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Alpha-Synuclein Abnormalities in Mouse Models of Peroxisome Biogenesis Disorders

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Abstract

alpha-Synuclein (αS) is a presynaptic protein implicated in Parkinson's disease (PD). Growing evidence implicates mitochondrial dysfunction, oxidative stress and αS-lipids interactions in the gradual accumulation of αS in pathogenic forms and its deposition in Lewy bodies, the pathological hallmark of PD and related synucleinopathies. The peroxisomal biogenesis disorders (PBD), with Zellweger syndrome serving as the prototype of this group, are characterized by malformed and functionally impaired peroxisomes. Here we utilized the PBD mouse models, Pex2-/-, Pex5-/- and Pex13-/-, to study the potential effects of peroxisomal dysfunction on αS–related pathogenesis. We found increased αS oligomerization and phosphorylation and its increased deposition in cytoplasmic inclusions in these PBD mouse models. Further, we show that αS abnormalities correlate with the altered lipid metabolism and specifically, with accumulation of long chain, n-6 polyunsaturated fatty acids, that occurs in the PBD models.

Keywords

Parkinson's disease; lipids; alpha synuclein; peroxisomes

Introduction

α-Synuclein (αS) is a presynaptic protein critically involved in the cytopathology and genetics of Parkinson's disease (PD) (reviewed in (Hardy et al. 2006; Lee and Trojanowski 2006; Moore 2005)). In PD and related human synucleinopathies (Duda et al. 2000), there is a progressive conversion of the normally highly soluble αS protein into insoluble, β-sheet rich filamentous assemblies, resulting in its intraneuronal deposition into Lewy bodies (LB) and Lewy neurites, the cytopathological hallmarks of this group of disorders (Goedert 2001; Spillantini et al.

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1997). However, a biochemical event that occurs prior to these pathological lesions is the cellular accumulation of various αS post translational modifications, including phosphorylation (Fujiwara et al. 2002) (Smith et al. 2005), ubiquitination (Iwatsubo et al. 1996), nitration (Giasson et al. 2000) and oligomerization (Sharon et al. 2003a). The cellular triggers that enhance the accumulation of modified αS proteins and how these pathogenic post-translational modifications enhance αS deposition into the Lewy bodies and Lewy neurites are unknown.

Peroxisomes are involved in cellular metabolic activities including a) β-oxidation of very long chain fatty acids (VLCFA) as well as long chain polyunsaturated fatty acids (PUFAs) (Poulos et al. 1989; Poulos et al. 1988; Sharp et al. 1987) and branched chain fatty acids, b) the conversion of cholesterol to bile acids, and c) the synthesis of ether phospholipids including plasmalogens (Poulos et al. 1988; Robinson et al. 1990; Sharp et al. 1987; Su et al. 2001). Peroxisome biogenesis disorders (PBD) are a group of human genetic disorders, with Zellweger syndrome being the most severe form, that are characterized by the absence of functional peroxisomes due to a disturbance in the peroxisomal protein import machinery (reviewed in (Wanders and Waterham 2005) (Gould et al. 2001)). PBD are caused by mutations in peroxin (Pex) genes encoding proteins involved in the biogenesis and assembly of peroxisomes. Three different Pex genes have been inactivated in the mouse, i.e., Pex2, Pex5 and Pex13 (Baes et al. 1997; Faust and Hatten 1997; Maxwell et al. 2003), in order to generate mouse models for PBD. Importantly, most of the major metabolic and neuronal abnormalities observed in ZS are recapitulated to varying degrees in these three PBD mouse models.

Three major biochemical insults attributable to peroxisomal dysfunction have been described in ZS and the related PBD mouse models: altered lipid metabolism, increased vulnerability to oxidative stress, and mitochondrial dysfunction ((Dirkx et al. 2005; Keane et al. 2007; Maxwell et al. 2003) reviewed in ((Baes and Van Veldhoven 2006; Wanders and Waterham 2005)). Interestingly, these types of insults have also been shown to be involved in PD pathogenesis (reviewed in (Abou-Sleiman et al. 2006; Halliwell 2001; Moore 2005; Scherzer and Feany 2004)). However, the role of peroxisome function in PD has not been tested before.

Here, we used PBD mouse models to study the potential cellular mechanisms underlying αS modification and toxicity in PD. We specifically focused on the shared insults between PD and PBD -- oxidative stress, mitochondrial dysfunction and lipid metabolism -- for their effect on αS oligomerization, phosphorylation and accumulation in Lewy-like inclusions. We show that αS toxicity correlates with altered lipid metabolism and specifically with an elevation of n-6 PUFA concentrations rather than with oxidative stress or mitochondrial dysfunction.

Materials and Methods

Mouse brains

Brains of newborn mice of Pex-/- and control Pex +/+ littermates were immediately frozen and shipped to the Hebrew University of Jerusalem. All protocols for animal use and experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Columbia University (Faust and Hatten 1997); University of Leuven (Baes et al. 1997) and Griffith University (Maxwell et al. 2003). Brains were fractionated (Sharon et al. 2001) and samples were processed for oligomers detection. Overall we tested 5-7 brains for each Pex-/- and control groups.

αS over expression

Pex5-/- and control fibroblasts were stably transfected with wt human αS cDNA in the pCDNA 3.1 vector or vector only using Lipofectamine 2000 (Invitrogen CA, USA). The clones were

tested for αS expression every week and maintained their stable αS expression for 3-4 months post transfection (Sharon et al. 2001).

Viral infection of αS expressing virus, the αS cDNA was amplified and sub-cloned into a pADlox vector and used for adenovirus production (Hardy et al. 1997). Recombinant adenovirus encoding αS was produced as described previously (Altschuler et al. 1998; Altschuler et al. 1999). Cells were transduced with αS- expressing adenovirus and GFP control virus (to compare gene transfer efficiency, at ~100 PFU/cells) for 2 hours and the unbound virus was removed by 3 washes in saline. Cells were then maintained in standard serum-supplemented medium for additional 16-18 hours, collected and fractionated as previously described (Sharon et al. 2001).

Western blot

Whole mouse brains were homogenized (20 up-and-down strokes with a Teflon homogenizer, followed by seven passages through a 27-gauge needle) in 1:10 (w/vol) homogenization buffer [20 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 0.32 M sucrose, 43 mM β-mercaptoethanol, protease inhibitor mix (Sigma), 25 mM β-glycerol phosphate, 20 mM Na-fluoride (Sigma)]. For the detection of αS oligomers and aggregates, protein samples of high speed supernatant (post 280,000 g) were incubated at 65°C for 16-18 hours (Sharon et al. 2003a) prior to loading on an 8-16% NuPAGE Bis-Tris (Invitrogen) or 14% SDS-PAGE. Immunoblots were reacted with H3C anti αS antibody (gift from Julia George, University of Illinois) (George et al. 1995) or Syn-1 (BD Transduction Labs, San Jose, CA), or anti phospho Ser129 αS antibody (Wako-Chem, Osaka, Japan).

Immunocytochemistry

For immunostaining, cells were pre- permeabilized with 0.002% Triton X-100 in PBS for 1 minute. Cells were then fixed with 4% paraformaldehyde for 10 minutes on ice and permeabilized with 0.2% Triton X-100 in PBS and 1% goat serum for 5 minutes at room temperature. The slides were next treated with 10% formic acid (pH 2.0) for 10 minutes at room temperature followed by extensive washes and blocking with 1.5% goat serum in PBS. Slides were then reacted with primary antibodies: anti αS monoclonal antibody (LB509 1:100 Zymed CA, USA); anti ubiquitin polyclonal antibody (1:100 Dako Glostrup, Denemark) and secondary ab at 1:200, anti mouse-Alexa flour 488 (Molecular probes CA, USA) and anti rabbit-Cy5 (Jackson ME, USA). Slides were sealed with mounting medium (cat# M1289 Sigma Rehovot, Israel) and analyzed by confocal microscopy with laser Argon 488 (filter BA 510 IF dichroic mirror filter) and laser helium neon 633 (filter BA 660 IF) (Laser Scanning microscope Zeiss 410, Germany). Total signal per cell was counted and using Image Pro/Image J softwares (Media Cybernetics Inc., Silver Spring, MD). The average count of n=10-13 cells is reported.

rtPCR

RNA was isolated from brain tissue using Tri-reagent (Sigma, Rehovot, Israel). Following isolation, RNA was treated with DNAse (RQ1 RNAse-free DNAse, Promega, Beit Ha'emek, Israel). cDNA was generated with GeneAmp RNA PCR kit (Applied Biosystems, CA, USA). Quantitative amplification of αS was performed with ABsolute QPCR SYBR Green ROX mix (ABgene, Epsom, UK) and normalized to 18S levels. The sequence of αS specific primers:

5' GTCTCAAAGCCTGTGGCATCT

3' TCCACACTTTCCGACTTCTG

18S specific primers:

5' GAGCGAGTGATCACCATCAT

3’ GCCAGAACCTGGCTGTACTT

Mitochondrial purification and respiratory chain complex I measurements

Mitochondria purification—Cells were grown to confluency, collected and washed. Cells were resuspended in buffer A (0.32 M Sucrose; 0.05 M Tris-HCl pH 7.4; 2 mM EDTA) and homogenized by five strokes in a Teflon dounce homogenizer and centrifuged at 1000 × g to remove nuclei. The supernatant was centrifuged at 12,000 × g and the pellet was resuspended in buffer A containing 0.02% digitonin to release mitochondria. Mitochondria were washed to remove digitonin by centrifugation at 12,000g , resuspended in minimal amount of buffer A and protein content was determined by Bradford (Bradford 1976). Frozen aliquots were stored at -70°C until use.

Enzymatic activities of the mitochondrial respiratory chain complex I were determined as rotenone sensitive NADH–cytochrome *c* reductase (I+III) and rotenone-sensitive NADH–coenzyme Q reductase (I). The activity of citrate synthase, a mitochondrial matrix enzyme was determined in parallel. The spectrophotometric assays were performed as previously described (Saada et al. 2004). Respiratory chain activities were normalized to citrate synthase activity in each sample.

Fatty acids analyses

Frozen brain tissue of newborn (P0) Pex2-/- and control mice was thawed briefly, weighed and homogenized in at least 20 volumes of chloroform:methanol 2:1 (Folch et al. 1957) containing 0.05% butyl hydroxytoluene (BHT) (w/vol). After 1 hour at room temperature the samples were centrifuged, and the supernatant was transferred to a weighed tube. 1/5 total volume of water was added and the mixture was centrifuged. The upper phase was removed and the lower phase was dried under N2 and weighed. The fatty acids were converted to their methyl esters with 1.5 ml 1N methanolic HCL at 75°C overnight. Following methanolysis, the fatty acid methyl esters were extracted in hexane, dried, solubilized in 50μl hexane, transferred to injection vials with glass inserts and 1μl was analyzed by capillary gas chromatography on an Agilent 5890 gas chromatograph. Two capillary columns differing in polarity were used to correctly identify and quantitate the fatty acid methyl esters. A 50 meter × .2u ID × .11u film OV-1 and a 100 meter ×.2u ID × .15u film SP-2560 capillary column. The columns are calibrated with the following standards that are injected at the beginning of each set of samples; NIH-F fatty acid methyl ester (fame) mix plus added known concentrations of phytanic, pristanic, C27:0, and C26:0 and the Supelco 37 fame mix. We also mapped out the retention time of the methyl ester of 24:6w3 that was a generous gift from Dr. Howard Sprecher. The samples are first injected on the polar column, the SP-2560; then after the first analysis the sample vials are decapped, hexane added to the original 50μl volume, and recapped before analysis on the second, non-polar OV-1 column. The results are collected from the chemstation, the integration and calibration is checked, the calibrated results are then transferred to Excel and the results are compared and merged using Excel macros that were developed for the preparation of combined reports based on the identification and quantitation of the fatty acid methyl esters. Lipids of known fatty acid composition, including brain phosophatidyl ethanolamine for mapping the 16:0, 18:0, 18:1w9 and 18:1w7 plasmalogens, and reagent blanks are analyzed with each set of samples. The lipids were purchased from Matreya (Pleasant Gap, PA. USA). The fatty acid methyl ester mixes and columns were purchased from Supelco Inc, (Bellefonte, PA. USA)

Cell Viability assay

To determine cell viability, cells were plated at a density of 150,000 cells per well in 96 wells in standard medium one day before they were treated with specific media for the time indicated.

The WST-1 (Roche Diagnostics, Germany) assay kit was used to determine the metabolic activity of the cells according to manufacturer recommendations.

Results

Increased αS oligomerization in brains of mice with defective peroxisome biogenesis

To evaluate the involvement of peroxisomes in endogenous αS oligomerization and potential cytotoxicity in vivo, we used three different mouse models of peroxisome biogenesis disorders (PBD), i.e., Pex 2 (Faust and Hatten 1997), Pex 5 (Baes et al. 1997) and Pex 13 (Liu et al. 1999). αS oligomerization was tested in newborn mouse brains (p 0), as Pex mutations are lethal and mice die soon after birth. Whole brains were fractionated (see Methods), and the soluble fraction was probed for the appearance of soluble αS oligomers by western blotting with anti αS antibodies, H3C and Syn-1 with similar results, using heat delipidation for oligomer detection (see Methods). While levels of the αS monomer (~17 kDa) were not altered, enhanced levels of αS oligomers, including dimers (~35 kDa), trimers (~53 kDa) and higher species, were readily detected in the brains of the three Pex -/- mouse models compared to the control Pex +/+ littermates (Fig. 1a-d). Therefore, enhanced levels of endogenous αS oligomers occur in mouse brains modeling PBD.

Overall, αS immunoreactivity (including monomer and oligomers) appeared higher in Pex-/- than control Pex +/+ brains (Fig. 1 a-c), whereas, tubulin or actin levels on the same blots were not affected (Fig. 1a-c). We quantified the total immuno-reactivity in the different Pex -/- models and found that while monomer levels were only slightly affected, oligomers levels were ~4 fold significantly higher in the different Pex-/- brains compared with the corresponding control Pex +/+ littermates (n=4-7 brains, ttest $p<0.01$) (Fig. 1a-c). Increased oligomer levels may result from increased αS expression or increased protein stability and decreased clearance. To exclude the possible effect of peroxisome deficiencies on αS expression levels, we analyzed αS mRNA levels in Pex5-/- and Pex13-/- brains compared with their littermate controls (n=5 each). We did not detect a difference in mRNA levels by quantitative RT-PCR (data not shown) and therefore concluded that the effect on αS protein levels observed in brains of PBD mouse models is mediated at the levels of translational/post translational mechanisms.

Increased oligomerization and phosphorylation in Pex 5-/- fibroblasts expressing human αS

We next over expressed human αS in immortalized fibroblasts from Pex5-/- and control cells (Amery et al. 1998). Cells were transduced with an αS expressing viral vector and conditioned in parallel in standard serum containing medium for 16 hours. To compare transduction efficiency in the two cell types, we co- transduced a GFP viral vector together with the αS vector and found similar efficacies in the two cell types. Cells were then collected and fractionated, and the soluble fraction was probed for the appearance of soluble oligomers by western blotting with H3C antibody. The result with human αS in Pex 5-/-fibroblasts was very similar to the result obtained for endogenous αS in mouse brains with PBDs. A marked accumulation of αS oligomers, including dimers (~35 kDa), trimers (~53 kDa) and higher species, was readily detected in αS over expressing Pex 5-/- fibroblasts compared to the normal control Pex 5+/+ fibroblasts over expressing αS (Fig. 2). Quantifications of the western blot indicated ~2 fold increase in the ratio of dimer to monomer in the Pex5-/- cells. To examine αS phosphorylation at serine 129 in Pex 5-/- and control cells, we probed an identical blot with anti phospho-S129 αS ab. Higher levels of phosphorylated monomer αS and dimers were detected in the αS over expressing Pex5-/-compared with the control cells (Fig. 2). Specifically, ~ six fold increase in the densimetric ratio of phospho-dimer to phospho-monomer was detected in the Pex5-/- cells than in control cells. Therefore, in Pex5-/- cells, both oligomerization and phosphorylation of αS are induced.

Induced inclusion formation in αS over expressing Pex5-/- fibroblasts

We wished to assess the cytopathological effects of peroxisomal dysfunction on accumulation of αS pathogenic forms, but endogenous mouse αS does not accumulate into Lewy-like inclusions (Kahle et al. 2001). We therefore performed the following experiments in Pex5-/- and control cells stably expressing human αS. The cells were conditioned in standard serum-supplemented medium in parallel and processed for icc using anti-αS and anti-ubiquitin antibodies, two major constituents of the Lewy bodies, as previously done (Assayag et al. 2007). Overall, we detected higher αS signal in the form of discrete granules in the cytoplasm of Pex5-/- than in control cells. This signal was not washed out by pre-permeabilizing the cells (Assayag et al. 2007). Cytoplasmic inclusions that were immunoreactive to both αS and ubiquitin were consistently observed in αS over expressing Pex5-/- cells and only occasionally occurred in the αS over-expressing control cells conditioned in parallel (Fig. 3). Quantification of total αS or ubiquitin signal per cell revealed that the average signal per cell rose ~4-10-fold in the pex-/- clones.

αS alterations in PBD do not correlate with mitochondrial dysfunction

To test the potential role of mitochondrial dysfunction in the accumulation of αS in pathogenic forms in the PBD models, we used the Pex5-/- and control cells with and without stable αS over expression. Previously, it was shown that complex I is inhibited in livers but not in fibroblasts of Pex5-/- mice (Baumgart et al. 2001). In line with these results, we did not detect differences in activities of complex I, measured by NADH- coenzyme Q reductase and normalized to citrate synthase activity (Figure 4a) or complexes I + III measured by reduction of NADH-cytochrome c (reductions by isolated mitochondria) and normalized to citrate synthase (not shown) in naïve Pex5-/- and control fibroblasts. Moreover, complex I (Figure 4a) and I + III (not shown) activities were not affected upon αS over expression. Therefore, the results suggest that mitochondrial dysfunction can not explain αS accumulation in modified forms in the Pex5 PBD model.

αS abnormalities in PBD are not due to induced oxidative stress

Peroxisomes harbor several enzymes that have dual functions, i.e., generation and detoxification of reactive oxygen species (ROS); these include catalase, xanthine oxidase, superoxide dismutase and glutathione peroxidase (Lopez-Huertas et al. 2000; Schrader et al. 1999; Singh 1996). To determine whether oxidative stress is involved in the accumulation of modified forms of αS in the PBD models, we again examined the untransfected and αS-over-expressing Pex5-/- vs. control cells. Cells were maintained in standard serum-supplemented medium in parallel and treated with H_2O_2 at the low concentration of 10 μM for 16 hours. To evaluate the effect of αS expression on cell viability we used the WST1 (Roche Diagnostics, Germany) cytotoxicity assay, relying on the cleavage of tetrazolium salts to produce soluble formazan salts. The assay, therefore, determines the metabolic activity of the cells by quantifying the specific enzymatic activity tested. Surprisingly, expressing αS in both control and Pex5-/- cells rescued the H_2O_2 effects, with cell viability values statistically significant from the corresponding control without H_2O_2. Thus, a consistent increase of 15-20% in cell viability values was observed with αS over-expression (Figure 4b). Therefore, oxidative stress occurring in the PBD fibroblasts does not correlate with αS accumulation in pathogenic forms.

Abnormal FA content in Pex2-/- brains suggests the involvement of altered n-6 PUFA in αS abnormalities

Previous reports have documented altered lipid metabolism in the different PBD mouse models, including accumulation of very long chain fatty acids (VLCFA) and an increased ratio of C26/C22, accumulation of branched chain fatty acids and marked deficiency of plasmalogens (Faust and Hatten 1997; Faust et al. 2001) (Baes et al. 1997; Maxwell et al.

2003). Further, in line with the proposed role of peroxisomes for generation of docosahexaenoic acid (DHA, 22:6 n-3), reduced levels of DHA were previously documented in newborn and early postnatal Pex2-/- and Pex5-/- brains (Faust et al. 2001) (Janssen et al. 2000). Additional analyses of PUFAs in newborn Pex2-/- brains demonstrated a similar level of the DHA precursor α-linolenic acid (18:3 n-3) in control and Pex2-/- brains (0.359 ± 0.019 versus 0.334 ± 0.038, respectively; % of total fatty acids), but a reduced level of most n-3 PUFAs that are intermediates in DHA synthesis in Pex2-/- brains (Table 1). Only 24:6 n-3 was increased in Pex2-/- brain, which is consistent with the need for peroxisomal β-oxidation to retroconvert this penultimate intermediate for DHA production (Su et al. 2001). In contrast to reduced levels of several n-3 PUFA, Pex2-/- brains had elevated levels of linoleic (18:2 n-6) and arachidonic (20:4 n-6) acid, as previously described in human Zellweger brains (Martinez 1995), along with increases in several other n-6 PUFA that are intermediates in arachidonate synthesis (Table 1). These changes in PUFA levels resulted in a higher n-6:n-3 ration in the pex2-/- brains (1.1 and 1.6 for wt and Pex2-/-, respectively). However, the concomitant increases in several n-6 PUFA along with decreases in n-3 PUFA in Pex2 mutant brain led to a similar level of total PUFA species in control versus Pex2-/- brains (32.22 ± 0.67 versus 32.08 ± 0.82, respectively; % of total fatty acids). Pex2-/- brains also had significant elevations in both long chain (16:0 – 20:0) and very long chain (23:0 – 26:0) saturated fatty acids, with a characteristic elevation in the ratio of C26:0/C22:0, and a significant increase in the level of total saturated FA species (Table 1).

While most n-6 PUFA and saturated FAs are elevated in Pex2-/- brains, the levels for several mono unsaturated fatty acids (MUFA) were reduced, including the most abundant species oleic acid (18:1 n-9) and vaccenic acid (18:1 n-7) (Table 1). Only 26:1 n-9 was elevated in Pex2-/- brain, representing a minor very long chain MUFA species that is not detectable in control brains. The level of total MUFA species was significantly decreased in Pex2-/- versus control brains (Table 1).

Discussion

In this study we examined the effects of peroxisomal deficiency on αS–related abnormalities, including αS oligomerization and aggregation; αS phosphorylation; inclusion formation and its effects on cell viability. We documented enhanced αS accumulation in phosphorylated oligomers in mouse brains harboring non-functioning peroxisomes due to specific null mutations in either Pex2, Pex5 or Pex13 genes. We also detected enhanced deposition of human αS in cytoplasmic ubiquitinated inclusions in αS over expressing Pex5 -/- but not in control fibroblasts maintained in parallel under standard serum-supplemented conditioning medium. We show a correlation between accumulation of several long chain n-6 PUFAs and reduced levels of MUFAs with the accumulation of αS pathogenic forms in the Pex2-/- brains. However, the accumulation of αS in pathogenic forms was not accompanied with oxidative stress neither with mitochondrial respiratory chain complex I or I+III dysfunction in Pex5-/- PBD model. Our results suggest a role for peroxisomes in αS oligomerization and toxicity.

Recent results suggested that upon interaction with membrane phospholipids, αS tends to acquire an α-helix-rich structure (Davidson et al. 1998) and enrichment of phospholipids with PUFA induced αS oligomerization in vitro (Perrin et al. 2001). In a series of recent studies we have documented different aspects of the interaction of neuronal αS with FA -- in particular with PUFAs -- under normal and pathological conditions (Assayag et al. 2007; Sharon et al. 2003a; Sharon et al. 2003b; Sharon et al. 2001). We found that the accumulation of αS in soluble oligomers and insoluble aggregates, is associated with alterations in PUFA. We specifically identified longer chained and unsaturated FAs as factors that induce αS oligomerization, aggregation (Sharon et al. 2003a; Sharon et al. 2003b), phosphorylation and Lewy-like inclusion formation (Assayag et al. 2007). Further, we documented accumulations

in certain PUFAs upon αS over expression in dopaminergic cell (Sharon et al. 2003b). These previous studies were performed on PD models with altered αS expression, i.e., over expression in dopaminergic cells, null αS mouse brains and human brains with PD and the related synucleinopathies, Dementia with Lewy Bodies (DLB). In contrast to our previous studies, we have now used the reciprocal approach, i.e., we show αS abnormalities in vivo, in brains of mice with a lipid metabolic disorder. These PBD models do not involve any manipulation of their endogenous αS expression. These models have enabled us to determine an involvement of lipid metabolism in the process of αS accumulations in pathogenic forms.

Our previous analyses of FA profiles in human brains with PD and DLB indicated accumulations of certain PUFAs in the cytosols (Sharon et al. 2003b). Specifically, significantly higher levels of 22:4 n-6 and 22:6 n-3 were measured in the PD and DLB than in control brains. Similar FA analyses in naïve and αS over expressing dopaminergic cells indicated accumulations of both n-3 and n-6 PUFAs in cytosols and membrane fractions upon αS over expression. Analyses of FA profiles of size exclusion chromatography fractions containing αS of wt and the corresponding fractions of αS -/- mouse brains revealed the related reductions in PUFAs in the αS -/- fractions, and specifically, reduced levels of 18:3 and 22:6 n-3 PUFAs. These previous analyses did not indicate a specific role for n-6 rather than n-3 PUFAs in αS pathogenesis. Here, we show that the accumulation of αS phosphorylated oligomers and its deposition in cytoplasmic, ubiquitinated inclusions is correlated mainly with the accumulation of n-6 rather than n-3 PUFA in Pex2-/- brains. It is important to stress that n-3 PUFAs metabolism is specifically affected in the PBD models due to the role of peroxisomes in the biosynthesis of DHA (22:6 n-3). Indeed the level of DHA is reduced while the level of 24:6 n-3, the penultimate precursor in 22:6 biosynthesis, is elevated in the Pex2 -/- brains. Therefore, it is possible that the finding herein, suggesting a role for n-6 but not n-3 PUFA in αS pathogenesis is strongly influenced by this specific effect of peroxisomes on n-3 PUFA metabolism. While further studies are needed to determine the precise role of n-6 vs n-3 in αS pathogenesis, the results herein support our previous observations that alterations in PUFA levels are correlated with αS expression and PD.

While the role of peroxisomes in neonatal neurodegeneration has been widely studied (reviewed in (Crumrine 2001)), only limited information concerning the potential role of peroxisomes in age-related neurodegeneration can be found in the literature. For example, in relation to Alzheimer's disease, peroxisomal proliferation attenuated Aβ-dependent toxicity and protected hippocampal neurons from Aβ-induced degenerative changes (Santos et al. 2005). Nevertheless, growing evidence correlates αS abnormalities, PD and lipid metabolism. Abnormal accumulations of αS were recently reported in neurons and glia of adrenoleukodystrophy (ALD) patients (Suzuki et al. 2007). ALD is a peroxisomal disorder due to loss of the ABCD1 gene, a peroxisomal membrane transporter (Moser et al. 2007). Abnormal αS accumulations were also observed in brains of patients with lysosomal storage diseases such as Sandhoff disease, Tay-Sachs disease, metachromatic leukodystrophy and β-galactosialidosis (Suzuki et al. 2007). Importantly, homozygous mutations in the glucocerebrosidase gene causing the glycosphingolipid-storage disorder, Gaucher's disease, were recently shown to be linked to familial forms of PD in diverse populations (Aharon-Peretz et al. 2004). These reports collectively suggest that brain lipid metabolism may have a role in specific forms of PD. Considering the profound effects of peroxisomal deficiency on lipid metabolism in general and on brain lipid metabolism in particular, it is now suggested that the involvement of peroxisome dysfunction in PD is resulting from its effects on brain lipid metabolism.

Mitochondrial complex-I inhibitors such as rotenone, maneb and paraquat clearly lead to aggregation and accumulation of αS in vitro and in animal models (Betarbet et al. 2000) (Manning-Bog et al. 2002) (Norris et al. 2007). The absence of a correlation between enhanced

αS oligomerization and mitochondrial dysfunction reported herein may indicate that αS toxic effects on mitochondrial activities are more complex than previously estimated. For example, it was recently shown that αS contains a cryptic mitochondrial targeting sequence at its N terminus (Devi et al. 2008) and that mitochondrial accumulation of αS has a role in mitochondrial dysfunction and PD pathogenesis. In this regard, it will be interesting to find out whether αS oligomers are also present in the mitochondria.

Zellweger syndrome is a fatal disease, with life expectancy of about 6 month. The peroxisomal dysfunction in ZS and the related PBD involves severe neurological abnormalities related to neural development and neuronal migration (reviewed in (Faust et al. 2001; Wanders and Waterham 2005)). Here we used this severe metabolic disorder to model an age-related neurodegenerative disorder. The rational being that in sporadic, age-related neurodegenerative diseases, gradual accumulation of cellular insults may progressively affect cellular metabolic activities in the brain to the point where neurodegeneration may occur.

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References

Abou-Sleiman PM, Muqit MM, Wood NW. Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat Rev Neurosci 2006;7(3):207–219. [PubMed: 16495942]

Aharon-Peretz J, Rosenbaum H, Gershoni-Baruch R. Mutations in the glucocerebrosidase gene and Parkinson's disease in Ashkenazi Jews. N Engl J Med 2004;351(19):1972–1977. [PubMed: 15525722]

Altschuler Y, Barbas SM, Terlecky LJ, Tang K, Hardy S, Mostov KE, Schmid SL. Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. J Cell Biol 1998;143(7):1871–1881. [PubMed: 9864361]

Altschuler Y, Liu S, Katz L, Tang K, Hardy S, Brodsky F, Apodaca G, Mostov K. ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. J Cell Biol 1999;147 (1):7–12. [PubMed: 10508850]

Amery L, Brees C, Baes M, Setoyama C, Miura R, Mannaerts GP, Van Veldhoven PP. C-terminal tripeptide Ser-Asn-Leu (SNL) of human D-aspartate oxidase is a functional peroxisome-targeting signal. Biochem J 1998;336(Pt 2):367–371. [PubMed: 9820813]

Assayag K, Yakunin E, Loeb V, Selkoe DJ, Sharon R. Polyunsaturated Fatty Acids Induce {alpha}-Synuclein-Related Pathogenic Changes in Neuronal Cells. Am J Pathol 2007;171(6):2000–2011. [PubMed: 18055555]

Baes M, Gressens P, Baumgart E, Carmeliet P, Casteels M, Fransen M, Evrard P, Fahimi D, Declercq PE, Collen D, van Veldhoven PP, Mannaerts GP. A mouse model for Zellweger syndrome. Nat Genet 1997;17(1):49–57. [PubMed: 9288097]

Baes M, Van Veldhoven PP. Generalised and conditional inactivation of Pex genes in mice. Biochim Biophys Acta 2006;1763(12):1785–1793. [PubMed: 17007945]

Baumgart E, Vanhorebeek I, Grabenbauer M, Borgers M, Declercq PE, Fahimi HD, Baes M. Mitochondrial alterations caused by defective peroxisomal biogenesis in a mouse model for Zellweger syndrome (PEX5 knockout mouse). Am J Pathol 2001;159(4):1477–1494. [PubMed: 11583975]

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3(12):1301–1306. [PubMed: 11100151]

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254. [PubMed: 942051]

Crumrine PK. Degenerative disorders of the central nervous system. Pediatr Rev 2001;22(11):370–379. [PubMed: 11691947]

Davidson WS, Jonas A, Clayton DF, George JM. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. J Biol Chem 1998;273(16):9443–9449. [PubMed: 9545270]

Devi L, Raghavendran V, Prabhu BM, Avadhani NG, Anandatheerthavarada HK. Mitochondrial import and accumulation of alpha -synuclein impairs complex I in human dopaminergic neuronal cultures and Parkinson's disease brain. J Biol Chem. 2008

Dirkx R, Vanhorebeek I, Martens K, Schad A, Grabenbauer M, Fahimi D, Declercq P, Van Veldhoven PP, Baes M. Absence of peroxisomes in mouse hepatocytes causes mitochondrial and ER abnormalities. Hepatology 2005;41(4):868–878. [PubMed: 15732085]

Duda JE, Lee VM, Trojanowski JQ. Neuropathology of synuclein aggregates. J Neurosci Res 2000;61 (2):121–127. [PubMed: 10878583]

Faust PL, Hatten ME. Targeted deletion of the PEX2 peroxisome assembly gene in mice provides a model for Zellweger syndrome, a human neuronal migration disorder. J Cell Biol 1997;139(5):1293–1305. [PubMed: 9382874]

Faust PL, Su HM, Moser A, Moser HW. The peroxisome deficient PEX2 Zellweger mouse: pathologic and biochemical correlates of lipid dysfunction. J Mol Neurosci 2001;16(23):289–297. discussion 317-221. [PubMed: 11478384]

Folch J, Lees M, SS GH. A simple mehod for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509. [PubMed: 13428781]

Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 2002;4(2): 160–164. [PubMed: 11813001]

George JM, Jin H, Woods WS, Clayton DF. Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. Neuron 1995;15(2):361–372. [PubMed: 7646890]

Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, Lee VM. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. Science 2000;290(5493):985–989. [PubMed: 11062131]

Goedert M. Alpha-synuclein and neurodegenerative diseases. Nat Rev Neurosci 2001;2(7):492–501. [PubMed: 11433374]

Gould, SJ.; Raymond, GV.; Valle, D. The peroxisome biogenesis disorders. Scriv, CR.; Sly, WS.; Beaudet, AL.; Valle, D., editors. New York: McGraw-Hill; 2001. p. 3181-3217.

Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 2001;18(9):685–716. [PubMed: 11599635]

Hardy J, Cai H, Cookson MR, Gwinn-Hardy K, Singleton A. Genetics of Parkinson's disease and parkinsonism. Ann Neurol 2006;60(4):389–398. [PubMed: 17068789]

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. Construction of adenovirus vectors through Cre-lox recombination. J Virol 1997;71(3):1842–1849. [PubMed: 9032314]

Iwatsubo T, Yamaguchi H, Fujimuro M, Yokosawa H, Ihara Y, Trojanowski JQ, Lee VM. Purification and characterization of Lewy bodies from the brains of patients with diffuse Lewy body disease. Am J Pathol 1996;148(5):1517–1529. [PubMed: 8623921]

Janssen A, Baes M, Gressens P, Mannaerts GP, Declercq P, Van Veldhoven PP. Docosahexaenoic acid deficit is not a major pathogenic factor in peroxisome-deficient mice. Lab Invest 2000;80(1):31–35. [PubMed: 10653000]

Kahle PJ, Neumann M, Ozmen L, Muller V, Odoy S, Okamoto N, Jacobsen H, Iwatsubo T, Trojanowski JQ, Takahashi H, Wakabayashi K, Bogdanovic N, Riederer P, Kretzschmar HA, Haass C. Selective insolubility of alpha-synuclein in human Lewy body diseases is recapitulated in a transgenic mouse model. Am J Pathol 2001;159(6):2215–2225. [PubMed: 11733371]

Keane MH, Overmars H, Wikander TM, Ferdinandusse S, Duran M, Wanders RJ, Faust PL. Bile acid treatment alters hepatic disease and bile acid transport in peroxisome-deficient PEX2 Zellweger mice. Hepatology 2007;45(4):982–997. [PubMed: 17393522]

Lee VM, Trojanowski JQ. Mechanisms of Parkinson's disease linked to pathological alpha-synuclein: new targets for drug discovery. Neuron 2006;52(1):33–38. [PubMed: 17015225]

Liu Y, Bjorkman J, Urquhart A, Wanders RJ, Crane DI, Gould SJ. PEX13 is mutated in complementation group 13 of the peroxisome-biogenesis disorders. Am J Hum Genet 1999;65(3):621–634. [PubMed: 10441568]

Lopez-Huertas E, Charlton WL, Johnson B, Graham IA, Baker A. Stress induces peroxisome biogenesis genes. Embo J 2000;19(24):6770–6777. [PubMed: 11118212]

Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem 2002;277(3):1641–1644. [PubMed: 11707429]

Martinez M. Polyunsaturated fatty acids in the developing human brain, erythrocytes and plasma in peroxisomal disease: therapeutic implications. J Inherit Metab Dis 1995;18(Suppl 1):61–75. [PubMed: 9053556]

Maxwell M, Bjorkman J, Nguyen T, Sharp P, Finnie J, Paterson C, Tonks I, Paton BC, Kay GF, Crane DI. Pex13 inactivation in the mouse disrupts peroxisome biogenesis and leads to a Zellweger syndrome phenotype. Mol Cell Biol 2003;23(16):5947–5957. [PubMed: 12897163]

Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of Parkinson's disease. Ann Rev Neurosci 2005;28:57–87. [PubMed: 16022590]

Moser HW, Mahmood A, Raymond GV. X-linked adrenoleukodystrophy. Nat Clin Pract Neurol 2007;3 (3):140–151. [PubMed: 17342190]

Norris EH, Uryu K, Leight S, Giasson BI, Trojanowski JQ, Lee VM. Pesticide exposure exacerbates alpha-synucleinopathy in an A53T transgenic mouse model. Am J Pathol 2007;170(2):658–666. [PubMed: 17255333]

Perrin RJ, Woods WS, Clayton DF, George JM. Exposure to long chain polyunsaturated fatty acids triggers rapid multimerization of synucleins. J Biol Chem 2001;276(45):41958–41962. [PubMed: 11553616]

Poulos A, Sharp P, Johnson D. Plasma polyenoic very-long-chain fatty acids in peroxisomal disease: biochemical discrimination of Zellweger's syndrome from other phenotypes. Neurology 1989;39(1): 44–47. [PubMed: 2462697]

Poulos A, Sharp P, Johnson D, Easton C. The occurrence of polyenoic very long chain fatty acids with greater than 32 carbon atoms in molecular species of phosphatidylcholine in normal and peroxisome-deficient (Zellweger's syndrome) brain. Biochem J 1988;253(3):645–650. [PubMed: 2845926]

Robinson BS, Johnson DW, Poulos A. Metabolism of hexacosatetraenoic acid (C26:4,n-6) in immature rat brain. Biochem J 1990;267(2):561–564. [PubMed: 2185745]

Saada A, Bar-Meir M, Belaiche C, Miller C, Elpeleg O. Evaluation of enzymatic assays and compounds affecting ATP production in mitochondrial respiratory chain complex I deficiency. Anal Biochem 2004;335(1):66–72. [PubMed: 15519572]

Santos MJ, Quintanilla RA, Toro A, Grandy R, Dinamarca MC, Godoy JA, Inestrosa NC. Peroxisomal proliferation protects from beta-amyloid neurodegeneration. J Biol Chem 2005;280(49):41057–41068. [PubMed: 16204253]

Scherzer CR, Feany MB. Yeast genetics targets lipids in Parkinson's disease. Trends Genet 2004;20(7): 273–277. [PubMed: 15219388]

Schrader M, Wodopia R, Fahimi HD. Induction of tubular peroxisomes by UV irradiation and reactive oxygen species in HepG2 cells. J Histochem Cytochem 1999;47(9):1141–1148. [PubMed: 10449535]

Sharon R, Bar-Joseph I, Frosch MP, Walsh DM, Hamilton JA, Selkoe DJ. The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. Neuron 2003a;37(4):583–595. [PubMed: 12597857]

Sharon R, Bar-Joseph I, Mirick GE, Serhan CN, Selkoe DJ. Altered fatty acid composition of dopaminergic neurons expressing alpha-synuclein and human brains with alpha-synucleinopathies. J Biol Chem 2003b;278(50):49874–49881. [PubMed: 14507911]

Sharon R, Goldberg MS, Bar-Josef I, Betensky RA, Shen J, Selkoe DJ. alpha-Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins. Proc Natl Acad Sci U S A 2001;98(16):9110–9115. [PubMed: 11481478]

Sharp P, Poulos A, Fellenberg A, Johnson D. Structure and lipid distribution of polyenoic very-long-chain fatty acids in the brain of peroxisome-deficient patients (Zellweger syndrome). Biochem J 1987;248(1):61–67. [PubMed: 3435449]

Singh I. Mammalian peroxisomes: metabolism of oxygen and reactive oxygen species. Ann N Y Acad Sci 1996;804:612–627. [PubMed: 8993576]

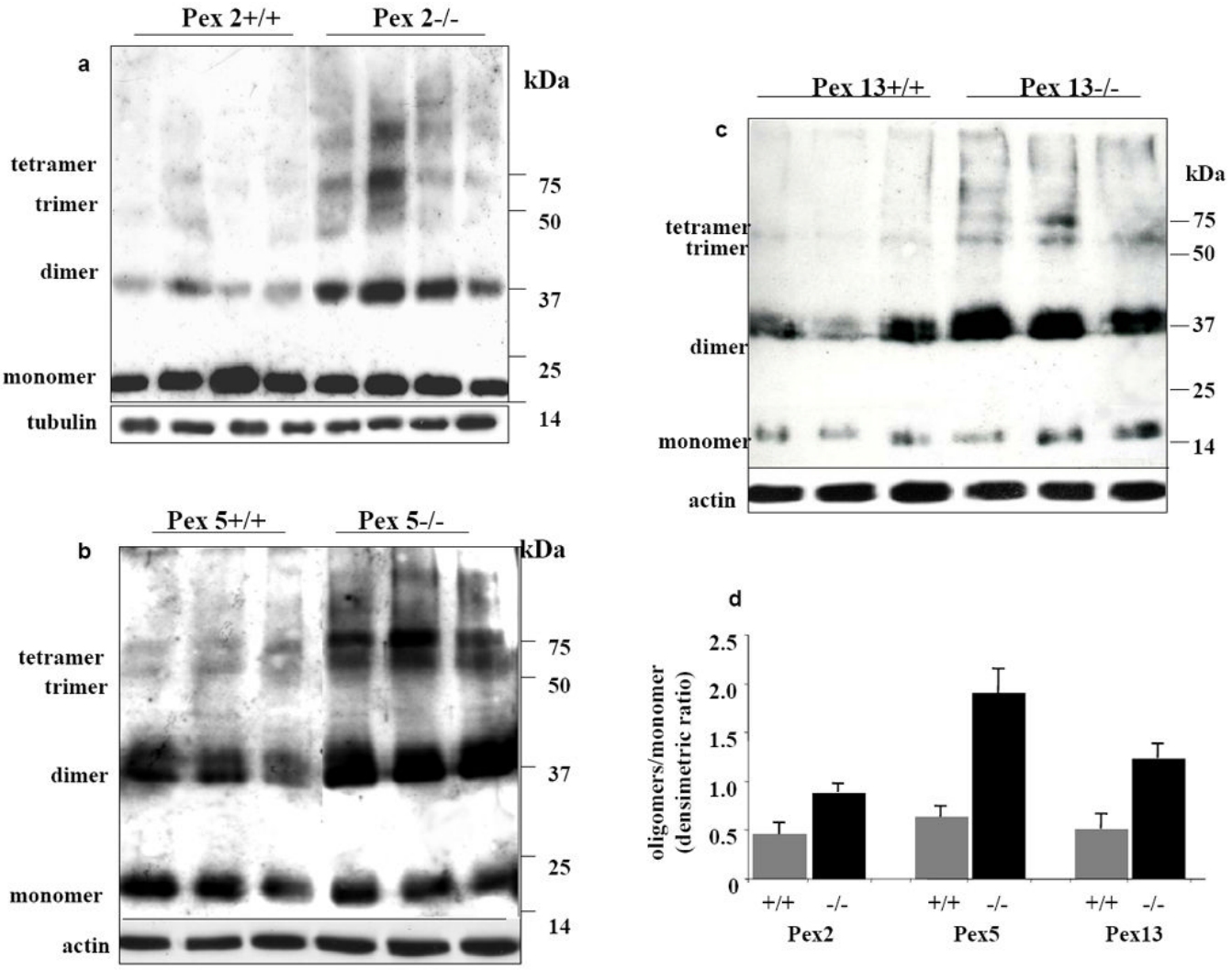
Smith WW, Margolis RL, Li X, Troncoso JC, Lee MK, Dawson VL, Dawson TM, Iwatsubo T, Ross CA. Alpha-synuclein phosphorylation enhances eosinophilic cytoplasmic inclusion formation in SH-SY5Y cells. J Neurosci 2005;25(23):5544–5552. [PubMed: 15944382]

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature 1997;388(6645):839–840. [PubMed: 9278044]

Su HM, Moser AB, Moser HW, Watkins PA. Peroxisomal straight-chain Acyl-CoA oxidase and D-bifunctional protein are essential for the retroconversion step in docosahexaenoic acid synthesis. J Biol Chem 2001;276(41):38115–38120. [PubMed: 11500517]

Suzuki K, Iseki E, Togo T, Yamaguchi A, Katsuse O, Katsuyama K, Kanzaki S, Shiozaki K, Kawanishi C, Yamashita S, Tanaka Y, Yamanaka S, Hirayasu Y. Neuronal and glial accumulation of alpha- and beta-synucleins in human lipidoses. Acta Neuropathol 2007;114(5):481–489. [PubMed: 17653558]

Wanders RJ, Waterham HR. Peroxisomal disorders I: biochemistry and genetics of peroxisome biogenesis disorders. Clin Genet 2005;67(2):107–133. [PubMed: 15679822]



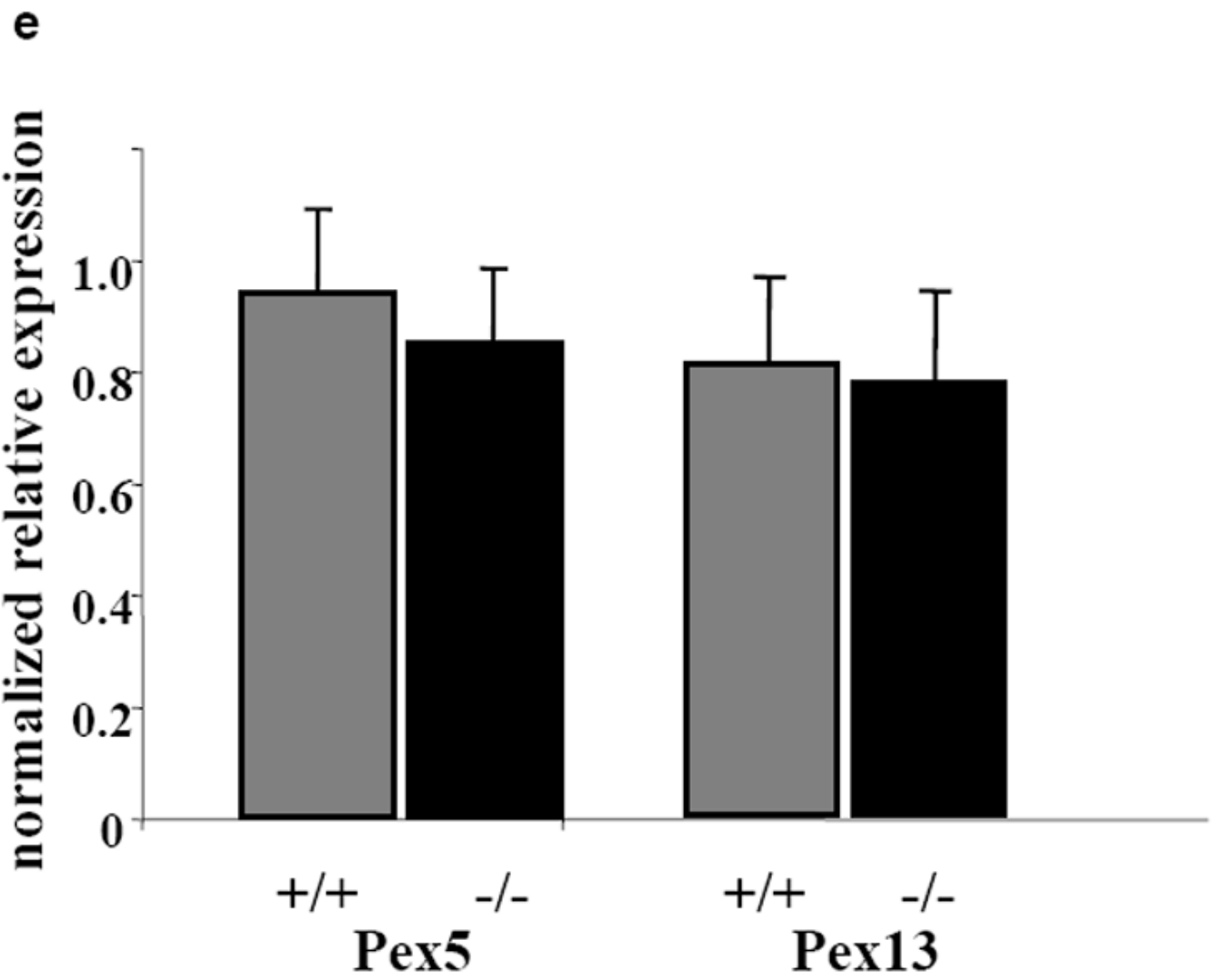


Figure 1. Induced accumulation of αS oligomers in vivo in peroxisome Biogenesis Disease mouse models

Samples of high-speed cytosol, post 100,000g (15 μg protein) extracted from whole newborn mouse brain. Samples were treated at 65°C overnight prior to gel loading and blotting with anti αS antibody.

Each lane represents a different brain processed in parallel. Representative immunoblots for a) Pex2-/- and control newborns; b) Pex5-/- and control newborns and c) Pex13-/- and control newborns. For each mouse model we tested four-seven individual brains with highly similar results. d) densimetric ratio of oligomers to monomer for each mouse model (mean ± SE, n=4-7 mice).

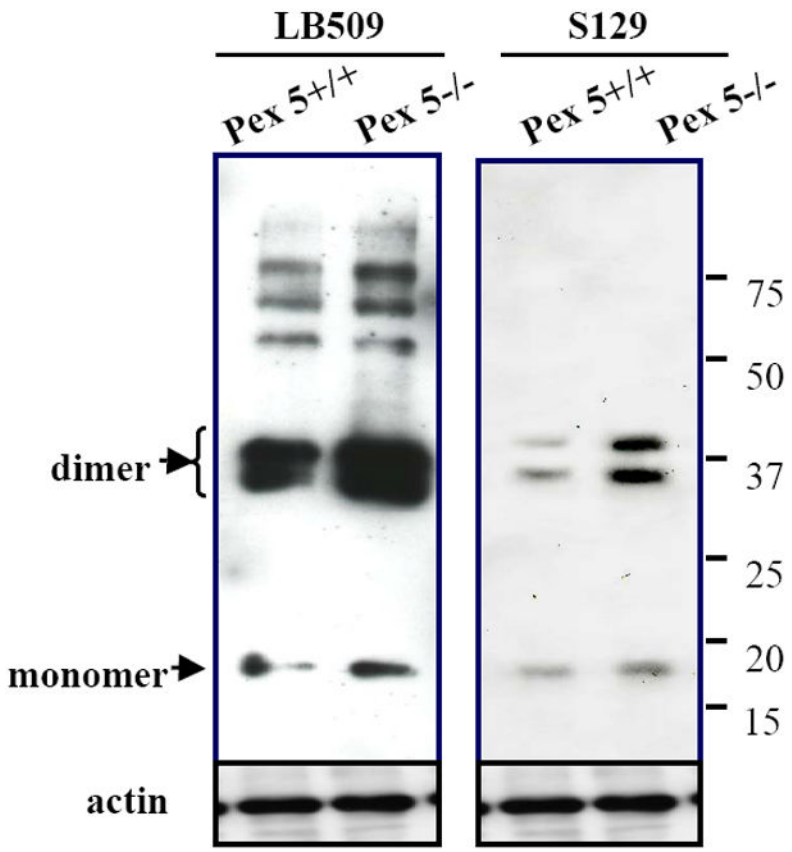


Figure 2. Induced oligomerization and phosphorylation of human-αS in Pex5-/- cells

High-speed cytosols (15 μg protein) of human wt αS-transduced Pex5-/- and control cells conditioned in standard serum-supplemented medium for 48 hours post trasduction. Samples were treated at 65°C overnight prior to gel loading and blotting with anti αS antibody LB509 or anti-phospho Ser129 αS antibody. Blot reacted with anti actin for loading control.

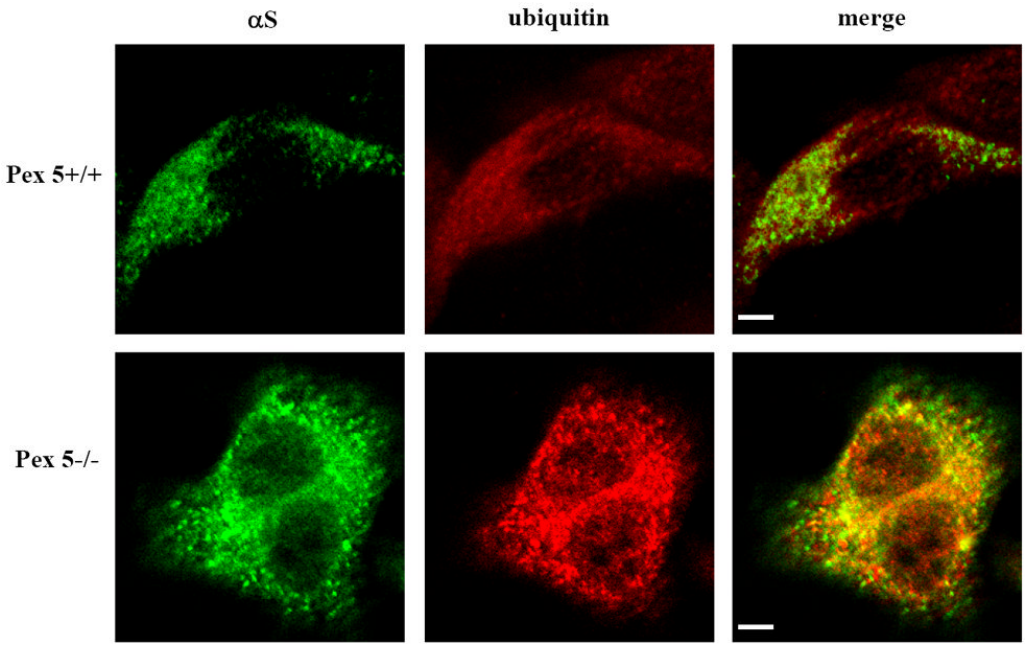


Figure 3. Induced Lewy-like inclusion formation in αS over expressing Pex5-/- fibroblasts

αS stably-transfected Pex5-/- and control cells were maintained in culture under standard serum conditions. Cells were pre-permeabilized with 0.002% Triton X-100 and then processed for icc using antibodies against αS (LB509) and ubiquitin (Dako), followed by Alexa 488 and Cy5, respectively. Bars represents 10 μm.

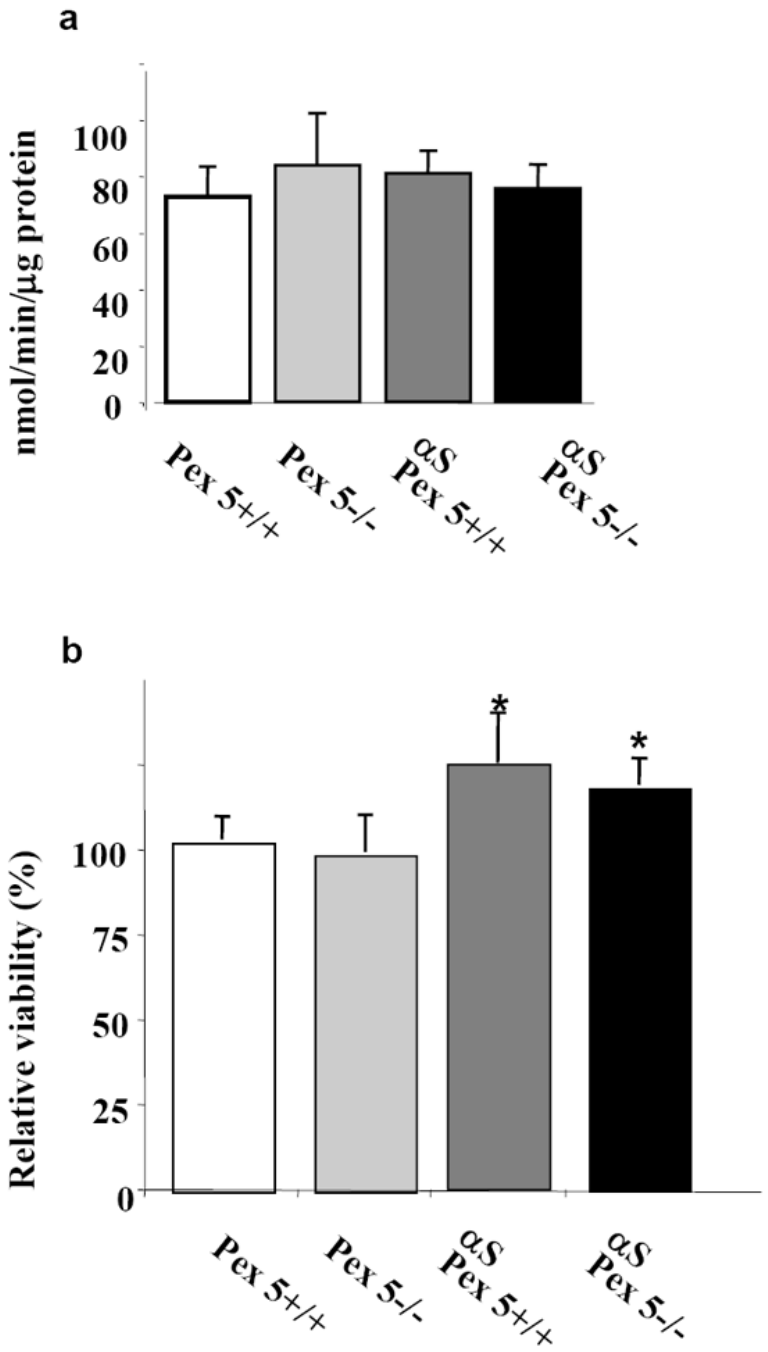


Figure 4. αS stable over expression did not inhibit mitochondrial complex I activity or induce oxidative stress in Pex5-/- fibroblasts

a) Mitochondrial complex I enzymatic activity was measured in isolated mitochondria of Pex5-/- and control cells, with and without stable overexpression of αS, and normalized to the activity of citrate synthase, a mitochondrial matrix enzyme, in the same sample. Mean ± SE of 2-3 mitochondrial preparations of each clone (2-3 different αS stable clones). b) Cell viability was measured in Pex5-/- and control cells without and with αS stable-overexpression in response to the addition of H_2O_2 (10 μM for 16 hours) to the conditioning medium. Results are presented as percent of viability of Pex5+/+ control cells (normalized to 100%). Mean ± SE 2-3 different αS stable clones.

Table 1

The relative amounts of SFAs, MUFAs and PUFAs in Control and Pex2-/- newborn mouse brains.

Elevated levels			**Reduced levels**		
FA	**Control**	**Pex2-/-**	**FA**	**Control**	**Pex2-/-**
16:0	24.87 ± 0.25	28.77 ± 1.07**	C14:0	0.993 ± 0.063	0.775 ± 0.058**
17:0	0.090 ± 0.12	0.104 ± 0.009*			
18:0	16.34 ± 0.12	18.81 ± 0.23**			
20:0	0.079 ± 0.05	0.131 ± 0.010**			
23:0	0.296 ± 0.20	0.849 ± 0.086**			
24:0	0.253 ± 0.15	0.302 ± 0.039**			
25:0	0.016 ± 0.09	0.031 ± 0.011*			
26:0	0.002 ± 0.00	0.031 ± 0.009**			
C26/C22	0.025 ± 0.03	0.363 ± 0.122**			
Total Sat.	**43.10 ± 0.33**	**49.96 ± 1.12****			
26:1n-9	0.000 ± 0.000	0.018 ± 0.009**	16:1n-9	1.19 ± 0.08	0.97 ± 0.14
			18:1n-9	13.07 ± 0.37	11.17 ± 0.91**
			18:1n-7	3.41 ± 0.08	2.63 ± 0.25**
Total MUFA	**20.61 ± 0.49**	**17.84 ± 1.50****			
18:2 n-6	1.070 ± 0.250	1.845 ± 0.173**	**20:3W9**	**0.51±0.04**	**0.16±0.04****
18:3 n-6	0.088 ± 0.025	0.114 ± 0.017*	22:4 n-6	2.70 ± 0.25	1.88 ± 0.31**
20:2 n-6	0.232 ± 0.086	0.446 ± 0.029**	22:5 n-6	0.780 ± 0.052	0.016 ± 0.046**
20:3 n-6	0.403 ± 0.055	0.715 ± 0.083**	18:4 n-3	0.136 ± 0.009	0.039 ± 0.007**
24:2 n-6	0.045 ± 0.005	0.064 ± 0.010**	20:5 n-3	0.111 ± 0.023	0.053 ± 0.023**
20:4 n-6	11.49 ± 0.21	14.06 ± 0.48**	22:5 n-3	0.402 ± 0.070	0.197 ± 0.045**
24:6 n-3	0.269 ± 0.025	0.811 ± 0.097**	22:6 n-3	13.56 ± 0.44	11.15 ± 0.29**
Total PUFA	**32.18±1.57**	**32.05±1.76**			

Mean ± SD of n=6 and 7 for controls and Pex2-/- respectively

* $P < 0.05$,

** $P < 0.001$, Student's t-Test