortical neural cells. Ongoing studies will provide insight into the underlying neuronopathic mechanisms in Gaucher disease and the relationship between GCase deficiency and the development of Parkinsonism manifestations. Understanding the mechanism of protein aggregation and the consequent neuronal toxicity/regeneration will be important for therapeutic strategy and new drug development.

MATERIALS AND METHODS

Materials

The following were from commercial sources: CBE, sodium taurocholate (Calbiochem, San Diego, La Jolla, CA), Triton X-100, Poly-D-Lys (P6407), OptiPrep (D1556), polyethyleneimine (PEI) (Sigma-Aldrich, St. Louis, MO), 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-Glc; Biosynth AG, Switzerland), Eagle’s minimum essential medium (ATCC, Manassas, VA), B27 (#17504), Gentamycin (# 15710), Glutamak (200 mM; #35050-061), NeurobasalA (#10888), Mouse FGF2 for mouse neurons (PMG0034) and 10% gradient Bis–Tris Gel, Dulbecco’s modified Eagle’s medium (DMEM), Hank’s Balanced Salt Solution (HBSS)-Ca (Invitrogen, Carlsbad, CA). HibernateA (HA), HA minus calcium (HA-Ca), (BrainBits, Springfield, IL). Papain (# 3119) (Worthington, Lakewood, NJ). Antibody sources are as follows: mouse monoclonal anti-α-synuclein, goat polyclonal anti-mouse α-synuclein, rabbit polyclonal anti-ubiquitin, anti-APP (ab2072), anti-Aβ (ab2539), anti-Lamp1 and anti-presenilin 1 (PS-1), mouse monoclonal to CNPase, and mouse monoclonal to Cox IV (Abcam, Inc. Cambridge, MA). Mouse monoclonal anti-APP (MAB348), anti-NeuN and anti-Map2 (Millipore, Temecula, CA). Goat polyclonal anti-mouse Tom20, anti-Tom40 (Santa Cruz Biotech, Santa Cruz, CA). Mouse monoclonal anti-GFAP (Sigma, St. Louis, MO), Goat anti-rabbit/rat [Alexa-488, fluorescein isothiocyanate (FITC)], Goat anti-mouse-biotinylated antibody (streptavidin-Alexa-610) (Molecular Probes, Irvine, CA). Peroxidase substrate kit DAB SK-4100, Alkaline phosphatase substrate kit II SK-5200 (Vector, Burlingame, CA). Peroxidase-conjugated goat anti-mouse IgG, Bicinchoninic acid (BCA) Protein Assay Kit, M-Per Mammalian Protein Extraction Reagent and ECL Kit (PIERCE, Rockford, IL). Hybrid ECL Nitrocellulose Membrane (Amersham, Piscataway, NJ). Pierce BCA Protein Assay Kit (Thermo Scientific). GentleMACS Dissociator, Separation Buffer, MACS column/separator and Anti-TOM22 MicroBeads (Miltenyi Biotec Inc. Auburn, CA). ATP Bioluminescence Assay Kit CLS II and protease inhibitor (Roche).

Gaucher disease mouse models

The nGD mouse models, 4L/PS-NA or 9H/PS-NA, are generated from backcrossing of the homozygous point-mutated Gba1 mice, V394L (4L) or D409H (9H) (40), respectively, with hypomorphic prosaposin (PS-NA) mice. These mice exhibit excesses of GC/GS accumulation in the CNS (9,26). Age-matched WT littersmates were controls. All mice were housed in microisolators within the barrier animal facility according to standard procedures approved by Institutional Animal Care and Use.
Isolation and culture of adult cerebral cortical neural cells

Adult cortical neural cells were isolated from the brains of 12-week 4L/PS-NA or 9H/PS-NA mice and their littermate WT controls (85). Briefly, the cortices were dissected in HibernateA/B27 medium (HABG) (1 × B27–0.5 mM Glutamax in Hibernate A) on ice, cut into 0.5-mm slices, and digested with 2 mg/ml papain [0.5 mM glutamax-Hibernate-Ca at 30 °C with gentle shaking (30 min, 170 rpm)]. Neural cells were released using a wide-bore pipette to triturate and the cells from the suspension were purified using an OptiPrep density gradient (800 g, 15 min, room temperature). The desired fractions were collected and the cells were washed in HABG. They were then plated onto sterile and Poly-d-Lys coated glass cover slips at 300 cells per mm² in 100 μl Neurobasal A/B27 (1 × B27–0.5 mM Glutamax–10 μg/ml Gentamycin–5 mg/ml mouse FGF2 in Neurobasal A) for 4–5 days. The cells then were fixed with 3% of paraformaldehyde for immunofluorescence studies. For ultrastructure, the isolated cerebral cortical neural cells were fixed in Karnovsky’s fixative.

Newborn cortical neural cell culture

Newborn mouse cortical neural cells were isolated from the WT postnatal pups (1–3 days) cortices (86,87). Briefly, the cerebral cortex was dissected in HBSS-Ca medium under the dissecting microscope and transferred to a 50 ml tube containing HBSS-Ca medium and cut into small pieces. Tissue slices were digested in HBSS-Ca medium containing 1 mg/ml papain and 0.1 mg/ml DNase I at 37 °C, suspended in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and frozen in liquid nitrogen. The newborn neural cells were seeded onto PEI pre-coated plates and chamber-slides for 1 h (healthy neural cells attach to the bottom) and then, changed to Neurobasal medium containing 1 × B27, 1 × glutamine, 1 × penicillin/streptomycin for desire culture. The next day, the cultured cells were treated with 2 mM CBE for 0–7 days (9). For GC/ GS and mitochondrial analyses, the cells were collected by gentle shaking (30 min, 170 rpm)

Histological studies

For histological and immunohistochemistry studies, three mouse brains were harvested from each genotype at the ages of 10–12 weeks, A minimum of two sections from each brain was examined (9). Tissue sections were permeabilized using 0.4% Triton X-100 in 10% NGS-1 × phosphate buffered saline (PBS). The primary antibody dilutions were as the follows: α-synuclein (1:100), APP (1:100), Aβ (1:100), TOM20 (1:100), TOM40 (1:100), Cox IV (1:500), P62 (1:100), LC3 (1:200), Lamp1 (1:200), PS-1 (1:100), Map2 (1:100), GFAP (1:100) and CNPase (1:100). Two anti-APP antibodies were used: (i) rabbit polyclonal anti-APP (ab2072) corresponding to amino acids 653–662 of APP; (ii) mouse monoclonal anti-APP (MAB348) corresponding to N-terminal amino acids 66–81 of APP. The rabbit polyclonal anti-Aβ (ab2539) is corresponding to amino acids 1–14 of human β-amyloid (AB). The secondary antibodies were appropriately biotin-conjugated anti-rabbit or anti-mouse antibodies. The signals were detected by Alexa-488, FITC, or streptavidin-Alexa-610. Cultured cells on slides were fixed with 3% paraformaldehyde, permeabilized using 0.2% Triton X-100 in 10% NGS-1 × PBS, and then followed the same procedure for immunohistochemistry or immunofluorescence analyses. For negative controls, respective sections were processed for background immunoreactivity without the primary antibody or with pre-immune (mouse or rabbit) serum as the primary antibodies. Immunofluorescence images were captured with a Zeiss Apotome microscope (Axiocam200) at excitation of 506 nm (for Alexa-488, FITC) or 590 nm (for Alexa-610, Rhodamine). Cell nuclei were stained with DAPI (blue). Co-localization of dual-signals was analyzed by Pearson correlation coefficient (r, ± 1) using Zeiss software. Immunohistochemistry images were captured with a Zeiss light microscope. Signal locations in brain sections were according to The Mouse Brain in Stereotaxic Coordinates (88). Pearson correlation coefficient software is incorporated in Apotome microscope and used to analyze the co-localization of two signals in cells. The average Pearson correlation coefficient number (from +1 to −1) were derived from multiple images and shown as the bar graphs in each figure. The derived signal pixel scatter diagrams for each image panel are given in Supplementary data.

Immunooblots

The cerebral cortex tissues from 4L/PS-NA, 9H/PS-NA and WT brains (n = 3) were micro-dissected and homogenized (1:5, mg:μl) in M-Per Mammalian Protein Extraction Reagent (9). Equal amounts of tissue lysate proteins were subjected to immunoblotting and quantified using β-actin signals as internal reference.

GCase enzyme activity

For GCase activities, neural cell pellets (∼10⁶ cells) were homogenized in 500 μl of 1% sodium taurocholate/1% Triton X-100, sonicated (4°C, 30 s × 3), and clarified (10 000g, 5 min, 4°C) (89). The assay mixtures were added to substrate (4 mM 4MU-Glc, final) and incubated (1 h, 37°C). Protein concentrations were determined using the BCA kit according to manufacturer’s instructions.

Tissue and cell lipid analyses

Anesthetized mice were perfused with three blood volumes of normal saline. The brains were immediately micro-dissected to isolate the cerebral cortices. Brain tissue samples (∼60 mg wet weight) were extracted (26). For cell lipid analysis, 1–2 × 10⁶ cultured cells were used. GC and GS in the tissue and cell extracts were analyzed by electrospray ionization-liquid chromatography-tandem mass spectrometry using a Waters
Quattro Micro API triple quadrupole mass spectrometer (Milford, MA) interfaced with an Acquity UPLC system (9,26). The GC and GS levels were normalized to extract protein mass in each sample and expressed as pmol/mg protein.

Isolation of mitochondria and function analyses

Tissue mitochondria were isolated (90). Briefly, the cerebral cortices (~250 mg) from 12 to 16-week mice were minced into 1-2 mm³ pieces in ice-cold isolation buffer (10 mM EDTA/0.05% trypsin in PBS), incubated for 30 min on ice and centrifuged at 300g for 5 min. The harvested pellets were homogenized in ice-cold PBS containing 10 mM EDTA and protease inhibitor using GentleMACS Dissociator. The homogenized samples were filtered with 70 μm filters and centrifuged (4°C, 5 min at 800g). The resultant supernatants were diluted with ice-cold Separation Buffer, mixed with anti-TOM22 MicroBeads, and enriched on a MACS column. The magnetically labeled mitochondria were eluted for assays. The ATP level in cerebral cortex mitochondria and cultured neural cells was determined using the ATP Bioluminescence Assay Kit CLS II (Hansatech Instrument, Ltd) and recorded for oxygen consumption. The instrument was calibrated with oxygen-saturated graph (Hansatech Instrument, Ltd) and recorded for oxygen consumption. The resultant supernatants were diluted with 1 ml warm (37°C) Medium A (Full deficient medium with 0.01% CaCl₂, 0.0105% isoleucine, 0.003% methionine, 0.0124% NaH₂PO₄.H₂O, 2% dialyzed FBS, 0.01% pyruvate). The supernatants were diluted with 1 ml warm (37°C) Medium A (Full deficient medium with 0.01% CaCl₂, 0.0105% isoleucine, 0.003% methionine, 0.0124% NaH₂PO₄.H₂O, 2% dialyzed FBS, 0.01% pyruvate). The samples were loaded in a closed chamber in Hansatech Oxygraph (Hansatech Instrument, Ltd) and recorded for oxygen consumption. The instrument was calibrated with oxygen-saturated distilled water (217 nmol/ml) at 37°C (95).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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REFERENCES


