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Susceptibility to cellular stress in PS1 mutant N2a cells is associated with mitochondrial defects and altered calcium homeostasis

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Abkürzungsverzeichnis

ΔΨm	mitochondrial membrane potential
2-APB	2-Aminoethoxydiphenylborane
Aβ	Amyloid beta
AβO	Amyloid beta oligomers
AD	Alzheimer's Disease
AEQs	Targeted aequorins
ANOVA	Analysis of variance
Ant	Antimycin
APP	Amyloid Precursor Protein and
BiP	Binding immunoglobulin protein
Cal	Calcimycin
CHOP	CCAAT-enhancer-binding protein homologous protein
CypD	Cyclophilin D
DAPT	N-[N(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
eIF2α	eukaryotic translation initiation factor 2α
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ER	endoplasmic reticulum
FAD	familial Alzheimer's Disease
FBS	Fetal Bovine Serum
GADD34	Growth arrest and DNA damage-inducible protein

GFP	Green Fluorescent Protein
HFIP	1,1,1,3,3,3-Hexafluoro-2-Propanol
hPS1	human Presenilin 1
LAMP1	Lysosome-associated membrane protein
LC3	Microtubule-associated protein 1A/1B-light chain 3
MEFs	Mouse Embryonic Fibroblasts
MPTP	Mitochondrial Permeability Transition Pore
MTCH1	Mitochondrial carrier homolog 1
N2a	Neuroblastoma cell line
PC12	rat pheochromocytoma cells
PDI	Protein disulfide isomerase
pRIPK3	phosphorylated Receptor-interacting protein kinase 3
PS1	Presenilin 1
PS1E280A	Mutant Presenilin 1 E280A
PS1Δ9	Mutant Presenilin 1 delta 9
PS2	Presenilin 2
PS1KD	Presenilin 1 knock-down
PS KO−/−	Presenilin double knock-out
qPCR	quantitative polymerase chain reaction
RFP	Red Fluorescent Protein
SEM	standard deviation of the mean
SHSY5Y	human neuroblastoma cell line
SynC	ATP synthase C
siRNA	small interfering ribonucleic acid
TMRM	tetramethyl rhodamine Methylester
Tun	Tunicamycin

UPR	unfolded protein response
WT	Wild-type
XBP1	X-box binding protein 1

1. Artikel

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Susceptibility to cellular stress in PS1 mutant N2a cells is associated with mitochondrial defects and altered calcium homeostasis

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Presenilin 1 (PS1) mutations are the most common cause of familial Alzheimer's disease (FAD). PS1 also plays a role in cellular processes such as calcium homeostasis and autophagy. We hypothesized that mutant presenilins increase cellular vulnerability to stress. We stably expressed human PS1, mutant PS1E280A and mutant PS1ΔE9 in mouse neuroblastoma N2a cells. We examined early signs of stress in different conditions: endoplasmic reticulum (ER) stress, calcium overload, oxidative stress, and Aβ 1–42 oligomers toxicity. Additionally, we induced autophagy via serum starvation. PS1 mutations did not have an effect in ER stress but PS1E280A mutation affected autophagy. PS1 overexpression influenced calcium homeostasis and generated mitochondrial calcium overload modifying mitochondrial function. However, the opening of the mitochondrial permeability transition pore (MPTP) was affected in PS1 mutants, being accelerated in PS1E280A and inhibited in PS1ΔE9 cells. Altered autophagy in PS1E280A cells was neither modified by inhibition of γ-secretase, nor by ER calcium retention. MPTP opening was directly regulated by γ-secretase inhibitors independent on organelle calcium modulation, suggesting a novel direct role for PS1 and γ-secretase in mitochondrial stress. We identified intrinsic cellular vulnerability to stress in PS1 mutants associated simultaneously with both, autophagic and mitochondrial function, independent of Aβ pathology.

Alzheimer Disease (AD) is the most common form of dementia, mainly attributed to altered processing and deposition of extracellular Aβ plaques and intracellular neurofibrillary tangles in the brain[1]. Current understanding of AD pathophysiology indicates impairment of several cellular processes such as lipid metabolism, mitochondrial function and autophagy, leading eventually to cellular stress and death. A multifactorial model for AD proposes a cellular phase in which Amyloid beta (Aβ) pathology drives Tau hyperphosphorylation inducing cellular damage[2].

Amyloid Precursor Protein (APP), Presenilin 1 (PS1) and Presenilin 2 (PS2) autosomal dominant mutations are causative of familial AD (FAD)[3]. FAD is characterized by its severity and earlier disease onset, together with severe brain atrophy indicating increased neuronal death[4]. Presenilins are the catalytic component of the γ-secretase complex, playing a role in Aβ generation. The pathological severity of FAD suggests a direct neurodegenerative role of PS1 mutations, whether by increased production of toxic Aβ or by other mechanisms[5]. Nevertheless, PS1 has also been related to other cellular functions, such as protein trafficking, Wnt/β-catenin signaling, apoptosis and the disruption of calcium homeostasis[6–8]. Accordingly, PS1 mutations have been associated to increased cellular stress or death responses such as endoplasmic reticulum (ER) stress[9], oxidative stress[10,11], autophagy[12], and apoptosis[13]. Abnormal calcium homeostasis and its pathological role (calcium overload) in AD have attracted attention during recent years. Calcium signaling is involved in different pathways, being essential for synaptic mechanisms, protein folding processes, cell survival and death, among many others[14]. Regarding

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FAD, PS1 and PS2 have been associated with altered calcium signaling and PS1 has been found to affect calcium dynamics in lysosomes and ER[8,14]. Those changes in neuronal and synaptic calcium could lead to synaptic and neuronal toxicity[14,15].

There is a large population carrying a single PS1 mutation, E280A, with ~6,000 individuals and ~600 affected carriers[16]. The PS1E280A mutation is localized in exon 8 of the PSEN1 gene and substitutes a glutamic acid for an alanine in the loop region of PS1[16]. It affects APP processing and Aβ generation[17]. Also, this mutation may affect the processing of other γ-secretase substrates[18,19]. On the other hand, mitochondrial dysfunction has been identified as one key process in AD pathophysiology. Altered processes include oxidative stress, mitochondrial dynamics and calcium dysregulation[20–22]. Our previous studies in brain tissue of PS1 E280A FAD patients and cellular models for PS1E280A mutation showed altered mitochondrial function and evidence of altered calcium homeostasis, associated with increased Purkinje cells loss and cerebellar damage[23]. These findings can indicate an increased cellular vulnerability to stress in PS1 mutant cells, induced by these mechanisms. Finally, it has been suggested that mitochondrial calcium dysregulation in AD can result from abnormal aperture of the mitochondrial permeability transition pore (MPTP) due to a modulatory effect of Aβ[24]. In the present study, we analyzed different cellular stress pathways in N2a cells overexpressing wild type human PS1 (hPS1WT), PS1 E280A (hPS1E280A) and PS1 exon 9 deletion (hPS1Δ9) mutations. We found that although PS1 overexpression has an impact in mitochondrial function, only PS1 mutations confer further vulnerability to autophagy and mitochondrial stress responses on a mutation-specific manner seemingly by γ-secretase dependent and independent mechanisms, including direct MPTP regulation.

Results

Establishment of a cellular stress model in N2a cells. First, we confirmed hPS1 overexpression in transfected cells using western blot and qPCR (Fig. S1A–C). We checked functional effects of hPS1 overexpression, showing increased APP production without modifying Aβ 1–40 or Aβ 1–42 production (Fig. S1D,E). As expected, overexpression of hPS1E280A increased Aβ 1–40 or Aβ 1–42 levels (Fig. S1E). Finally, we observed that hPS1 overexpression did not modify significantly the subcellular distribution of PS1 (Fig. S2A,B). For our cellular stress model, we selected tunicamycin as an ER stress inducer and serum starvation as an autophagy inducer. Given PS1 role in calcium homeostasis, we treated cells with calcimycin to see the effect of calcium overload in ER stress and autophagy. Also, we evaluated antimycin as a mitochondrial stressor given possible mitochondrial stress crosstalk with ER stress and autophagy[25]. Finally, we assessed the effect of Aβ 1–42 (Aβ 1–42) as a positive control for putative mechanisms of stress in AD. To define the optimal time and dosage for each treatment, we incubated wild type N2a cells with each stressor or in the absence of FBS (for serum starvation) and extracted total protein after 0 h, 8 h, 16 h and 24 h. After 8 h of treatment we could identify that tunicamycin, calcimycin and antimycin already elicited ER stress in wild type N2a cells as evidenced by the induction of BiP, CHOP and GADD34. ER stress remained at 16 h and 24 h. Also, at 16 h some degree of LC3B-II conversion was seen with all treatments, with serum starvation depicting more (Fig. 1a). At 24 h, starvation showed clear LC3B-II conversion and some crosstalk between pathways became evident with tunicamycin leading to higher LC3B-II conversion. Additionally, N2a cells can start to differentiate under FBS starvation conditions at 24 hours. Consequently, we selected 16 h as the optimal time for inducing ER stress and autophagy in our cellular model.

PS1 mutations do not modify ER stress in N2a cells. We aimed to assess if PS1E280A mutation affects mechanisms of cellular stress, with ER stress and autophagy as the first targets given that alterations in both processes have previously been associated with PS1 mutations[9,12]. As expected, tunicamycin induced markers of ER stress (BiP, CHOP and GADD34) in mock, hPS1WT and hPS1E280A cells. However, in hPS1Δ9 cells only CHOP was reproducibly induced by tunicamycin (Fig. 1b,c). Previously, varied, sometimes contradictory effects of PS1 on ER stress signaling have been published using diverse markers. Reported findings are inconsistent regarding whether ER stress response is enhanced or dysfunctional due to mutations in PS1 (for references see Supplementary Table 1). This might reflect the use of differing cellular models or strategies involving modified PS1. To shed light on this, we silenced murine PS1 expression in N2a cells (PS1KD) using siRNA targeting exon 3 of the PSEN1 gene and treated them with tunicamycin to generate ER stress on a timeline up to 16 hours. Although transfection of murine PS1 and control siRNA could have affected basal levels of GADD34 and BiP, tunicamycin treatment elicited increased levels of BiP after 8 h, and of GADD34 after 8 h and 16 h of treatment in both groups without visible differences between PS1KD or control cells (Fig. S3A,B). Additionally, we evaluated whether the total absence of Presenilins affected the induction of ER stress. For that we used MEFs generated from Presenilin double knock out mice (PS KO−/−)[26]. By western blot, we observed some differences in the activation of BIP and GADD34. PS KO−/− cells showed a slight delayed activation of both proteins (Fig. S3C). Next, we evaluated ER stress markers XBP1 and CHOP by RT-PCR. Only CHOP showed some difference with increased levels by tunicamycin treatment after 8 hours in PS KO−/− cells (Fig. S3D). Finally, we evaluated GADD34, BiP, and phosphorylated eIF2α steady state levels with western blot in WT cells and PS1 KO−/− cells, with and without stable rescue for hPS1WT and hPS1E280A (Fig. S3E). We found no statistically significant differences within cell lines for ER stress markers (Fig. S3F). Taken together, these results indicate that in N2a and MEF cells, PS1 mutations or downregulation do not affect ER stress responses.

Autophagic system impairment in hPS1 E280A N2a cells. To assess the autophagic response in PS1 overexpressing N2a cells, we measured first steady state levels of LC3B-I and its conjugate, LC3B-II which serves as an indirect measurement of autophagic activity[27]. LC3B-II/I ratio was increased in hPS1E280 cells after tunicamycin, calcimycin and serum starvation treatments, whereas hPS1Δ9 cells showed no statistically significant differences (Figs. 2a,b and S4A). Increased levels of LC3B-II in hPS1E280A might reflect increased autophagic activity or impaired autophagic flux. Therefore, we evaluated the autophagic system in our cell lines in basal

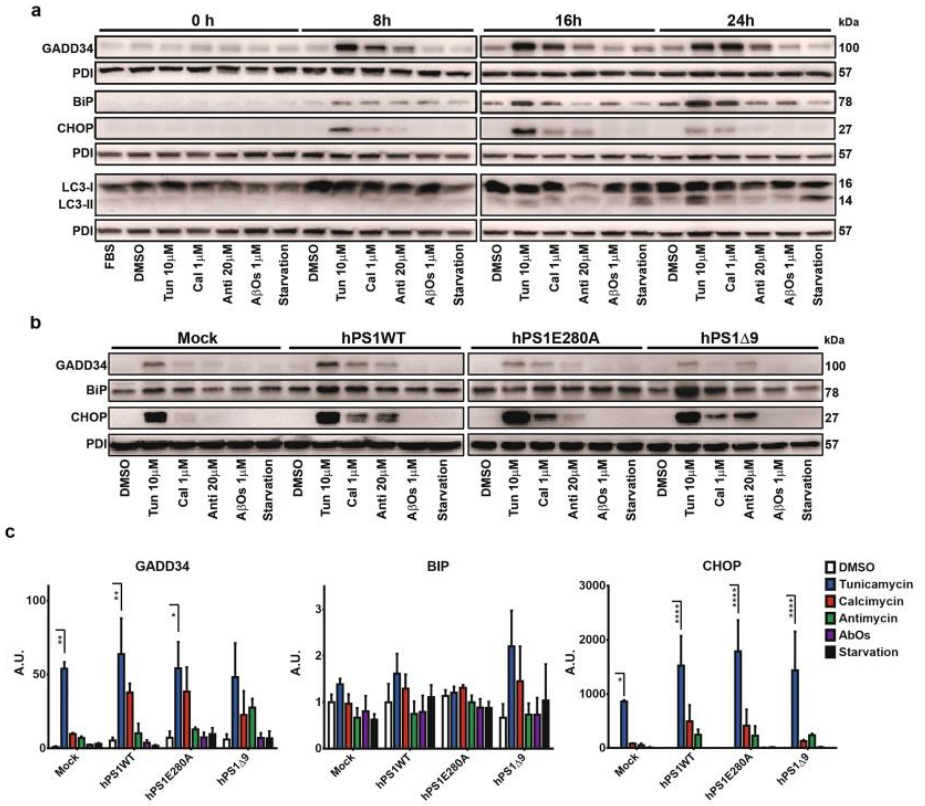

Figure 1. PS1 mutations do not affect ER stress response. (**a**) N2a mock transfected cells were treated with FBS, DMSO, tunicamycin 10 μM, calcimycin 1 μM, antimicyn 20 μM, Aβ 1–42 oligomers (AβOs) 1 μM, and serum starvation during 8, 16, and 24 h. Representative western blots for ER stress and autophagy markers are depicted. PDI was used as loading control. (**b**) N2a mock and PS1 stably transfected cells were treated with FBS, DMSO, tunicamycin 10 μM, calcimycin 1 μM, antimicyn 20 μM, Aβ 1–42 oligomers (AβOs) 1 μM, and serum starvation during 16 h. Representative western blots of protein steady state levels of GADD34, BiP, and CHOP in N2a cells overexpressing hPS1, hPS1-E280A or hPS1Δ9 are depicted. (**c**) Densitometric analysis of GADD34, BiP, and CHOP steady state levels. Mean ± SEM is presented for all experiments, Two-Way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $n = 3$.

conditions, treated with positive stressors (calcimycin and serum starvation) or with calcimycin vehicle (DMSO) as its control; to finally transfect them transiently with a LC3B-GFP-RFP tandem construct. LC3B-RFP signal is more resistant to low pH, therefore the transition from the autophagosome to the autolysosome is reflected by the specific loss of GFP fluorescence upon acidification of the autophagolysosome (Figs. 2c and S4B). After 16 h of calcimycin and serum starvation, there were no significant changes in the autophagic turnover in mock, hPS1WT, and hPS1Δ9 cells. Only hPS1E280A cells showed significant differences between treatments with serum starvation by showing a lower proportion of red puncta when compared to basal conditions represented by serum presence or vehicle application (Figs. 2d and S5A). This difference was not reflected by the total number of puncta when normalized against puncta number in mock cells (Fig. 2d). It is possible that fewer red puncta indicates an alteration of autophagic flux, with the autophagosome not fusing successfully with the lysosome for degradation. To test this, we analyzed LC3B and LAMP1 co-localization as a marker of autophagolysosome fusion. There were no significant differences between un-starved or starved hPS1E280A N2a cells regarding LC3B-LAMP1 co-localization (Fig. S5B), suggesting that observed changes in red puncta numbers are due to pH modulation in those compartments rather than to autophagic flux deficiency. Finally, ultrastructural analysis of mock, hPS1WT and hPS1E280A overexpressing cells indicated that serum starvation lead to autophagic vacuoles formation in all cells but that only overexpression of hPS1E280A lead to increased lysosomal degradation of mitochondria under starvation and the presence of abnormal mitochondria (Fig. 2e,f). Previously, a role for PS1 in autolysosome acidification was reported, and defective autophagy was identified in PS1 mutants[28]. Our findings confirm this effect in hPS1E280A but not in hPS1Δ9 cells hinting towards a γ-secretase independent role regarding autophagy.

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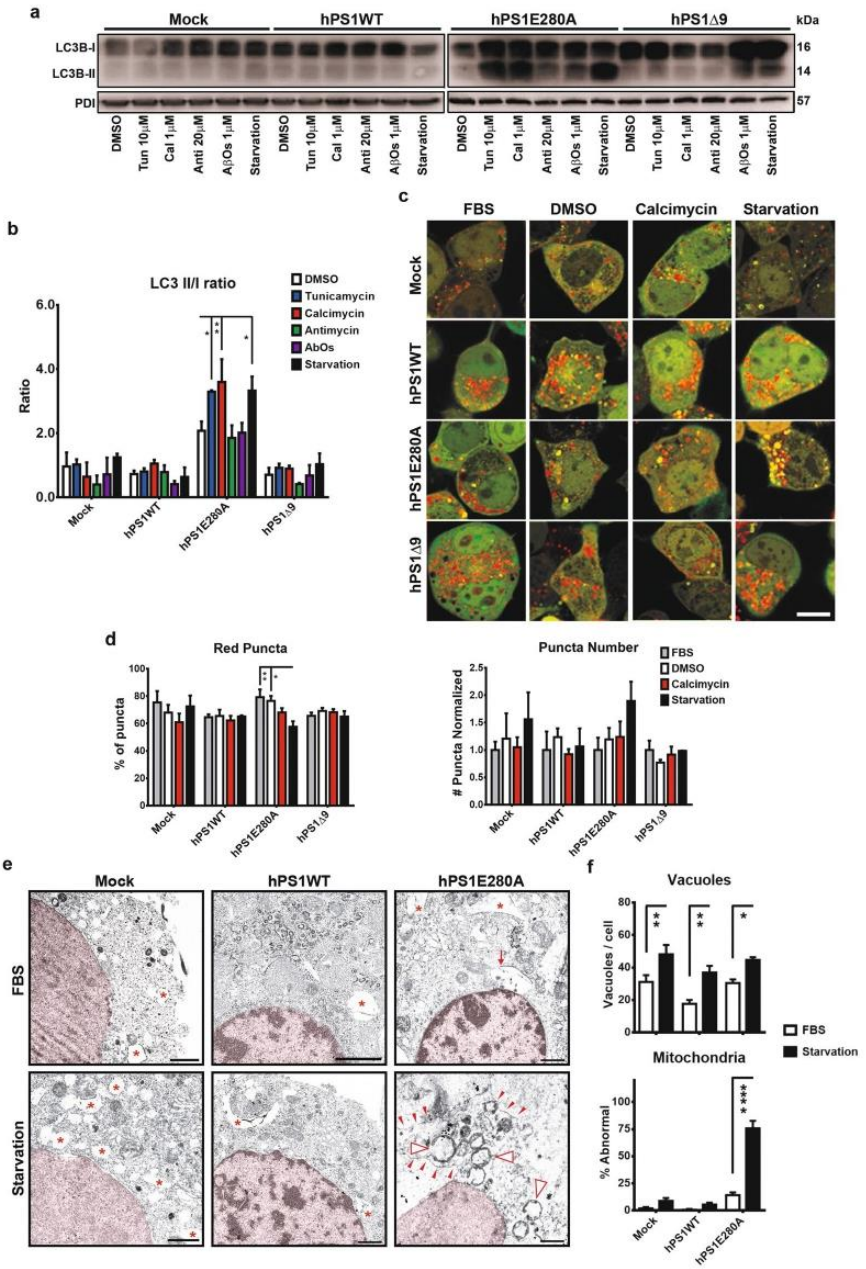

Figure 2. Abnormal autophagy in hPS1E280A N2a cells. (**a**) Steady state levels of LC3B I and II in N2a cells overexpressing hPS1, hPS1-E280A or hPS1Δ9 after tunicamycin, calcimycin, antimycin, AβOs, or serum starvation treatment. (**b**) LC3B densitometric quantification normalized to PDI and compared to basal conditions. LC3B conjugation (assessed by measuring LC3B-II/LC3B-I ratio) was increased in hPS1E280 cells after tunicamycin, calcimycin, and serum starvation treatments, while PS1Δ9 mutation showed no statistically significant differences. Mean ± SEM Two-Way ANOVA *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 3$. (**c**) Monitoring of autophagy turnover using N2a cells transfected with the RFP-GFP-LC3B construct. Transfected cells were treated with vehicle (DMSO) and stressors (calcimycin and serum starvation). The transition from autophagosomes (yellow) to autolysosomes (red) is detected by the loss of GFP fluorescence upon acidification of the autophagolysosome (for visualization of green and red channels, see Fig. S4). Bar = 1 μm. (**d**) Puncta quantifications showed significant differences in hPS1E280A cells by measuring whether red (decreased lysosomal degradation) or yellow (increased autophagosome formation) puncta. *$P < 0.05$,

data are mean ± SEM, Two-Way ANOVA. (**e**) Ultrastructure analysis of basal and serum starved (16 h) mock, overexpressing hPS1WT, and hPS1E280A N2a cells (n = 3 independent experiments for each cell line). In basal conditions, hPS1E280A cells can show autophagic vacuoles but after 16 h of serum starvation, mitochondrial lysosomal degradation can be observed. Red asterisks = vacuoles, Red arrows = autophagic vacuoles, red arrowheads = lysosomal membrane, open arrowheads = degrading mitochondria. (**f**) Serum starvation significantly increased the number of vacuoles in all evaluated cell lines and only hPS1E280A cells it presented a significantly increased percentage of abnormal mitochondria (at least 10 cells and at least 130 mitochondria were evaluated from each cell line and condition). *$P < 0.05$, **$P < 0.01$, ****$p < 0.0001$; data are mean ± SEM, Two-Way ANOVA.

Abnormal calcium homeostasis in hPS1 overexpressing N2a cells. We have previously reported evidence of mitochondrial dysfunction and calcium dyshomeostasis in the cerebellum of FAD patients carrying PS1E280A mutation[23]. A cellular stress mechanism involving both processes could explain this observation. First, we used compartment specific calcium reporters to examine calcium homeostasis in our cellular model. In effect, cytosolic calcium concentrations were significantly increased in hPS1WT and hPS1E280A, when compared to mock N2a cells. hPS1Δ9, in contrast, showed decreased cytosolic calcium concentrations (Fig. 3a,b). ER calcium concentration was significantly higher only in hPS1WT N2a cells when compared to the others (Fig. 3c,d); and mitochondrial calcium concentration was significantly increased in all hPS1 overexpressing cells when compared to mock cells (Fig. 3e,f). Only hPS1WT cells presented elevated calcium levels in all compartments when compared to controls. Although increased ER calcium in hPS1WT could be attributed to PS1 overexpression, cells overexpressing mutant hPS1 did not present that effect in the ER. In fact, cytoplasmic and mitochondrial calcium levels showed a similar trend in which hPS1E280A calcium levels were significantly increased in both compartments when compared to controls. Finally, the overexpression of hPS1 seems to have a direct effect in mitochondrial calcium levels. The divergence between ER and mitochondrial calcium levels in hPS1E280A cells can potentially be associated to a mitochondria-specific alteration in this mutation. Interestingly, when PS1 is knock down in N2a cells, only mitochondrial calcium is affected with PS1KD cells showing significantly lower levels (Fig. S6A), indicating a direct role for PS1 in mitochondrial calcium homeostasis.

Basal mitochondrial dysfunction in PS1 Mutant N2a cells and cellular stress. Alongside their multiple metabolic functions, mitochondria play a role in calcium homeostasis in the cell[20]. Following mitochondrial calcium measurements, we evaluated basal mitochondrial membrane potential ($\Delta\Psi_m$) in mock and stably transfected hPS1N2a cells as a measurement of mitochondrial health. hPS1 overexpressing cells presented with significantly increased $\Delta\Psi_m$ when compared to mock and hPS1Δ9 cells showed significantly higher $\Delta\Psi_m$ when compared to hPS1WT and hPS1E280A cells (Fig. 4a). Furthermore, PS1KD N2a cells showed significantly decreased $\Delta\Psi_m$ when compared to control cells (Fig. S6B). This, together with altered mitochondrial calcium concentrations, suggest a possible activation of mitochondrial permeability transition pore (MPTP). We evaluated MPTP opening in mock and PS1 overexpressing N2a cells, using two different methods. Assessment of MPTP-dependent alteration of the mitochondrial transmembrane potential showed that only hPS1E280A cells accelerated MPTP opening (Fig. 4b). By using the calcein-quenching assay, we found that only PS1 mutants, hPS1E280A and hPS1Δ9 cells, presented with significantly accelerated and delayed MPTP openings, respectively, when compared to mock cells (Fig. 4c,d). Both results indicate a PS1 mutation-dependent effect on MPTP activity in N2a cells. Furthermore, MPTP assessment using both methods showed no significant differences between PS1KD and control cells (Fig. S6B). Finally, we also applied our stress model and measured steady state levels of cyclophilin D and ATP synthase C, as putative MPTP components[29], together with MTCH1, a mitochondrial PS1 interacting protein involved in apoptosis signaling[30]. Although no treatment-specific differences were identified (Fig. 4e,f), cyclophilin D showed significantly increased levels in hPS1 mutated cell lines when compared to mock and hPS1WT cells. Meanwhile, MTCH1 levels were significantly increased in hPS1WT but not in PS1 mutants and ATP synthase C was significantly increased in hPS1Δ9 cells (Supplementary Table 2). In summary, mitochondrial health findings support a role for PS1 in mitochondrial homeostasis with PS1 mutations potentially leading to mitochondrial stress characterized by abnormal MPTP opening in basal conditions.

γ-secretase dependent and independent cellular stress phenotypes in PS1E280A overexpressing cells. In our present study, we have found PS1E280A specific changes in autophagy, calcium homeostasis and mitochondrial health. To define if those changes are related to γ-secretase dysfunction due to this mutation and to differentiate between γ-secretase dependent and independent processes, we evaluated LC3B conversion and MPTP opening using N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor (Fig. S7A) and 2-Aminoethoxydiphenylborane (2-APB), an ER calcium channels inhibitor. Regarding LC3B conversion, neither DAPT nor 2-APB reduced LC3B conjugation in PS1E280A cells overloaded with calcium or serum starved. On the contrary, LC3B conversion was significantly elevated in starvation conditions with DAPT and 2-APB treatments, and after combined treatment with calcimycin and 2-APB (Fig. 5a,b). Regarding MPTP modulation and similar to Cyclosporin A, DAPT was able to significantly inhibit MPTP opening in mock, hPS1WT and hPS1E280A cells but not in hPS1Δ9 cells. On the other hand, 2-APB only affected MPTP opening in PS1 mutants, inhibiting it in hPS1E280A cells and accelerating it in hPS1Δ9 cells (Fig. 5c). The effect of γ-secretase inhibition on MPTP opening was confirmed by using another γ-secretase inhibitor, Compound W, in mock, hPS1WT and hPS1E280A cells (Fig. S7B). Given the possible effect of mitochondrial calcium levels in MPTP opening, we evaluated mitochondrial and cytoplasmic calcium concentration under DAPT and 2-APB treatments in hPS1 overexpressing cells. As expected, only 2-APB had an impact in calcium

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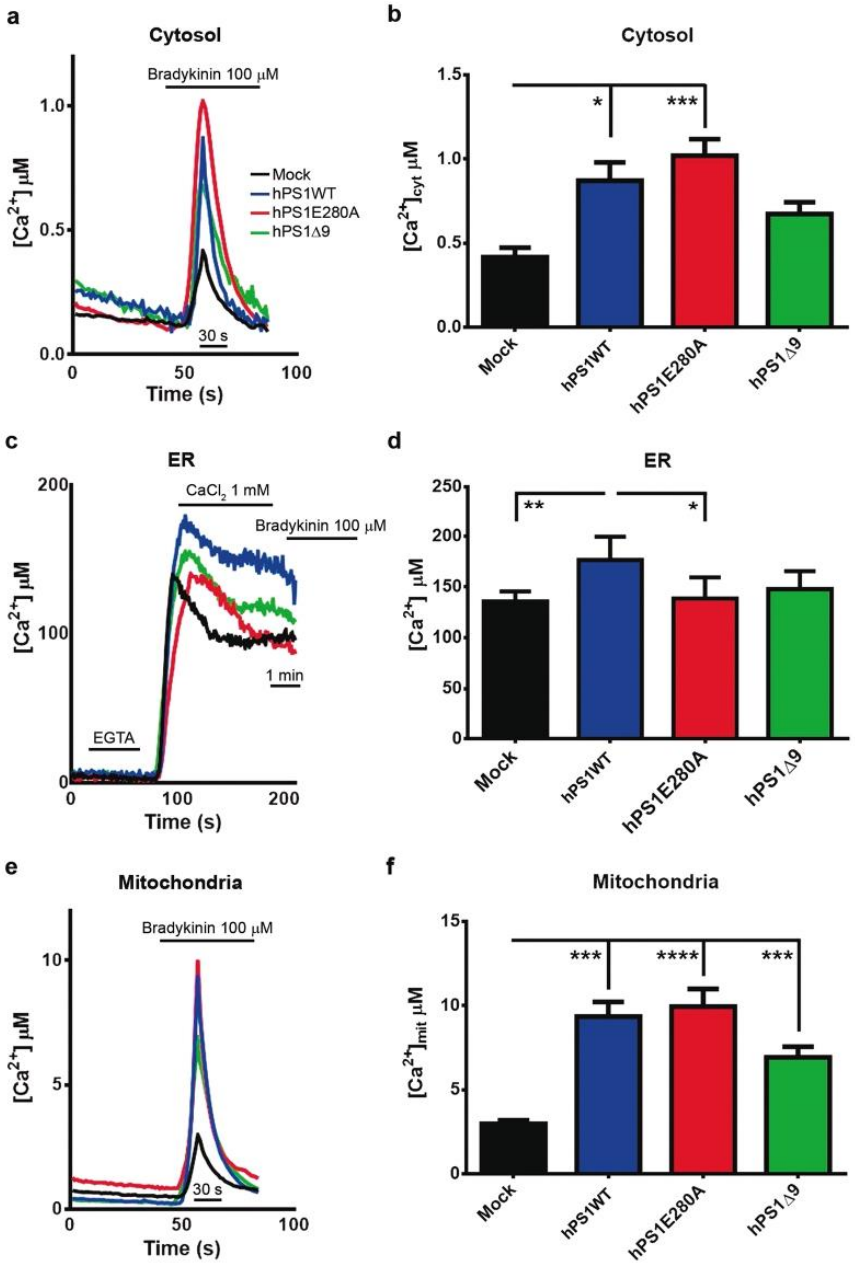

Figure 3. Abnormal calcium concentration in cellular compartments of N2a cells overexpressing wild type and mutanthPS1. N2a stably transfected cells overexpressing hPS1WT, hPS1E280A and hPS1Δ9 were used to measure intracellular calcium (Ca^{2+}) concentrations in basal conditions using transient transfection of a compartment-specific aequorin constructs. Ca^{2+} intracellular levels were perturbed with the addition of 100 μM bradykinin for cytosolic and mitochondrial light recordings; for ER Ca^{2+} measurements, cells were first depleted of extracellular Ca^{2+} with EGTA, then intracellular Ca^{2+} concentrations were perturbed with the addition of 1 mM CaCl2. Finally, 100 μM bradykinin was added to deplete Ca^{2+} stores. (**a**) Representative averaged recordings of cytosolic calcium in the different cell lines, showing the maximum calcium concentration reached after Bradykinin addition. (**b**) Bar graphs of the maximum cytosolic calcium showing increased calcium concentration in hPS1WT and hPS1E280A cells. (**c**) Representative averaged recordings of ER calcium in N2a mock and hPS1 overexpressing cells. (**d**) Bar Graphs of maximal calcium concentration detected in the Endoplasmic Reticulum, hPS1WT showed increased calcium levels in the ER. (**e**) Representative

averaged recordings of maximal mitochondrial calcium concentration in the different cell lines. (**f**) Bar graphs of the maximal mitochondrial calcium concentration after Bradykinin addition; mean and ±SEM are presented for all experiments, Two Way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001,****p < 0.0001 n = 3.

levels in both, mitochondria and cytoplasm, although mitochondrial calcium levels were unaffected by 2-APB in mock cells (Fig. 5d,e). These results indicated that the autophagic phenotype in hPS1E280A cells relies neither on γ-secretase activity nor on the availability of cytoplasmic calcium. It can also be concluded that γ-secretase activity influences MPTP opening and that this role seems to be affected by PS1 mutations. Finally, ER retention of calcium affecting MPTP opening after 2-APB treatment was only observed in PS1 mutants. This last mechanism possibly occurred due to calcium regulation in the ER and mitochondria.

Discussion

Mutations in PS1 confer a 100% penetrance for familial Alzheimer's disease, characterized by earlier disease onset, more severe pathology and associated to increase of Aβ 1–42 levels in brain tissue[2–4]. Presenilins are the catalytic subunit of γ-secretase and their mutations affect APP processing, favoring the generation of longer Aβ peptides. Thus, the severe pathology found in FAD cases is often considered to be a consequence of Aβ aggregation and toxicity[1]. Nevertheless, collected evidence has suggested cellular roles for PS1 independent on Aβ generation that could also be affected by its mutations, modulating cellular responses to stress[31–33]. For instance, some previous reports showed contradictory evidence for a role of PS1 or PS1 mutations in unfolded protein response (UPR) and ER stress[34–36] (Supplementary Table 1). Our results indicate that the downregulation or mutation of PS1 do not affect ER stress in two independent cellular models. Also, it is known that ER stress is susceptible to calcium modulation in the cell[37]. It should be noted that in our PS1 overexpressing model, abnormal calcium levels in diverse cellular compartments were not enough to induce ER stress or differential activation of it. Thus, previous evidence in favor or against a role of PS1 in ER stress should be further reassessed according to the cellular model used.

Autophagy is a physiological process for recycling diverse cellular constituents. However, under stress conditions, autophagy can be considered a pathogenic factor as seen in several neurodegenerative disorders. Disrupted autophagy leads to accumulation of autophagic vacuoles inside swollen dystrophic neurites in AD. Lysosome system failure during autophagy has been also associated to several AD pathological outcomes such as amyloidogenesis, neuritic dystrophy, apoptosis, and tauopathy[38]. Furthermore, autophagy dysfunction in AD is considered to be an effect of Aβ accumulation and toxicity[39]. We did confirm an effect of mutated PS1 in autophagy. PS1E280A showed a clear phenotype for autophagic dysfunction in N2a cells. Previous reports indicated that PS1 modulates autophagosome acidification regulating calcium homeostasis in the lysosome[12]. Interestingly, PS1Δ9 did not affect autophagy in our model. Deletion of exon 9 in PS1 has shown to affect γ-secretase dependent functions without affecting γ-secretase independent ones[40]. Lee *et al.* attributed PS1-mediated autophagic dysfunction to two γ-secretase independent processes: calcium homeostasis maintenance and lysosomal proteolysis[28]. We agree with this model, given that PS1Δ9 mutants do show impairment of γ-secretase dependent functions and that autophagy was unaffected in hPS1Δ9 N2a cells. Furthermore, increased LC3B conjugation was not rescued to normal levels by DAPT or 2-APB in hPS1E280A cells. Lack of effect of 2-APB also supports an independent regulatory function for PS1 in lysosomal calcium, independent on ER calcium retention. Previous studies[12,28] and our results indicate that an underlying autophagic dysfunction can be an ongoing cellular process in PS1 mutation carriers, turning affected cells more vulnerable to other stressors, such as Aβ accumulation itself.

One of the mechanisms proposed for autophagy regulation via PS1 is the maintenance of lysosomal calcium homeostasis[28]. This mechanism is only one among several cellular calcium-related processes in which PS1 can play a role[7]. In fact, organelle calcium homeostasis seems to be a very relevant function for PS1, considering our findings on mitochondrial calcium levels and MPTP opening. Our measurements of total calcium and ER calcium for hPS1E280A cells went along with similar findings with other PS1 point mutations[41] and confirmed the findings for PS1Δ9[42]. Regarding mitochondrial calcium concentration, PS1 overexpression in general showed an opposite effect to that reported for PS2 overexpression, which decreases mitochondrial calcium concentration in SHSY5Y cells[43]. It should be noted that increased mitochondrial calcium levels in hPS1 overexpressing cells could be associated to elevated $\Delta\Psi_m$, and both phenomena could be associated to hPS1 overexpression in this cellular model. This association was further confirmed by decreased mitochondrial calcium and $\Delta\Psi_m$ in PS1KD cells. However, PS1 mutant cells responded abnormally to calcium overload with PS1E280A accelerating and PS1Δ9 decelerating MPTP opening, while it was unaffected in PS1KD cells. Assembly and activation of MPTP is part of mitochondrial stress responses and can potentially lead to apoptosis signaling. However, transient MPTP activation seems to be a homeostatic mitochondrial response[44]. It has been suggested that Aβ toxicity can affect MPTP, whether indirectly via oxidative stress or directly, increasing Cyclophilin D translocation to the internal mitochondrial membrane and favoring MPTP opening[24]. Also, a mathematical model has been proposed linking abnormal calcium signaling, Aβ deposition and MPTP related apoptosis[45]. Remarkably, Aβ toxicity and pathology are absent in our model. Yet, cells overexpressing mutant hPS1 present with abnormal MPTP activity.

More to the point, γ-secretase inhibitors such as DAPT and Compound W effectively inhibit MPTP opening in mock, hPS1WT and hPS1E280A cells pointing to a role for γ-secretase in MPTP modulation. PS1 mutations effect on MPTP opening could be attributed to gain of abnormal function more than to loss of function, given that decreased PS1 levels did not modify MPTP opening in PS1KD cells, while γ-secretase inhibition successfully neutralized it. Decreased MPTP in hPS1Δ9 cells can be associated with significantly higher $\Delta\Psi_m$. However, γ-secretase dysfunction in hPS1Δ9 cells could also explain MPTP inhibition. Additionally, 2-APB affected MPTP opening only in PS1 mutants, again with contrasting effects between the two PS1 mutations. In those cells, 2-APB

10.

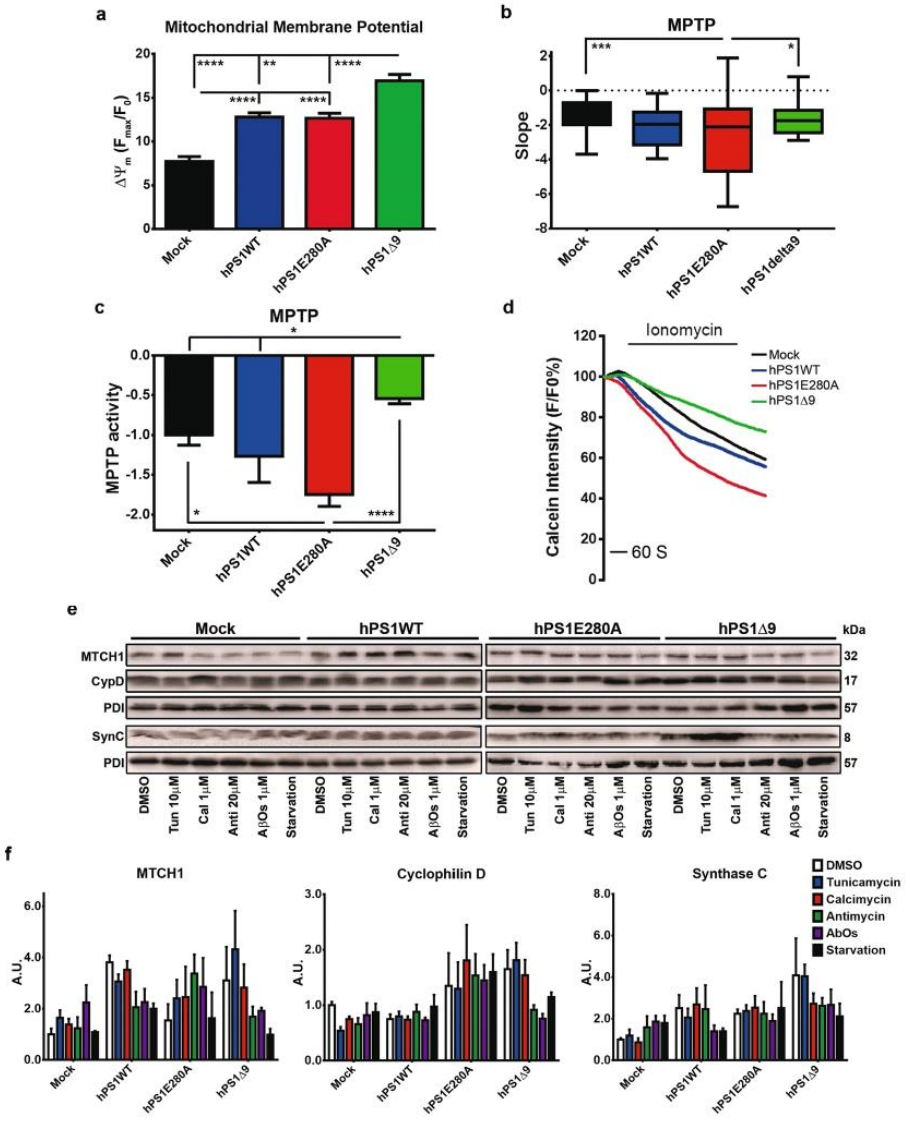

Figure 4. PS1 mutations lead to mitochondrial stress and abnormal mitochondrial permeability transition pore opening. (**a**) Bar graphs representing TMRM intensity as measurement of mitochondrial membrane potential ($\Delta\Psi_m$) during live imaging. All hPS1 overexpressing cells showed increased potential compared to mock and hPS1Δ9 showed increased potential when compared to all other cell lines. (**b**) Cells were challenged with H_2O_2 500 μM to induce mPTP opening and depolarize mitochondria, using TMRM intensity for assessment. hPS1E280A MPTP opening was accelerated compared to mock. SEM, $^{**}p < 0.01$, $^{***}p < 0.001$, n = 93–158. (**c**) MPTP opening in N2a cells assessed with the Co^{2+} -calcein assay in three independent cell cultures of N2a cells overexpressing hPS1, hPS1-E280A or hPS1Δ9. Cells were challenged with 1 μM ionomycin (Sigma-Aldrich, Hamburg, Germany) to induce MPTP opening and quenching of the calcein signal. PS1 mutant cells showed altered MPTP opening, accelerated in hPS1E280A and inhibited in hPS1Δ9 cells. (**d**) Representative timeline of calcein intensity quenching after the addition of ionomycin in hPS1 overexpressing and mock N2a cells. (**e**) Representative western blots for MPTP (Cyclophilin D, CypD and ATP synthase C, SynC) or PS1 associated (MTCH1) mitochondrial proteins in N2a cells after stress treatments. (**f**) Bar graphs of densitometric analysis of steady state levels of CypD, SynC and MTCH1. Only hPS1WT overexpressing cells presented significantly increased MTCH1 levels. PS1-Mutants showed increased general steady state levels of CypD and SynC independent of stress. All data are mean ± SEM, Two-Way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

12.

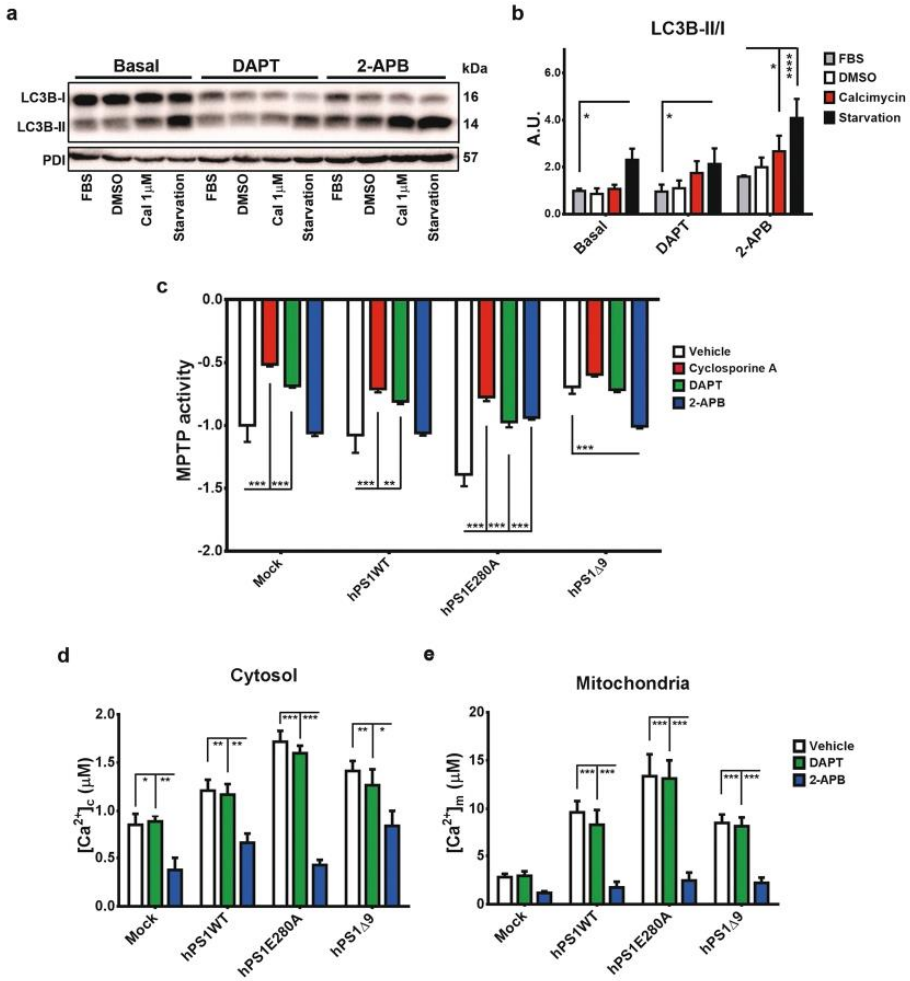

Figure 5. γ- secretase dependent and independent cellular stress response in hPS1E280A cells. (**a**) LC3B conjugation was evaluated in hPS1E280A cells treated with γ-secretase inhibitor DAPT and ER calcium channels inhibitor 2-APB. Representative western blot for LC3B from hPS1E280A N2a cells treated with FBS, DMSO, calcimycin and serum starved, all for 16 h. Cells were assessed with and without concurrent 16 h exposure to DAPT or 2-APB. (**b**) Bar graphs for densitometric analysis of hPS1E280A N2a cells. DAPT increased LC3B conjugation when compared to basal conditions and 2ABP increased it after calcymicin treatment or starvation in hPS1E280A cells. (**c**) Mock transfected, hPS1WT, hPS1E280A, and hPS1Δ9 N2a cells were challenged with 1 μM ionomycin to induce MPTP opening and quenching of the calcein signal. Cells were treated with Cyclosporin A, DAPT, or 2-APB. Cyclosporin A and DAPT inhibited MPTP opening in mock, hPS1WT, and hPS1E280A cells while 2-APB only showed an effect in PS1 mutants, inhibiting MPTP opening in hPS1E280A cells and accelerating it in hPS1Δ9 cells. (**d**) Bar graphs of maximum cytosolic calcium concentration and (**e**) mitochondrial calcium concentration in the different N2a cell lines, treated with DMSO (vehicle), DAPT and 2-APB for 16 h. 2-APB decreased mitochondrial calcium levels in PS1 overexpressing cells and cytoplasmic calcium levels in all cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are mean ± SEM, Two-Way ANOVA.

effectively compensated abnormal basal MPTP profiles while decreasing mitochondrial and cytoplasmic calcium levels. Therefore, calcium retention in the ER relieves mitochondrial calcium overload in PS1 mutants which avoids abnormal MPTP opening. This effect can be achieved via mitochondria-ER interaction, given calcium exchange between these two organelles as previously suggested by Toglia *et al.* in their model[46] and the known effect of 2-APB in mitochondrial calcium[47]. Mitochondrial function seems to be also altered in AD affecting lipid synthesis, respiratory chain, and calcium homeostasis[21]. Previous studies suggest increased ER-Mitochondrial apposition leading to augmented calcium trafficking between the two organelles as one possible explanation for

mitochondrial dysfunction in AD[48], that could lead to mitochondrial stress and cell death. This model of mitochondrial dysfunction in AD also portrays Aβ accumulation and toxicity as a causative factor[49,50].

Independently of its wide use, hPS1 overexpression in otherwise unmodified murine cells can always bring unexpected effects given specific functional profiles of human vs murine Presenilins[51]. Our N2a hPS1 overexpression model has this limitation and our results should be interpreted within this context. Nevertheless, we observed specific γ-secretase dependent and independent dysfunction in overexpressing hPS1 mutants, with direct impact in some stress pathways. Also, although our experimental design aimed to induce cellular stress but not cellular death, findings regarding autophagy dysfunction in hPS1E280A cells and mitochondrial calcium-dependent stress in both hPS1 mutants suggested the possibility that cellular death pathways could be activated whether basally or via cellular stress induction. Remarkably, neither the treatment with antimycin nor Aβ 1–42 oligomers presented evidence for increased cellular stress in our model and assessed markers after 16 h. Doses used for both treatments are within range for stress induction in N2a cells, according to previous reports[52,53], and oligomeric synthetic Aβ 1–42 is known to induce different types of cellular stress, sometimes simultaneously, in various cellular models[54,55]. It is possible that 16 h of treatment were not enough to allow these reagents to induce visible effects in our assays. As a confirmation of this, levels of the apoptotic marker pRIPK3 were significantly increased in mock N2a cells after 24 h of treatment with both, antimycin and Aβ 1–42 oligomers (Fig. S7C). Given that our purpose was to examine early stress events caused by PS1 mutations, we consider Aβ negative findings at 16 h to be a confirmation of a role for PS1 independent of Aβ toxicity in cellular stress. Likewise, lack of effect of antimycin and 16 h shows, at this point, that mutant PS1 effects in mitochondria are not directly related or modified by oxidative stress. Finally, additional limitations of our study include the use of a single cellular model, N2a cells, and of single stably transfected clones in our cell lines. These limitations should be taken in account for the interpretation of the results and for further exploration of our findings.

Previously, an elegant study by Guo *et al*. in a murine knock-in model determined that PS1 mutations confer increased neuronal vulnerability to excitotoxicity via apoptosis in hippocampal cells. They also showed that impaired mitochondrial function increased susceptibility to Aβ-induced stress[56]. The same group determined in PS1 transfected PC12 cells increased sensitivity to mitochondrial stress with associated elevated calcium in PS1 mutants. Interestingly, Cyclosporin A was used successfully to prevent apoptosis caused by oxidative stress in their model and already then, MPTP activation was suggested as a possible mechanism of increased mitochondrial stress in PS1 mutants[57]. More to the point, recently Toglia *et al*. suggested a theoretical model for the involvement of MPTP in PS1 mutants as a result of IP3R altered activity and increased mitochondrial calcium uptake[46]. We have confirmed both, previous findings and the theoretical model, but we bring in the novel finding of γ-secretase as a direct modulator of MPTP independent of mitochondrial calcium homeostasis.

In this study we presented an alternative mechanism for mitochondrial damage in PS1 FAD with altered MPTP as a driving force behind it, and we showed how PS1 mutations affect cellular stress responses simultaneously with specific mutations presenting different profiles. Although several of these stress mechanisms have been reported previously to be altered in different cellular models, our experimental design allowed us to integrate them on a single picture with PS1 mutations simultaneously affecting calcium homeostasis, lysosomal function and mitochondria. As with autophagy, PS1 mutation-dependent alterations in mitochondria detected in our model occur under basal conditions or as a result of acute non-lethal cellular stress. We have previously reported evidence of mitochondrial dysfunction in PS1E280A patients, reproduced on animal and cellular models[23]. Our results confirm that PS1 mutations render the cell more susceptible to different kinds of cellular stress, such as autophagy or mitochondrial dysfunction, on a mutation-specific manner. Even in one single mutation, stress pathways involving γ-secretase dependent and independent mechanisms simultaneously can be identified without the participation of Aβ toxicity. These Aβ-independent stress mechanisms point to a larger role of PS1 on cellular homeostasis and death beyond APP processing. We suggest that in PS1 FAD patients basal cellular stress could be taking place throughout life, increasing vulnerability to damage in susceptible cells and presenting eventually with cumulative effects leading to the complex end-point pathology found in AD. The identification of cellular stress mechanisms in human post mortem brain tissue after a long degenerative process is complicated further by multiple events that lead to the end-point pathology found in the tissue. Therefore, we consider that the study of such mechanism in cellular models is still a valid approach to reconstruct the pathophysiology observed in FAD. Although further studies in cellular and animal models need to be done, we suggest the possibility that earlier disease onset, more severe brain atrophy and underlying neurodegeneration in FAD are a result not only of earlier Aβ pathology but of latent cellular vulnerability to stress due to other PS1 functions.

Materials and Methods

Cellular culture and transfection. Murine Neuroblastoma N2a cells were stably transfected with pcDNA 3.1 Zeo + vector with resistance to Zeocin. 1 μg of 4 different plasmids: Mock (empty vector), human PS1WT, PS1E280A and PS1Δ9 were used to transfect the cells with lipofectamine™ 2000 (Thermo Fisher Scientific, Schwerte, Germany), according to manufacturer's instructions. The clones were established by selection with Zeocin (Invitrogen, Carlsbad, CA, USA). The dose used for selection was 200 μg/ml. Positive clones were isolated after approximately 30 days. Overexpression of human PS1 was assessed via western blot and qPCR for human PS1 (Fig. S1A–C). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and treated with different reagents to induce cellular stress and death (see below). *PS KO−/−* MEFs and *PS KO−/−* MEFs stably transfected with wildtype or mutated (E280A) human PS1 were kindly donated by Bart de Strooper (VIB, Leuven, Belgium). This cellular model has been used previously to study γ-secretase function[58] and to study Presenilins impact in mitochondrial function[59]. MEFs were cultured in DMEM supplemented with 10% FBS at 37 °C and 5% CO_2 and treated with tunicamycin and calcimycin 10 μM.

Reagents. Cell stress inducers were dissolved first in DMSO (maximal concentration 0.1%) and added to cells as follows: ER stress with 10 μM tunicamycin (Sigma-Aldrich, Hamburg, Germany), calcium overload with 1 μM calcimycin (Sigma-Aldrich, Hamburg, Germany). Oxidative stress with 20 μM antimycin (Sigma-Aldrich, Hamburg, Germany), autophagy induction via serum starvation (DMEM without FBS), Aβ toxicity with 1 μM Aβ 1–42 oligomers (see oligomers preparation). Also, 250 nM of N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma-Aldrich, Hamburg, Germany) and 10μM of Compound W (Tocris Biosciences, Bristol, England), γ-secretase inhibitors, were used to evaluate the role of γ-secretase activity in cellular stress and MPTP opening. Furthermore, 100 μM of 2-Aminoethoxydiphenyl borate (2-APB, Sigma-Aldrich, Hamburg, Germany), an ER calcium channels inhibitor, was used to evaluate the influence of store operated calcium and 1.6 μM Cyclosporin A, was used as a MPTP inhibitor (Sigma-Aldrich, Hamburg, Germany) (see Supplementary Table 3 for mechanisms of action).

Aβ oligomers preparation. Aβ 1–42 synthetic peptide oligomerisation was conducted according to published protocols[60]. Lyophilized powder of Synthetic human Aβ 1–42 (GenicBio, Shanghai, China) was first dissolved to 1 mM solution by adding 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP). Aβ-HFIP solution was incubated at room temperature (RT) for at least 30 min and then the tubes were opened to allow HFIP evaporate overnight. Tubes were transferred to a SpeedVac and dried down without heating for 1 h to remove any remaining traces of HFIP. A thin clear film at the bottom of the tubes was obtained and diluted again whether by adding DMSO (final concentration 5 mM) to store at −20 °C or DMSO (5 mM) plus Medium (DMEM + FBS 10%) for a final concentration 1 μM for cell treatment.

Western blots. Cells were harvested after 16 h of treatment for each reagent and lysed with a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 10% glycerol,1% NP-40 and sodium azide. Protein concentration was determined with the bicinchoninic acid method, and equal amounts of protein were loaded into a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a Polyvinylidene difluoride membrane. Membranes were blocked with 5% non-fat milk + 0.1% Tween 20 in tris-buffered saline for 1 hour and then incubated with specific primary antibodies overnight at 4 °C. Early and late markers for ER stress, mitochondrial proteins, and autophagy were assessed by immunoblotting (see Supplementary Table 4). Afterwards blots were incubated with secondary antibodies for 1 hour and detected by chemiluminescence. Densitometric analysis was performed using Image Studio Lite 5.2 (LI-COR, Lincoln, NE, USA).

Autophagic turnover assessment. All cell types were transiently transfected using Lipofectamine™ 2000 with a tandem LC3B fluorescent construct including Green fluorescent protein (GFP) and Red fluorescent protein (RFP) (pMRX-IP-GFP-LC3-RFP Plasmid, Addgene, Cambridge, MA, USA) following the manufacturer´s instructions. Autophagic turnover assessment was performed after 16 hours of incubation with calcimycin or serum starvation, according to methods described above, cells treated with DMSO or only culture medium were used as controls. The cells were visualized using a Leica LCS-SP5 confocal microscope system, all experiments were performed under live imaging conditions (SP5 confocal microscopy chamber at 37 °C and 5% CO_2). Transfected LC3B could be detected as emitting in the green and red channels (yellow signal) if it was located in the cytoplasm or autophagosomes; and as emitting in the red channel only in autolysosomes[27].

Ultrastructural analysis. Mock, hPS1WT and hPS1E280A N2a cells were collected and centrifuged in PBS at 1000 g for 5 minutes at 4 °C. The resulting pellets were prefixed with 0.25% glutaraldehyde and 4% paraformaldehyde in PBS, for 24 h at 4 °C. Prefixed pellets were further fixed in glutaraldehyde and chrome-osmium at least for 2 hours at room temperature, dehydrated in ethanol, and embedded in Epon 812 (Serva Electrophoresis GmbH). After polymerization, 1-μm-thick sections were cut, stained with toluidine blue, and checked for adequate cell morphology. For further processing for electron microscopy, relevant specimens were cut into 60- to 80-nm-thick sections, which were contrasted with uranyl acetate and lead solution. Sections were visualized with a LEO EM 912AB electron microscope (Carl Zeiss GmbH, Jena, Germany).

Intracellular calcium measurements. Targeted aequorins (AEQs) were used to measure intracellular calcium concentrations in N2a cells. 50000 cells were grown in coverslips and transfected with 0.5 μg aequorin-cDNA construct directed to different cellular compartments (cytAEQ, mitAEQ and ERAEQ, described in[61]). Mitochondrial and cytosolic responses were induced by the addition of 100 μM Bradykinin (Sigma-Aldrich, Hamburg, Germany). ER calcium re-uptake was measured in calcium depleted cells. Cells were lysed with a 10 mM calcium and 0.1% Triton containing buffer at the end of the experiment to estimate the efficiency of transfection as described previously[61].

Mitochondrial membrane potential measurement. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by loading cells with 10 nM tetramethyl rhodamine Methylester (TMRM, Thermo Fisher Scientific, Schwerte, Germany) for 30 min at 37 °C. Images were taken with an inverted microscope (NikonLiveScan Swept Field Confocal Microscope (SFC) Eclipse Ti equipped with NIS-Elements microscope imaging software, Nikon Instruments, Amstelveen, Netherlands). TMRM excitation was performed at 560 nm and emission was collected through a 590 to 650 nm band-pass filter. Images were taken every 5 s with a fixed 20 ms exposure time. 10 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, Sigma-Aldrich, Hamburg, Germany), an uncoupler of oxidative phosphorylation, was added after 5 minutes of acquisitions in order to completely collapse the

electrical gradient established by the respiratory chain. $\Delta\Psi_m$ was determined as the ratio of basal TMRM fluorescent signal intensity (F_{max}) divided by TMRM fluorescent signal intensity after FCCP addition (F_0).

Assessment of mitochondrial transition pore opening. *MPTP assessment by Calcein/Co^{2+}quenching assay*. Permeability transition pore complex opening was assayed as previously described[62]. Cells were loaded with 1 mM calcein acetoxymethyl ester (Sigma-Aldrich, Hamburg, Germany) and Co^{2+} as instructed by the Image-IT® LIVE Mitochondrial Transition Pore Assay Kit (Thermo Fischer Scientific, Schwerte, Germany). Cells were then imaged based on 490 ± 20 nm excitation and 525 nm long pass emission filters with an Axiovert 200 M fluorescence microscope equipped with a 40X water immersion objective (N.A. 1.2, from Carl Zeiss Microscopy, Jena, Germany). Finally, images were analyzed with MetaMorph® (Molecular Devices, LLC, San Jose, CA, USA), and quenching rate was calculated as the slope of the fluorescence trace over a period of 60 sec after stimulation.

MPTP assessment by Mitochondrial transmembrane potential. Assessment of MPTP opening via $\Delta\Psi_m$ was performed as previously described[63]. Cells were loaded with 10 nM TMRM (Thermo Fischer Scientific, Schwerte, Germany) in Krebs-Ringer buffer supplemented with 250 μM sulfinpyrazone, then placed in a humidified chamber at 37 °C and imaged with a LiveScan Swept Field Confocal Microscope (Nikon Instruments, Inc.) equipped with a 60× oil immersion (N.A. 1.4, from Nikon Instruments, Inc.) every 30 sec for 30 min. TMRM fluorescence was analysed with the NIS Elements software package (Nikon Instruments, Amstelveen, Netherlands), and depolarization rate were calculated as the slope of the fluorescence trace over a period of 10 min after stimulation.

Statistics. Data were analyzed by GraphPad Software (La Jolla, CA, USA) using one-way or two-way ANOVA, when required, followed by Holms-Sidak post-hoc correction, values are given as mean ± standard error of mean (SEM). Differences with p-value < 0.05 were considered significant.

Data availability

Relevant data for the interpretation of the results is included in the manuscript and supplementary files. More detailed data and specific materials such as cell lines used, are available from the corresponding author on reasonable request.

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D.S.-F., P.P., M.G. and S.J.M. designed the study, L.R.-C., S.C.-M., C.M., G.M., K.P., C.H. and D.S.-F. performed experiments. L.R.-C., C.M., G.M. and D.S.F. performed statistical analysis. L.R.-C., S.C.-M. and D.S.-F. drafted the manuscript with editorial help from C.H., S.J.M., M.G. and P.P. All authors have read and approved the manuscript.

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2. Zusammenfassende Darstellung der Publikation

2.1. Introduction

Alzheimer disease (AD) is the most common form of dementia. Most of patients suffering from AD start symptoms in the elderly, this population corresponds to the sporadic cases of AD. Less than 5 % of the patients with Alzheimer developed early symptoms, caused by gene mutations in presenilins (PS), amyloid precursor protein (APP) or apolipoprotein E (apoE) (Querfurth and LaFerla 2010). Some pathological hallmarks of this disease are neuritic plaques, neurofibrillary tangles (NTFs) and neuronal loss. Nonetheless, the pathophysiological mechanism of AD remains unclear. While it is a neurodegenerative disease, it could have a multifactorial cause where different cellular mechanisms are compromised, for instance alteration in lipid metabolism, mitochondrial disfunction, calcium disturbances and activation of death pathways. From a clinical point of view, patients suffering from FAD have increased morbimortality. Microscopically those patients show increased neuronal death and brain atrophy (Ryan and Rossor 2010).

Presenilin 1 and 2 (PS1 and PS2) are main parts of the γ-secretase complex and their mutations cause familial forms of Alzheimer's disease (FAD). Although PS mutations have been always related to an alteration in the γ-secretase complex, some PS functions are independent of the γ-secretase activity and still be related to the mechanism of disease in AD (De Strooper and Karran 2016). PS1 plays an important role in stabilizing β-catenin in Wnt signaling pathway, regulating calcium homeostasis and synaptic transmission (Duggan and McCarthy 2016). Nevertheless, its precise role in Ca 2+ homeostasis remains controversial. When mutations in PS1 lead to calcium overload, disrupting Calcium related mechanism such us signaling, protein folding processes, even cell survival (Honarnejad and Herms 2012, Pchitskaya et al 2018).

The largest and most studied FAD population (PS1E280A) has been selected for key therapeutic studies on the prevention of AD, assuming that both, sporadic and familial AD, share a common amyloidogenic etiology with Aβ deposition. In previous studies it was found that PS1E280A mutation affects Ca 2+ homeostasis leading to cellular damage (Bezprozvanny 2012, De Strooper et al. 2012, Honarnejad et al. 2012, McBrayer et al. 2013, Sepulveda-Falla et al 2014 and Pchitskaya et al. 2018). Our previous findings suggest that some neurodegenerative mechanisms are independent on Aβ deposition and more related to Ca2+ dysregulation, affecting organelles inside the neurons and around, among other processes (Sepulveda-Falla et al. 2014). In other words, it is possible that PS1 mutations lead to cellular stress through different pathways including intracellular calcium dysregulation.

Here, we studied the roll of PS1E280A in the dysregulation of calcium homeostasis. These results will help to clarify PS1 FAD pathophysiology.

2.2. Background

Alzheimer´s Disease

Alzheimer´s disease (AD) was first described by Alois Alzheimer in 1906. The AD International Group defines it as a progressive neurodegenerative disorder and estimates that AD accounts for 50-60% of all cases of dementia, placing it as the most common cause of dementia. The symptoms in a patient suffering from AD general include memory loss (i.e. difficulty recalling events that happened recently), difficulty in finding the right words or understanding what people are saying, difficulty in performing previously routine tasks and personality and mood changes. AD-symptoms progress in time, reducing the expectancy of life as much as 2-11 years after the onset of symptoms (Todd et al. 2013). Therefore, Dementia in general and especially AD is considered a public health issue and consequently as a priority in public health policies worldwide (Alzheimer´s Disease International, global information on Alzheimer´s Disease, online version 2020).

The cause of AD is poorly understood, different hypotheses have tried to explain the pathophysiological mechanism of AD, namely cholinergic hypothesis, amyloid hypothesis, Tau hypothesis, calcium dysregulation, mitochondrial dysfunction, among others. Only 1-5 % of the Alzheimer´s cases were identified as familial AD (FAD), those are attributed to mutations in Presenilin 1, 2 (PS1, PS2) and amyloid precursor protein (APP). Patients suffering FAD develop symptoms before 65 years of age. In contrast, the most common form of AD is its sporadic form, here the major risk factor is the age. Patients with the sporadic form of AD will develop symptoms in the elderly.

Pathological Mechanism of FAD

Cholinergic hypothesis

Alterations in the cholinergic system has been correlated to the impairment of cognitive processes. The hypothesis described a presynaptic reduced neurotransmitter, acetylcholine, inducing an alteration in the neurotransmission in the brain cortex and therefore altering the cognitive function seen in patients with Alzheimer's disease. With this hypothesis many drugs were released as a treatment for AD. Nowadays, this hypothesis as a cause for AD has only a historical purpose, since further evidence could not support this theory (Bartus et al. 1982, Francis et al. 1999). Nevertheless, the therapeutical benefits of cholinesterase inhibitors, although only temporal and side effects, remain approved to treat symptomatic AD. Many authors and clinical data support the benefits of this treatment as "disease modifier" (Hampel et al. 2019)

Amyloid hypothesis

The histopathological changes depicted in a brain with AD show accumulation of extracellular Aβ-plaques and intracellular neurofibrillary tangles, that leads macroscopically to brain atrophy. These observations started the Amyloid hypothesis, which was postulated in 1991 (Hardy and Allsop 1991). Here, the amyloid beta (Aβ) peptides are originated from the cleavage of APP by the enzyme beta-site amyloid precursor protein–cleaving enzyme 1 (BACE-1). The resulting Aβ peptides, if not cleared, accumulate in the extracellular space, restraining neurotransmission and activating cellular death pathways (Querfurth and LaFerla 2010). One of the Aβ products after the metabolism of APP are monomers of Aβ40, however other longer products, like Aβ42, could be also produced. This type is more toxic for the neurons. Aβ42 are more prone to aggregate. It seems that Aβ peptides alone cannot induce cellular damage. When Aβ peptides aggregates, especially in soluble oligomers and

intermediate amyloids, the pathological process starts, those oligomers were associated to synapse loss and neuronal toxicity, altering neurotransmission and neuronal activity (Lue et al. 1999, Selkoe 2001, Stine et al. 2003). Aβ Oligomers in the extracellular space will attach themselves to the cellular surface, altering the function of several cellular receptors, for instance the N-methyl-D-aspartate receptor (NMDAR) related to synaptic, calcium and mitochondrial function, p75 neurotrophin receptor (p75NTR) related to cell death, neuronal insulin receptor (IR) associated to signaling in dendrites, Frizzled (Fz) cysteine-rich domain associated to cell-cell communications and Wnt signaling pathway, among others (Kayed and Reeves 2013). Aβ Oligomers were also correlated to disruption of plasma membrane integrity, increasing calcium within the cell and cellular organelles (Arispe et al. 1993). Other mechanisms in cellular dysfunction induced by Aβ products are a result of their accumulation inside the cell, Almeida et al. described the inhibition of the proteasome, impairing the endocytic trafficking of neuronal receptors and consequently the synaptic function. The accumulation of Aβ peptides in the intracellular compartment remains elusive, it could be whether by back up of the already secreted Aβ products or the intracellular deposits that, as in the outside environment, aggregate and accumulate promptly inside the cell in toxic forms (Almeida et al. 2006, Kayed and Reeves 2013). Although some variations and more mechanisms on how Aβ product could damage the cell and lead to AD are still in research, the core of this hypothesis for almost 30 years now remains the same: Amyloid beta formation (from peptides to oligomers, soluble and insoluble forms) leads through different pathways to cognitive impairment and at the end in dementia, specifically AD. However, other clinical data that evaluate amyloid burdens with PET-Scans or other tests in many individuals showed a substantial Aβ burdens in their brains but still being healthy. Similarly, individuals, to whom plaque loads were reduced with immunization, showed after years persistent deterioration of their cognitive status,

meaning that the disease progress while less plaque deposits were seen in their brains (Herrup 2015).

Tau Hypothesis

Tau is a soluble cytoplasm or axonal protein, which facilitates the vesicular transport and microtubular stabilization in neurons (Querfurth and LaFerla 2010). In AD, Tau is the essential component of the intracellular deposits or neurofibrillary tangles, those are paired helical filaments of hyperphosphorylated tau. In the normal human brain 6 isoforms of tau are expressed, they differ by the presence or absence of N-terminal inserts. In AD, those isoforms are equally present, and the pathological mark is related to the hyperphosphorylation of tau and their aggregation. Those filaments tau deposits are present not only in AD but also in other neurodegenerative diseases, defining a group of neurodegenerative disorders called the Tauopathies (Goedert and Spillantini 2006, Goedert et al. 2017). AD is a 3R+4R tauopathy, meaning 3 isoforms with absence of the N-Terminal insert but with inclusion of 3 repeats of the 31 amino acid repeat in the C-Terminal half, and four isoforms with the N-Terminal and with the repeats, meaning all the possible isoforms of tau. Those forms are also present in other diseases and how the tau-filaments induce a specific neurodegenerative disease may depend in their own fold formation (Falcon et al. 2018). Regarding tau genotype, although more than 30 tau-mutations were determined, none of them are present in AD (Querfurth and LaFerla 2010, Falcon et al. 2018).

Genetic Hypothesis

More than a hypothesis, it describes the different mutations found in familial AD (FAD). FAD is an autosomal dominant inheritance. The protein mutations are related to the components of the γ-Secretase complex APP, PS1 and PS2. Since there is a genetic

background associated to the neurodegenerative process, one expects the symptoms in an early age (early onset of FAD or EOAD). However, there are also cases for some protein mutations where the onset of symptoms starts in the elderly and those individuals still belong to the sporadic AD or late onset of AD (LOAD), here the most common predisposition factor account for sporadic AD is the inheritance of the ε4 allele of the apolipoprotein E (APOE) (Blennow et al. 2006). Nowadays, more studies unfavor the amyloid hypothesis, even they propose a presenilin-based hypothesis, where presenilin mutations, that induce its loss of function, are the origin of all pathological events involved in FAD (Sun et al 2017).

Proteins involved in FAD

γ-Secretase complex

The cleavage of APP is performed by multi-subunit proteases, called secretases (Haas et al. 2011), one of them is the gamma Secretase (γ-Secretase). γ-Secretase has 4 different subunits: Presenilin 1 (PS1) or 2 (PS2), presenilin enhancer 2 (PEN2) anterior pharynx-detective 1 (Aph1) and nicastrin. Since there are two types of presenilins and two types of Aph1 (Aph1a and 1b), are at least 4 different types of γ-Secretase. However, if they differ in functions is not well known (De Strooper et al. 2012). APP is not the only integral membrane protein processed by γ-Secretase, γ-Secretase has been related to the metabolism of other substrates such as Notch, ErbB4, Cadherins, among others. Mutations in the γ-Secretase subunits have been related to FAD, especially PS1-Mutations (De Strooper et al. 2012, Wolfe 2019, Xia et al. 2019).

Amyloid precursor protein (APP)

First, APP is proteolytically processed by β-secretase (BACE1) and generates a 12 kDa C-terminal remnant of APP (C99); then, C99 is cleaved by γ-secretase to yield

two major species of Aβ ending at residue 40 (Aβ40) or 42 (Aβ42). Xia et al described APP mutations as beneficial or non-beneficial. Genetic studies showed that missense mutations in APP that increases the ratio of Aβ42, may cause EOAD, while the beneficial mutation decreases the Aβ production, and the patients remains cognitive intact in advance age (Xia et al. 2019).

Presenilin 1 (PS1)

Presenilin 1 is the catalytic component of the γ-secretase complex, it cleaves a variety of type 1 transmembrane proteins, notably including the amyloid precursor protein (APP). It is encoded by *PSEN1* gene, located in the chromosome 14q24.2. The genetic information encoded in the mRNA of PSEN1 is read by the ribosome, producing a 50 kDa mature transmembrane protein with a 30 kDa amino-terminal fragment (NTF) and a 20 kDa carboxy-terminal fragment (CTF) (Laudon et al 2005). Mutations of PSEN1 lead not only to FAD, but they are also related to other forms of dementia, including Parkinson (Larner et al. 2013). Wolfe et al described that the mutagenesis of presenilin in two aspartyl residues, in the TMDs VI and VII of PSEN1, results in a dominant negative effect on the γ-secretase activity (Wolfe et al. 1999). According to the amyloid hypothesis, the PS mutations increased Aβ42 production by enhancing APP processing, remaining an overproduction of Aβ42 (Duff et al 1996, Strooper et al 2012 and Wolf 2019). Nevertheless, Sun et al demonstrated, that 138 distinct FAD-causing mutations had a reduced production of Aβ40 and 42, because of the impaired γ-secretase function to cleaved APP (Sun et al 2017). Beyond the γ-secretase function, PS1 has been also involved in other pathways and functions, for instance Notch and Wnt signaling pathways, beta-catenin stabilization, calcium homeostasis, insulin signaling, among others (Honarnejad et al 2012 and Duggan et al 2016).

E280A-PS1 Mutation

Special mention deserves the E280A-PS1 or “Paisa” mutation, not only because of its role in this study, but also because it is by far the most common cause of FAD. Currently, the pedigree spans over 5 generation with more than 1000 confirmed carriers (alzforum.org, Lopera et al 1997). It was first documented almost 20 years ago, Lopera et al. described early symptoms in young patients (less than 30 years old) even before dementia onset (Lopera et al 1997). Genetically, the E280A-PS1 mutation carriers bear the codon 280 Glu to Ala substitution (Glu280Ala) in the exon 8.

Presenilin 2 (PS2)

Similar to PS1, PS2 is a transmembrane protein. PSEN-1 gene is located in the chromosome 1, encoding a 448 aminoacids protein (Levy-Lahad et al. 1995). Nowadays, there are at least 200 mutations of PSEN-2 reported worldwide (Alzheimer Disease & Frontotemporal Dementia Mutation Database, AD&FTDMDB, http://www.molgen.vib-ua.be/ADMutations, https://omim.org/entry/600759#geneMap, https://medlineplus.gov/genetics/gene/psen2/#resources), few of them associated to AD (Canevelli et al. 2014 and Marín-Muñoz et al. 2016).

Apolipoprotein E (APOE)

ApoE has three major isoforms, ApoEε2, ε3 and ε4. ApoEε4 allele is the strongest known risk factor for AD. Brains of sporadic AD patients carrying ApoEε4 allele were found to have increased density of Aβ deposits, limited capability to clear Aβ, and enhanced neuroinflammation (Xia 2019)

2.3. Materials and Methods

A more detailed description of all materials and methods for this study can be found in the text and supplementary material of the published paper identified as PMID: 32296078. Briefly, N2a cells were used to establish the cellular model for the study. These cells were stably transfected with pcDNA 3.1 Zeo + vector with resistance to Zeocin. Four different lines were obtained after plasmid transfection: Mock (empty vector), human PS1WT, PS1E280A and PS1Δ9. The overexpression of human PS1 was assessed via western blot and qPCR for human PS1. The positive PS1 transfected cells and mock were cultured in DMEM supplemented with 10% FBS, after more than 80% growth in culture plates the cells were treated with different reagents to induced cellular stress as follow:

1. 10μM Tunycamicin for ER stress
2. Calcium overload with 1μM Calcimycin
3. 20μM antimycin to induce oxidative stress
4. Autophagy was induced via starvation (cells were cultured in DMEM without FBS).
5. Aβ toxicity was induced after treating the cells with 1μM Aβ 1-42 oligomers (Aβ oligomers preparation protocol by Stine et al 2003).

Standardization for an adequate treatment doses and time was required for all reagents. We treated N2a cells with different reagents to produce cellular stress, namely ER Stress (Tunicamycin), calcium overload (Calcimycin), mitochondrial stress (Antimycin), Aβ-related cellular stress (Aβ1-42 oligomers) and autophagy induced by starvation (serum deprivation). To determine the optimal time to induce cellular stress without inducing crossing over to multiorganelle intracellular disfunction/stress and/or activation of cellular death, the cell cultures were harvest after 0-8-16-24h of treatment.

At 8 hours of treatment, proteins related to ER stress started to be induced (BiP, CHOP and GADD34). This effect remained after 16 and 24h of treatment. The autophagy marker LC3B converts from LC3B-I to -II when autophagy is activated. After 16h LC3B started to convert to LC3B-II (Manuscript Fig 1a). Thus, 16 hours was selected as the optimal time to treat the cells with the different reagents.

The evaluation of γ-secretase activity was performed using γ-secretase inhibitors such as 250nM DAPT and 10uM Compound W. An ER calcium inhibitor, 100uM 2-APB, was used to evaluate the influence of store operated calcium. For MPTP inhibition, 1,6uM Cyclosporin A was used.

The cells were harvest after 16 hours of treatment, after lysis the protein concentration from the cultures was measured and stored at -20°C. Western blotting was used to determine protein markers of the induced stress. Densitometric analysis was performed using Image Studio Life 5.2 (LI-COR, Lincoln, NE, USA). Autophagy was measured using immunofluorescence. All cell lines were transient transfected with the GFP-RFP-LC3B tandem-Plasmid (Kimura 2007). Then, they were treated with the reagents for 16 hours and lively visualized using a Leica LCS-SP5 confocal microscope system.

To analyze the role of mutant-PS1 in the mitochondria, we used the mitochondrial permeability transition pore assay, according to the manufacture instructions (Thermo Fischer Scientific, Schwerte-Germany) and stablished protocols (Bonora et al 2016).

2.4 Results and Discussion

The mechanism of disease behind familial Alzheimer Disease is unclear and has been associated with multiple causes. The amyloid hypothesis, although is still worldwide accepted, more evidence showed that the real cause could be multifactorial. Mutant presenilins and amyloid precursor protein are the key players in FAD. In the present study we intent to test our hypothesis, that PS1 mutations induced a stronger susceptibility to cellular stress through different mechanisms including mitochondrial defects and calcium dysregulation in a cellular model.

The results of this thesis are part of the attached publication "**Susceptibility to cellular stress in PS1 mutant N2a cells is associated with mitochondrial defects and altered calcium homeostasis"** PMID: 32296078**,** by Sergio Calero-Martinez as a co-first author.

2.4.1 Cellular stress

After optimal time and doses were established, four groups of N2a cells were selected:

(1) Control group -Mock-

(2) N2a cells overexpressing human PS1 wild type -hPS1WT-

(3) N2a cells overexpressing the human PS1-Mutation E280A -hPS1E280A-

(4) N2a cells overexpressing the human PS1-Mutation Delta 9 -hPS1Δ9-.

Previous reports claimed that ER stress is among the mechanism of disease behind AD, activated by Aβ aggregation (Katajama et al. 1999, Fonseca et al. 2013 and De Strooper and Karran 2016). Thus, we intended to induce cell stress first through ER Stress in our cellular model. After treating the cells with Tunicamycin, Calcimycin and Antimycin, ER-stress was induced in all cell lines, being CHOP constantly significant when compared to the control of every group. Nevertheless, we did not find significant

differences in BiP, GADD34 and CHOP between the groups. (Manuscript Fig 1b). Since ER is the main calcium stored inside the cell, we were waiting for altered response to ER Stress after Tunicamycin or Calcimycin treatment. Nevertheless, no differences were seen after inducing ER stress in PS1-Mutant or hPS1 WT N2a-Cells.

Autophagy disruption

Disturbances in autophagy have been described as a consequence of Aβ deposition and toxicity. Glulielmoto et al, showed that AβO has an inhibitory effect in apoptosis but also in the accumulation of autophagosomes inside SK-N-BE neuroblastoma cells (Glulielmoto et al. 2014). In our study, LC3B conversion was significantly induced in the hPS1E280A group after starvation and less with Tunicamycin and Calcimycin, but not with AβOs. Autophagy was then evaluated with the LC3B-GFP-RFP tandem construct. hPS1E280A was the only group with a smaller number of red puncta after starvation compared to the other groups. Likewise, this mutation showed in the ultrastructural analysis an increased vacuoles formation after starvation showing considerably an altered response in the autophagy process (Manuscript Figure 2). Those findings are similar to previous studies, where lysosomal failure was described in the AD pathology, maybe through calcium dysregulation (McBrayer and Nixon 2013). Other authors have proposed functions of PS1 in autophagy as part of a γ-secretase independent process (Woodruff et al. 2013, Nixon 2013 and Lee et al. 2015).

Intracellular calcium dysregulation

As mentioned before, the role of presenilin in calcium homeostasis has been stressed out in several studies (Bezprozvanny 2012, McBrayer and Nixon 2013, Sepulveda-Falla et al. 2014 and Lee et al. 2015). It is also important to mention that PS1 could be involved in calcium homeostasis, accomplishing a function beyond the γ-secretase

activity (Honarnejad and Herms J 2012, Dugan and McCarthy 2016). After measuring cytosolic calcium concentrations, we observed in our experiments comparable results in N2a cells, where PS1E280A overexpressing cells showed higher amounts of calcium in the cytoplasmic and mitochondrial compartment, compared to the mock (Manuscript figure 3). Similarly essential was to observe, that PS1E280A had less calcium levels in the ER compared to the hPS1WT. hPS1WT and PS1mutants showed an increased mitochondrial calcium concentration. This causes us to believe in a PS1 function in the mitochondrial calcium homeostasis.

Mitochondrial alterations

Using the MPTP-Assay, we were able to induces the opening of the MPTPs in N2a cells. hPS1E280A exhibited an accelerated MPTP opening, meanwhile hPS1Δ9 showed a delayed reaction (Manuscript figure 4c,d). In western blotting those differences were sustained, by finding that cyclophilin D, the most studied component of the MPTP, was significantly higher in the mutant groups compared to the controls (Manuscript figure 4e). MPTP formation activates apoptosis via generation of reactive oxygen species (ROS), dissipation of mitochondrial membrane potential, mitochondrial calcium alterations, among others. Finally, we were able to probe a role of Mutant-PS1 in mitochondrial dysfunction. MPTP formation induces severe mitochondrial dysfunction with release of pro-apoptotic factors (Morais et al. 2002, Abramov et al. 2004 and Chauhan and Chauhan 2006). Some authors have related Aβ deposition to MPTP formation. Due and Yan reviewed the interaction of Aβ oligomers with CypD. This interaction might induce the formation of MPTP, consequently mitochondrial stress will end activating apoptosis and cell death (Du and Yan 2010). Nevertheless, in our understanding this reaction could be more related to the malfunctioning of PS1, and not to Aβ deposition.

PS1 functions beyond γ-secretase

We have shown up to now a multiorganelle dysfunction related to PS1 mutations. It is not clear which of them is dependent only to PS1 as a lonely functionally protein or as a part of the γ-secretase complex. The literature is also contradictory regarding this. To elucidate that, we inhibited the γ-secretase function with DAPT. Conversion of LC3B was still significantly higher after starvation and DAPT treatment in hPS1E280A cells. So that we confirmed a γ-secretase independent function of PS1 in autophagy. We appointed also to proof this as an independent reaction from calcium dysregulations, for that we measured the cytoplasmatic calcium after the treatment with DAPT and 2 APB -an ER calcium Inhibitor- in N2a cells. Likewise, only 2 APB showed elevated calcium in the cytoplasm and mitochondria of the hPS1 groups, where DAPT did not show differences when compared every group to its vehicle.

MPTP opening was delayed in the mock, in hPS1WT and in hPS1E280A cells but not in hPS1Δ9 after DAPT treatment. Meanwhile, 2 APB treatment altered the MPTP formation in both mutant PS1, blocking it in PS1E280A and accelerating it in hPS1Δ9. Subsequently, the role of PS1 in MPTP formation could be then associated to a γ-secretase dependent function.

From cellular stress to AD in PSEN1 mutants

In this study, we showed how PS1 mutations affect the response to cellular stress. Remarkably, this altered response affects simultaneously calcium homeostasis, lysosomal and mitochondrial function, on a mutation-specific manner. Here, the activation of different stress pathways was identified with or without AβOs. With our results, we can suggest, that PS1 plays a greater role in cellular homeostasis beyond its γ-secretase function. For FAD, the PSEN1 mutants render the cells more

susceptible to damage with a cumulative effect throughout life, inciting a multiorganelle intracellular dysfunction, that ends in AD.

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3. Zusammenfassung

Deutsch

Sporadische und familiäre AD haben möglicherweise einen gemeinsamen Mechanismus, der von der Aβ-Hypothese unabhängig ist. Neue Literaturrecherchen und mehr Veröffentlichungen zeigten unterschiedliche Mechanismen, die verdeutlichen, wie PS1 die neuronale Verschlechterung spezifisch bei FAD induziert. Wir haben Schritt für Schritt die Rolle von PS1 in einer γ-Sekretase-abhängigen und unabhängigen Weise bewiesen, die die normale zelluläre Wirkung auf Stress beeinflusst, unter Berücksichtigung der Einschränkungen eines zellulären Modells. Ein möglicher Mechanismus wurde im ER beschrieben, wo ER-Stress den UPR-Mechanismus aktivieren könnte. Obwohl wir in der Lage waren, ER-Stress zu induzieren, schien sich die zelluläre Reaktion darauf in der PS1-Mutante im Vergleich zu hPS1WT in N2a-Zellen nicht zu unterscheiden. Selbst eine durch Calcimycin induzierte Calciumveränderung reichte nicht aus, um Änderungen in der zellulären Reaktion auf ER-Stress zu induzieren. Autophagie war auch ein Schlüsselmechanismus, der seit vielen Jahren in AD verwandt ist. Unsere Ergebnisse korreliert Autophagie mit einer γ-Sekretase-unabhängigen Funktion von PS1, da wir nach Verwendung eines γ-Sekretase-Inhibitors (DAPT) nicht den gleichen Effekt sahen. Zuletzt hatten wir in unseren Experimenten PS1 und mitochondrialen Stress verbunden. Wir haben gezeigt, dass die mitochondriale Calciumdysregulation von PS1-Mutationen durch MPTP-Aktivierung abhängt und die mitochondriale und neuronale Toxizität im Kontext einer γ--Sekretase-abhängigen Funktion fördert. Zusammenfassend müssen weitere Studien zur zugrunde liegenden Veränderung der Zellorganellen und ihrer normalen Funktion durchgeführt werden, die sich nicht nur auf eine Aβ -Toxizität, sondern auch auf PS1-veränderte Funktionen beziehen

English

Sporadic and familiar AD might have a common mechanism independent to the Aβ-Hypothesis. Reviewing the literature more and mor publications showed different mechanism that clarifies how PS1 induces the neuronal deterioration specifically in FAD. We have step-by-step proof the role of PS1 in a γ-secretase dependent and independent manner, that affects the normal cellular action to stress, considering the limitations of a cellular model. One possible mechanism was described in the ER, where ER-stress could activate the UPR mechanism. Although we were able to induce ER stress, the cellular reaction to it, did not appear to be different in PS1 mutant compared to hPS1WT in N2a cells. Even calcium alteration induced by calcimycin was not enough to induced changes in the cellular response to ER Stress. Autophagy was also a key mechanism that has been related for many years in AD. We consider that our findings regarding autophagy correlates to a γ-secretase independent function of PS1, since we don't see the same effect after using a γ-secretase inhibitor (DAPT). Our last contribution was linked to PS1 and mitochondrial stress. We showed that mitochondrial calcium dysregulation depends on PS1 mutations through MPTP activation, promoting mitochondrial and neuronal toxicity in the context of a γ-secretase dependent function. Summing up, more studies in the underlying alteration in cellular organelles and their normal function must be done, not only related to a Aβ toxicity but also to PS1 altered functions.

4. Erklärung des Eigenanteils

Sergio Calero-Martinez (S.C-M). designed specific experiments of the study, namely starvation, mitochondrial stress and cell death, always under the direction and guidance of his Supervisor Diego Sepulveda-Falla and his Doctoral Advisor Markus Glatzel. Also, he standardized the reagents to induce ER and mitochondrial stress (Tunicamycin and Antimycin), starvation (Deprivation) and toxicity (AßO). S.C-M performed all the experiments regarding cell stress and death by western blotting and immunofluorescence, including the experiments with the γ-secretase inhibitors. He proposed and designed the first experiments that includes MPTP and cell death, with the corresponding statistical analysis. Sergio Calero-Martinez and Liliana Rojas-Charry drafted the manuscript supported by Diego Sepulveda-Falla. Finally, with the approval of all authors the manuscript was submitted to publication.

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6. Lebenslauf

Lebenslauf

Lebenslauf

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Lebenslauf

6. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....  .....