RESEARCH ARTICLE

Insulin Resistance Is a Modifying Factor for Parkinson's Disease

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ABSTRACT: Background: Parkinson's disease (PD) is the second most common, and the fastest-growing neurodegenerative disorder with unclear etiology in most cases. Therefore, the identification of non-genetic risk factors for PD pathology is crucial to develop effective preventative or therapeutic strategies. An increasing number of evidence suggests that central insulin resistance might have an essential role in PD pathology. Nevertheless, it is not clear whether insulin resistance arises from external factors/lifestyle, comorbidities such as type 2 diabetes or it can occur in a PD patient's brain independently from peripheral insulin resistance.

Objective: We aimed to investigate insulin resistance and its role in *GBA1* mutation-associated PD pathogenesis and phenotype severity.

Methods: Midbrain organoids, generated from induced pluripotent stem cells (iPSCs) of PD patients carrying the *GBA1*-N370S heterozygous mutation (GBA-PD) and healthy donors, were exposed to different insulin concentrations to modify insulin signaling function. Transcriptomics analysis was performed to explore insulin signaling gene expression patterns in GBA-PD and to find a potential target for GBA-PD-associated phenotype rescue.

Results: The insulin signaling pathway genes show dysregulation in GBA-PD. Particularly, we highlight that a knockdown of *FOXO1* mitigates the loss of dopaminergic neurons and cellular death in GBA-PD. Additionally, our findings suggest a promising therapeutic potential of the anti-diabetic drug Pioglitazone in decreasing dopaminergic neuron loss associated with GBA-PD.

Conclusion: Local insulin signaling dysfunction plays a substantial role in GBA-PD pathogenesis, exacerbating dopaminergic neuron death. © 2024 The Author(s). *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: comorbitity; diabetes; GBA; Parkinson's disease; pioglitazone

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Parkinson's disease (PD) and type 2 diabetes (T2D) are both age-related, complex diseases with fast-growing incidence over the last decades.[1-3] Recent studies have established a connection between both diseases through multiple shared disease mechanisms[4] and genetic links.[5] Insulin resistance is one of the pathophysiological molecular processes shared between these diseases. It has been shown to play a central role in disease pathophysiology, increasing oxidative stress, promoting mitochondrial damage, and leading to cell death.[6-8] We have previously shown in the midbrain organoid model from healthy donors that prolonged high insulin concentration in cell cultures results in cellular insulin resistance and leads to reduced amounts of dopaminergic neurons and significant metabolic alterations, which might predispose a healthy midbrain to PD pathology.[9] Furthermore, Alzheimer's disease (AD), another common neurodegenerative disorder, has been named type 3 diabetes because of the discovery of central insulin signaling dysfunction contributing to AD progression.[10] In addition, it has been reported that insulin resistance is highly prevalent among non-diabetic PD patients,[11] implying that insulin resistance can also occur independently or before a T2D diagnosis.

Insulin binding to its receptor activates the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway, which is highly complex and through diverse phosphorylation/dephosphorylation events regulates important cell functions including cellular growth, survival, and metabolism.[12,13] Moreover, several downstream proteins of insulin signaling are shown to be involved in the pathophysiology of PD.[14-16] In particular, forkhead box O (FOXO) transcription factors are linked to metabolic alterations, tyrosine hydroxylase (TH) levels[14] and synuclein alpha (αSyn) accumulation.[17] Depending on protein levels and activity regulated by posttranscriptional modifications, FOXOs can be both neuroprotective and neurotoxic.[18] Particularly, *FOXO1* and genes under its transcriptional regulation have been associated with PD pathology in genome-wide association studies (GWAS).[19] Target genes of *FOXO1* are involved in pathways, such as cell cycle, apoptosis, p53 signaling, metabolism, and oxidative stress management,[19,20] indicating the importance of *FOXO1* functional regulation in health and disease.

Another evidence that T2D and PD are connected is provided by the beneficial effects of T2D drugs on PD phenotypes.[21-24] Glucagon-like peptide-1 (GLP-1) receptor agonists exenatide and liraglutide, as well as the thiazolidinedione derivate pioglitazone, have completed phase 2 of clinical trials.[25-27] Although GLP-1 agonists showed PD-modifying properties, pioglitazone clinical trial results were rather inconclusive and controversial to other studies, which demonstrate pioglitazone association with reduced risk of PD.[23,26,28] In addition, the antihyperglycemic drug metformin has been associated with a decreased risk of PD in a dose-dependent manner.[24]

In this study, to investigate how insulin resistance affects PD phenotype severity and explore the potential cause of insulin signaling dysregulation in PD, we generated midbrain organoids from PD patients carrying the *GBA1*-N370S mutation (GBA-PD), which is one of the most common genetic variants in the *GBA1* gene associated with PD.[29] We cultured midbrain organoids under two different insulin concentrations to promote insulin resistance or activate insulin signaling.[9] In addition, we compared insulin signaling transcriptomic signatures between healthy and GBA-PD midbrain organoids to investigate the insulin signaling state at the gene expression level. We identified *FOXO1* as a potential target in GBA-PD and showed that its downregulation rescues GBA-PD-associated dopaminergic neuron loss. Eventually, we treated GBA-PD midbrain organoids with known anti-diabetic drugs—pioglitazone and metformin to explore their potential beneficial effects on GBA-PD. Overall our results demonstrate that insulin signaling is dysregulated in GBA-PD and that it is a major factor in determining the severity of PD-associated phenotypes.

Materials and Methods

The approval from the Ethics Review Panel of the University of Luxembourg and the national Luxembourg Research Ethics Committee (CNER no. 201901/01; ivPD) to work with induced pluripotent stem cells (iPSCs) is in place.

Cell lines used in this study are summarized in Supplementary Table S1. As a starting population for midbrain organoid generation, we used iPSC-derived neuroepithelial stem cells (NESCs) described in Reinhardt et al.[30] Organoids were differentiated following a previously published protocol by Nickels et al[31] until day 30 or day 60 of organoid culture depending on the experiment. Insulin resistant organoids were cultured in N2B27 base medium containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Thermo Fisher Scientific, Waltham, MA, cat. no. 21331046) and Neurobasal (Thermo Fisher Scientific, cat. no. 10888022) 50:50, supplemented with 1:200 N2 supplement (Thermo Fisher Scientific cat. no. 17502001), 1:100 B27 supplement without vitamin A (Life Technologies, Carlsbad, CA, cat. no. 12587001), 1% GlutaMAX (Thermo Fisher Scientific, cat. no. 35050061), and 1% penicillin/streptomycin (Thermo Fisher Scientific, cat. no. 15140122). For insulin-sensitive cultures, the N2 supplement was substituted by a self-made N2 supplement without insulin starting from day 2 of organoid culture. Self-made N2 was prepared as described in Harrison et al[32] adding human apo-transferrin (Sigma, St. Louis,

MO, cat. no. T1147), putrescine dihydrochloride (Sigma, cat. no. P5780), sodium selenite (Sigma, cat. no. S5261), and progesterone (Sigma, cat. no. P8783) in the cell culture media in the same concentrations as in the commercial N2 supplement. Midbrain organoid treatment with 100 nM of customized LNA-enhanced antisense oligonucleotides against *FOXO1* (Qiagen, Hilden, Germany), 10 µM pioglitazone (Sigma, cat. no. E6910), and 500 µM metformin (STEMCELL Technologies, Vancouver, Canada, cat. no.73252) was performed from day 2 organoid culture until day 60. A detailed description of all other procedures can be found in Supplementary Data S1. Antibodies used for immunofluorescence stainings are given in Supplementary Table S2.

Data Sharing

All data supporting the conclusions of this article are publicly available at: DOI 10.17881/d22s-x010. RNA sequencing data is available on Gene Expression Omnibus (GEO) under the accession code GSE237116. MATLAB and R scripts used for data analysis are available on GitHub at: https://gitlab.com/uniluxembourg/lcsb/developmental-and-cellular-biology/zagare_2024.

Results

GBA-PD Organoids Show Dopaminergic Neuron Loss and an Altered Lipid Profile

To assess whether insulin resistance accelerates PD phenotypes, we followed the workflow described in Zagare et al[9] and cultured healthy control (WT) and GBA-PD midbrain organoids under insulin-resistant (IR) or insulin-sensitive (IS) conditions. IR conditions are equivalent to standard cell culture conditions that are used in most studies. We have previously shown that IS conditions activate insulin signaling by increasing the expression of insulin receptor substrate 1 (IRS1), elevating AKT phosphorylation and increasing glucose uptake.[9] Here, we confirmed that under IR conditions, GBA midbrain organoids exhibit a significantly reduced number of TH-positive dopaminergic neurons and dopamine depletion compared to WT midbrain organoids (Fig. 1A,B), as also shown before.[33] IS conditions led to a significant increase of TH-positive dopaminergic neurons in WT organoids, but not in GBA-PD midbrain organoids (Fig. 1A–C). Nevertheless, both WT and GBA-PD midbrain organoids cultured under IS conditions showed significantly fewer apoptotic events compared to IR counterparts, suggesting that insulin signaling function is essential for the overall cellular viability (Fig. 1D,E).

Mutations in the *GBA1* gene are associated with lipid changes because of the altered *GBA1*-encoded glucocerebrosidase (GCase) enzymatic activity.[33,34] In line with previous studies, we observed a strongly altered lipid profile in GBA-PD midbrain organoids (Supplementary Fig. S1). Although, in a principal component analysis (PCA) analysis, we observed that the lipidomics profiles of IR and IS GBA-PD samples were not completely separable, contrary to WT samples, IR GBA-PD organoids demonstrated a tendency to cluster more with IR WT organoids, whereas IS GBA-PD organoids were closer to IS WT organoid cluster, suggesting a role of insulin signaling in the regulation of lipid metabolism (Supplementary Fig. S1A). Overall, most of the lipid species in GBA-PD samples were at significantly lower levels compared to WT organoids, confirming severe *GBA1* mutation-dependent lipid metabolism alterations (Supplementary Fig. S1B). Similarly, to WT organoids, cholesterol esters were the most differentially abundant lipid class between IR and IS GBA-PD samples (Fig. 1F), implying that cholesterol ester levels strongly depend on the insulin signaling functional state. Another lipid class, which demonstrated insulin function-dependent changes in both WT and GBA-PD organoids, were cell membrane phospholipids–1-alkenyl, 2-acyl phosphatidylcholine (PC-Os) (Fig. 1G), which is the prevailing phospholipid class in mammalian cells, essential for many cellular functions, including protein trafficking, endoplasmic reticulum (ER) health, and membrane fluidity.[35-37] Although we did not detect a *GBA1* mutation-associated increase of total ceramide or hexosylceramide levels[38] in GBA-PD organoids, we observed a significant increase in the relative abundance of those species, derived from myristic (C14) and palmitic acid (C16) compared to other ceramide structures (Fig. 1H). Particularly, ceramide species with a palmitic acid backbone have been considered as a shared biomarker between several neurodegenerative diseases.[39] IS GBA-PD samples showed lower levels of C14 and C16-derived ceramide species, suggesting an insulin resistance contribution, particularly to toxic ceramide accumulation.

Overall, these results show that healthy midbrain organoids are more responsive to a change in insulin signaling activity than GBA-PD midbrain organoids. As a result, when comparing IR to IS conditions, the effects are more pronounced in healthy midbrain organoids than in GBA-PD ones.

FOXO1 as a Potential Drug Target in GBA-PD

To further evaluate if insulin signaling plays a role in GBA-PD pathogenesis, we analyzed transcriptomic signature differences of insulin signaling genes between WT and GBA midbrain organoids at IR culturing conditions. Based on the expression of insulin signaling-associated genes from the Kyoto Encyclopedia of Genes and Genomes database[40] we observed that IR WT and IR GBA-PD organoid samples cluster separately, suggesting

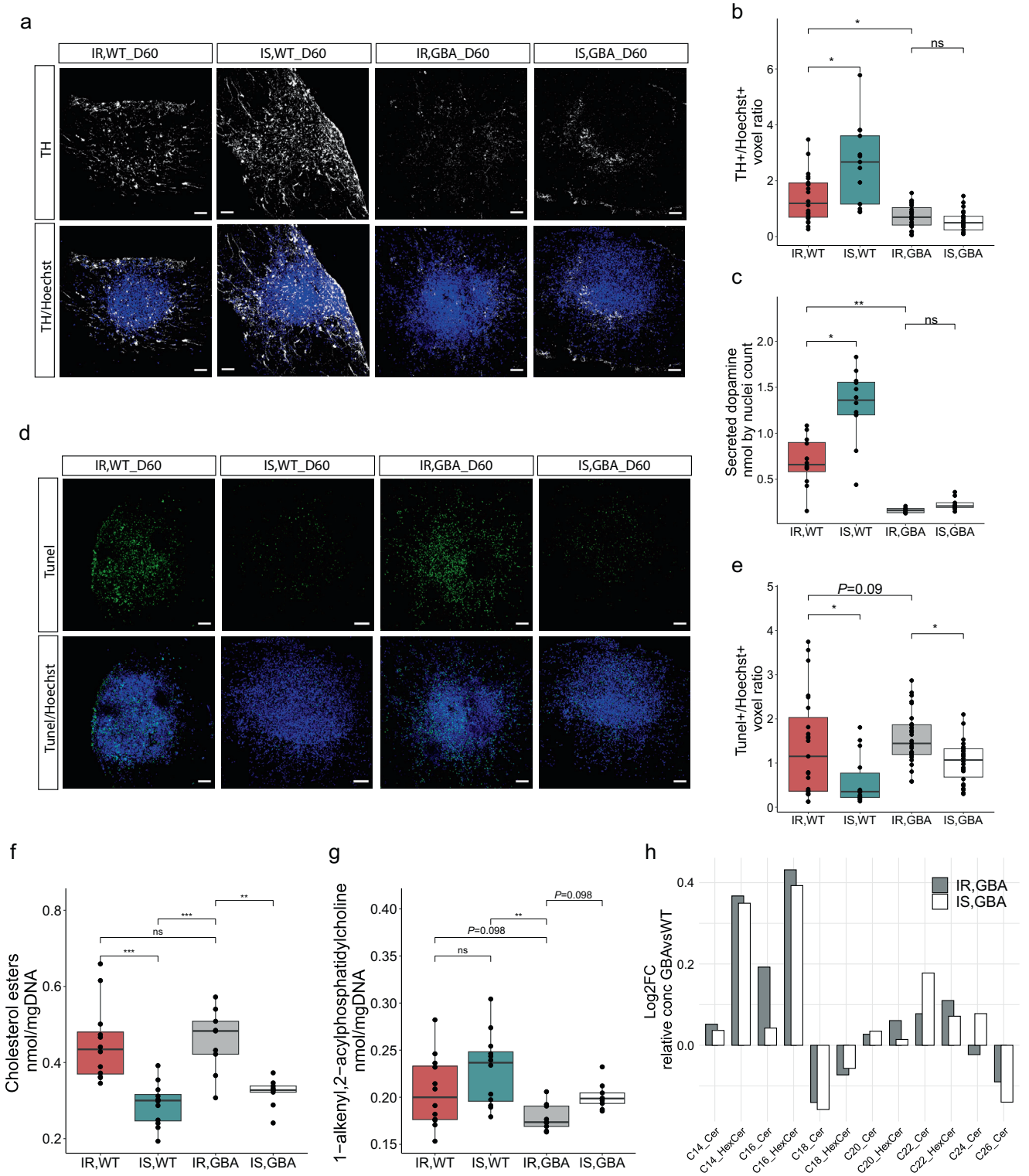

FIG. 1. *GBA1*-N370S mutation (GBA-PD) organoids show dopaminergic neuron loss and altered lipid profile. (**A**) Representative immunofluorescence images of MAP2 and tyrosine hydroxylase (TH) staining in sections of 60 days old GBA-PD and healthy control (WT) midbrain organoids cultured in insulin resistant (IR) or insulin sensitive (IS) conditions. Scale bars: 100 μm. (**B**) Quantification of TH-positive area normalized to the Hoechst area in 60 days old midbrain organoids. (**C**) Extracellular dopamine levels measured in spent media of 60 days old midbrain organoids. (**D**) Representative immunofluorescence images of Tunel staining in sections of 60 days old midbrain organoids. Scale bars: 100 μm. (**E**) Quantification of Tunel-positive area normalized to the Hoechst area in 60 days old midbrain organoids. (**F**) Quantification of cholesterol esters. Concentration normalized to the metagenomic DNA. (**G**) Quantification of 1-alkenyl, 2-acylphosphatidylcholine. Concentration normalized to the metagenomic DNA. **(H)** The relative concentration of the sum of ceramide and hexosylceramide species sharing the same carbon number expressed as log2 fold change (FC) in GBA-PD samples against insulin-resistant (IR) healthy control (WT) samples. Statistical significance was tested using the non-parametric Kruskal-Wallis test for multiple comparisons, followed by Dunn's post hoc test. *P* values are adjusted using Benjamini-Hochberg method (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). [Color figure can be viewed at wileyonlinelibrary.com]

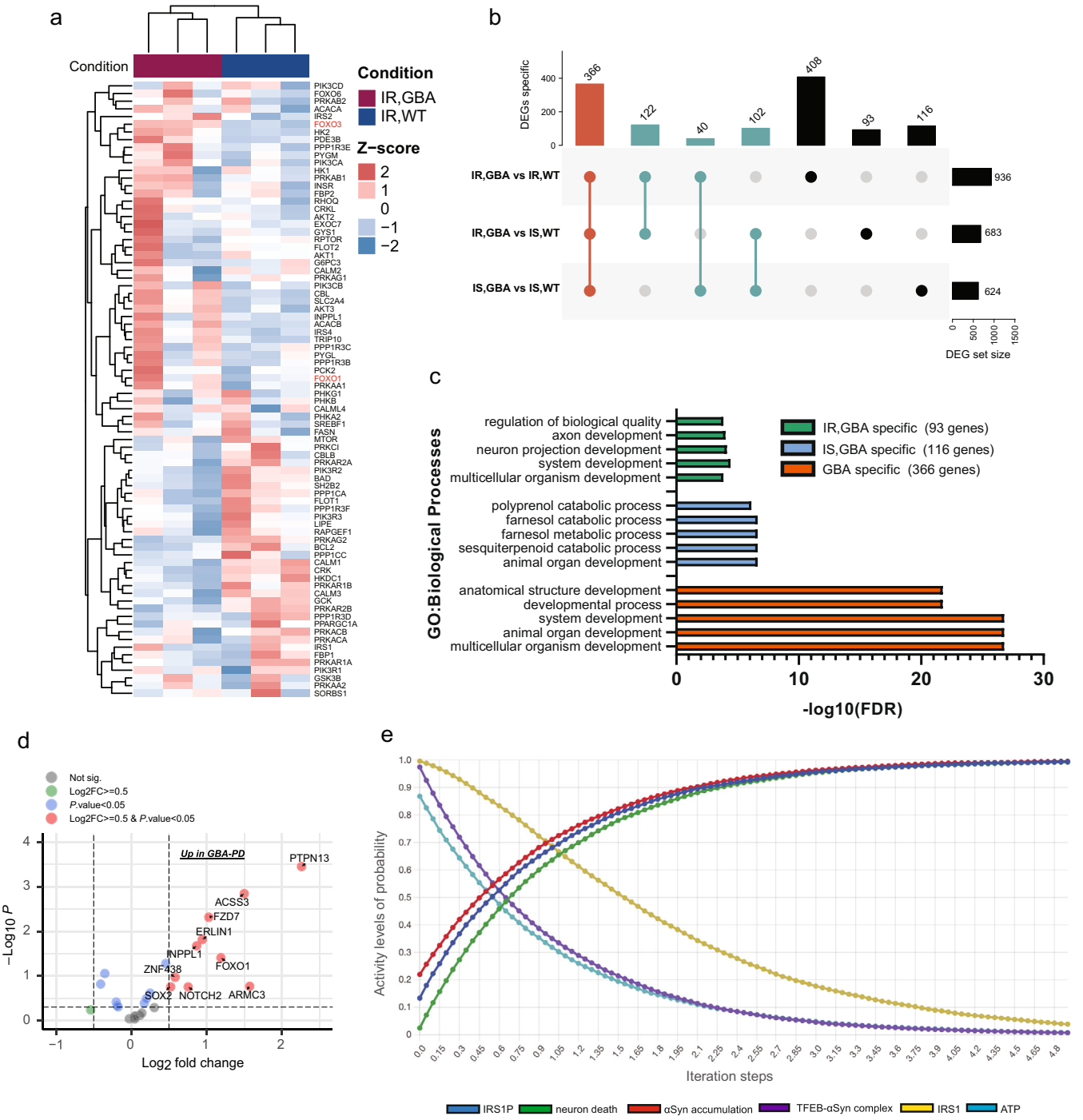

FIG. 2. Transcriptome analysis of *GBA1*-N370S mutation (GBA-PD) midbrain organoids. (**A**) Unsupervised clustering of insulin signaling genes between insulin-resistant (IR), GBA, and IR, healthy control (WT) midbrain organoids. Gene expression was normalized using Z-score transformation. (**B**) UpSet plot demonstrating the number of unique differentially expressed genes (*P*.adjust < 0.05) found in each comparison. (**C**) Gene enrichment analysis of the selected specific genes. (**D**) Volcano plot demonstrating expression of a set of *FOXO1* target genes in IR, GBA versus IR, WT midbrain organoids. (**E**) A probabilistic Boolean model with defined perturbations: *AKT1*-OFF and *FOXO1*-ON, demonstrating dynamic behavior of downstream effects associated with Parkinson's disease (axis-Y) over the number of iterations (axis-X). [Color figure can be viewed at wileyonlinelibrary.com]

insulin signaling dysregulation already at gene expression level in GBA-PD (Fig. 2A). Among the major insulin signaling effectors downstream of the central protein AKT, only *FOXO1* and *FOXO3* genes were significantly differentially expressed between IR WT and IR GBA-PD midbrain organoids (Fig. 2A and Supplementary Fig. S2A), indicating that in GBA-PD insulin resistance effects might be governed by FOXOs. Further, to assess the potential contribution of the defective insulin signaling in GBA-PD, we compared differentially expressed genes

(DEGs) between IR GBA-PD versus IR WT; IR GBA-PD versus IS WT and IS GBA-PD versus IS WT organoid samples. We visualized distinct genes for each comparison (Fig. 2B) and performed gene functional enrichment analysis for the DEG clusters of interest (Fig. 2C). A total of 366 DEGs that overlapped between all comparisons, representing biological processes dysregulated in GBA-PD independently of IR and IS state, were enriched in developmental processes. A total of 116 DEGs that were distinct to the comparison IS GBA-PD versus IS WT, demonstrating biological processes dysregulated in GBA-PD assuming normal insulin signaling function, were enriched in specific plant-derived metabolite catabolic processes with neuroprotective properties.[41] Finally, 93 DEGs that were specific for the comparison IR GBA-PD versus IS WT, representing biological processes dysregulated in GBA-PD accelerated by insulin resistance, were enriched in organ development and particularly in neuronal developmental processes.

Next, we evaluated the possible role of *FOXO1* in governing insulin resistance effects in GBA-PD by exploring the expression of *FOXO1* and its targets reported to be found highly expressed in PD patient brains.[19] We confirmed significantly increased expression of nine of 29 reported *FOXO1* target genes (Fig. 2D). Additionally, we applied probabilistic Boolean modeling (PBM) to the transcriptomics data to predict *FOXO1*-associated phenotypes with simulations reflecting insulin resistance-dependent *FOXO1* activity upregulation (*AKT1*:OFF and *FOXO1*:ON) in GBA-PD midbrain organoids (Fig. 2E). Based on this model, impaired *FOXO1* inhibition by *AKT1* because of insulin resistance potentially leads to an energy crisis resulting in reduced ATP levels. Consequently, insulin resistance would increase by activation of the feedback loop because of accelerated IRS1 phosphorylation leading to its subsequent degradation. Additionally, the model predicted the accumulation of αSyn and neuronal death. Altogether, the transcriptomics data analysis and PBM-derived predictions suggest that multi-faceted assault driven by insulin resistance-dependent *FOXO1* overactivation likely contributes to the severity and progression of GBA-PD.

FOXO1 Depletion and Pioglitazone Rescue GBA-PD Associated Dopaminergic Neuron Loss

To experimentally confirm *FOXO1* contribution to the GBA-PD progression regarding dopaminergic neuron loss, dopamine secretion and cell death, we treated GBA-PD midbrain organoids with antisense oligonucleotides (ASOs) targeting *FOXO1* (Fig. 3A and Supplementary Fig. S2B–G). The efficiency of *FOXO1* knockdown by ASOs was confirmed in WT organoids, showing that 28-day-long treatment downregulates FOXO1protein by ∼25% (Supplementary Fig. S2D,E). To achieve higher knockdown efficiency, we treated GBA-PD organoids for a longer time—until day 60 of organoid culture. We observed that the downregulation of *FOXO1* by ASOs, significantly restored dopaminergic neuron numbers and increased dopamine levels in GBA-PD midbrain organoids (Fig. 3B–D). Moreover, *FOXO1* downregulation significantly reduced apoptosis in GBA-PD midbrain organoids (Fig. 3B,E), further highlighting *FOXO1* as a potential target in PD modifying therapy.

Finally, we treated GBA-PD midbrain organoids with two widely used anti-diabetic drugs—metformin and pioglitazone, to evaluate their potential in PD phenotype amelioration (Fig. 3A). We did not observe a significant increase in dopaminergic neuron amount and dopamine levels on metformin treatment (Fig. 3F,G). Nevertheless, metformin reduced overall cell death in GBA-PD midbrain organoids (Fig. 3H). The treatment of GBA-PD midbrain organoids with pioglitazone led to a significant increase in TH-positive dopaminergic neuron numbers and elevated dopamine levels (Fig. 3I,J). In addition, also here we observed a reduction in cell death in GBA-PD midbrain organoids when compared to IR GBA-PD condition (Fig. 3K). Taken together, these results demonstrate pioglitazone's potential to halt or delay dopaminergic neuron loss in GBA-PD and suggest *FOXO1* as an important contributor to the severity of GBA-PD dopaminergic neuron loss and cellular death phenotypes.

Discussion

Central insulin signaling is crucial for energy homeostasis, synaptic plasticity and overall neuronal survival.[8,42,43] Particularly, here, we investigated how insulin signaling dysfunction associated with insulin resistance affects main GBA-PD-associated phenotypes—dopaminergic neuron loss, reduced dopamine levels, increased cell death, and changed lipid metabolism. We have previously described that a more physiological insulin concentration in cell culture media can increase dopaminergic neuron amount and functionality.[9] In this study, we observed that GBA-PD midbrain organoids cultured in a more physiological insulin concentration show improved cellular viability and normalization in the levels of some important lipids.

Altered lipid metabolism seems to present an important link between T2D and PD. Mutations in the *GBA1* gene lead to the loss of the enzymatic function of GCase, which is crucial in sphingolipid, ceramide and hexosylceramide metabolism. Impaired activity of GCase is associated with the accumulation hexosylceramides that promotes αSyn pathology.[34,44] It has been reported that particularly short-chain fatty acid

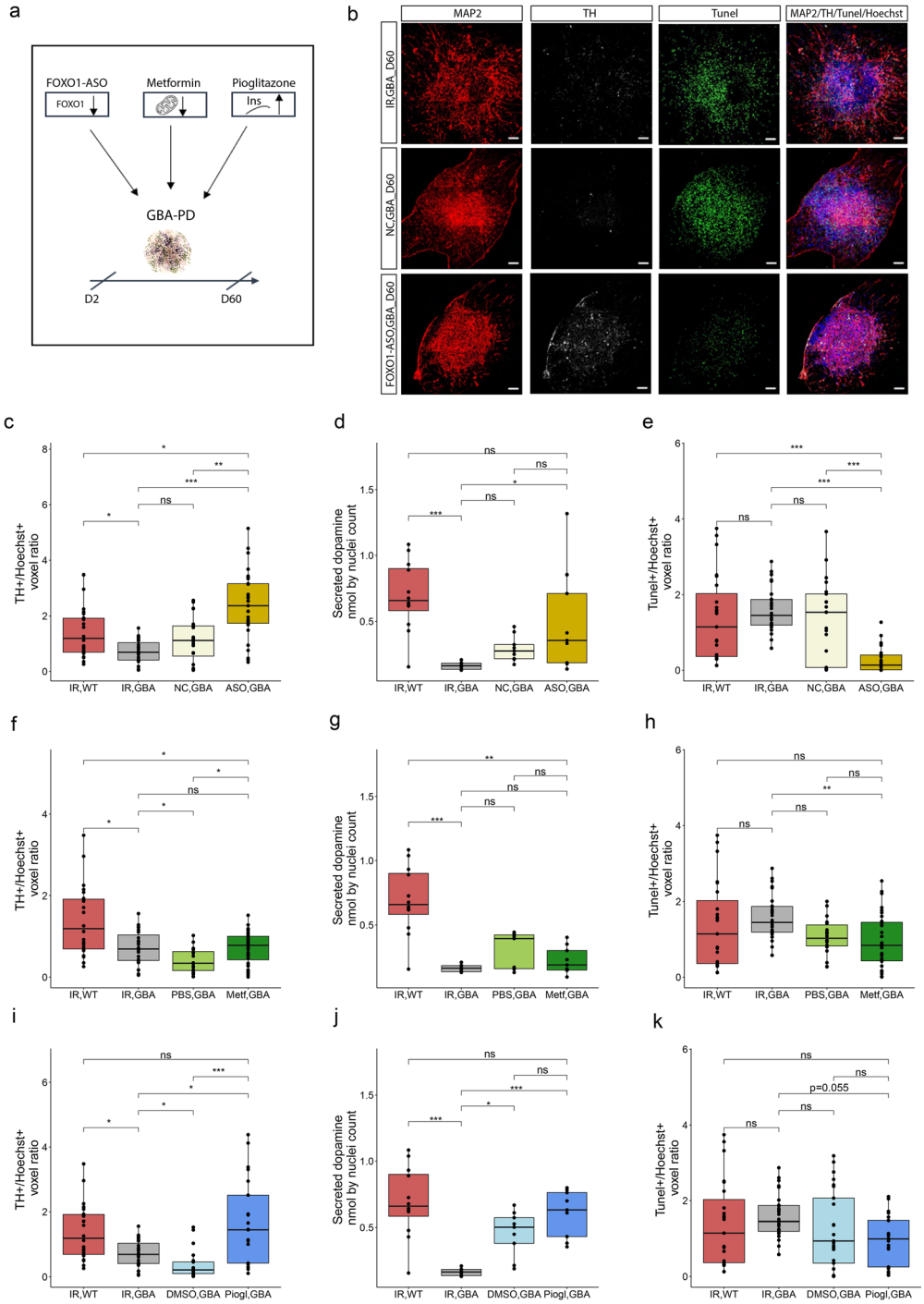

FIG. 3. Rescue experiments of *GBA1*-N370S mutation (GBA-PD) associated phenotypes using antisense oligonucleotides (ASOs) and anti-diabetic drugs. (**A**) Organoid treatment strategy with ASOs, pioglitazone, and metformin. (**B**) Representative immunofluorescence images of MAP2, tyrosine hydroxylase (TH) and Tunel staining in sections of 60 days old GBA-PD midbrain organoids treated with negative control (NC), and ASO targeting *FOXO1* transcript (FOXO1-ASO). Scale bars: 100 μm. (**C**) Quantification of TH-positive area normalized to the Hoechst area in 60 days old GBA-PD midbrain organoids treated with NC, and ASO targeting *FOXO1* transcript. (**D**) Extracellular dopamine levels measured in spent media of 60 days old GBA-PD midbrain organoids treated with NC, and ASO targeting *FOXO1* transcript. (**E**) Quantification of Tunel-positive area normalized to the Hoechst area in 60 days old GBA-PD midbrain organoids treated with NC, and ASO targeting *FOXO1* transcript. (**F**) Quantification of TH-positive area normalized to the Hoechst area in 60 days old GBA-PD midbrain organoids treated with vehicle (dimethylsulfoxide [DMSO]) and pioglitazone (Piogl). (**G**) Extracellular dopamine levels measured in spent media of 60 days old GBA-PD midbrain organoids treated with vehicle (DMSO) and Piogl. (**H**) Quantification of Tunel-positive area normalized to the Hoechst area in 60 days old GBA-PD midbrain organoids treated with vehicle (DMSO) and Piogl. (**I**) Quantification of TH-positive area normalized to the Hoechst area in 60 days old GBA-PD midbrain organoids treated with vehicle (phosphate buffered saline [PBS]) and metformin (Metf). (**J**) Extracellular dopamine levels measured in spent media of 60 days old GBA-PD midbrain organoids treated with vehicle (PBS) and Metf. (**K**) Quantification of Tunel-positive area normalized to the Hoechst area in 60 days old GBA-PD midbrain organoids treated with vehicle (PBS) and Metf. Statistical significance was tested using the non-parametric Kruskal-Wallis test for multiple comparisons, followed by Dunn's post hoc test. *P* values are adjusted using Benjamini-Hochberg method (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). [Color figure can be viewed at wileyonlinelibrary.com]

ceramides and hexosylceramides are increased in GBA-PD and are also found in lipid extracts from αSyn fibrils.[39,44] Our findings were consistent with these previous studies and demonstrated higher relative levels of C14 and C16 ceramides and hexosylceramides in GBA-PD, whereas, for instance, C18 species were relatively decreased compared to WT.[33] Ceramides with 18 carbons are the most abundant in the brain and reduction of this ceramide species has been associated with neurodegeneration.[45] Interestingly, insulin resistance seems to accelerate the increase, particularly of toxic ceramide species of C14 and C16. Moreover, we showed increased levels of cholesterol esters in IR WT and IR GBA-PD samples compared to IS samples, which is consistent with previous studies that have reported elevated cholesterol ester levels in neurodegenerative diseases.[46-48]

We found that GBA-PD midbrain organoids have different transcriptome profiles of insulin signaling-associated genes, suggesting that insulin signaling dysfunction has a local cause and is implicated in GBA-PD pathology, possibly preceding central insulin resistance. Interestingly, DEG enrichment between GBA-PD and WT samples, independently from the insulin signaling state, showed an association of GBA-PD with developmental processes, which has also been recently described.[33] DEGs between GBA-PD IR and IS conditions suggested that insulin signaling plays a role, particularly in neuronal development. Furthermore, the *GBA1* mutation alone, without additional extracellular insulin burden, affects the metabolism of sesquiterpenes. These plant-derived bioactive molecules exhibit neurotrophic effects and have been linked to cognitive improvements in AD.[49,50] Moreover, farnesol, which is also a sesquiterpene, is an intermediate of cholesterol synthesis, crucial for the development and proper function of neurons.[51] These results suggest that mutation in the *GBA1* alone would cause essential metabolic changes, leading to a disrupted cellular developmental process. However, insulin signaling dysfunction locally in the midbrain might amplify the *GBA1*-mutation-caused phenotypes, particularly affecting neuronal development.

Overall, insulin signaling is a highly complex signaling pathway, therefore, we focused on major effectors of the pathway downstream of AKT. We found that *FOXO1* and *FOXO3* transcription factors were significantly differentially expressed between WT and GBA-PD midbrain organoids. We further focused on *FOXO1*, based on a study demonstrating of not only *FOXO1* but also its target gene upregulation in PD patient brain samples.[19] To predict *FOXO1* overexpression effects in GBA-PD, we performed PBM. The model highlighted that insulin resistance with specific perturbations (*AKT1*-OFF, *FOXO1*-ON) is a significant contributing factor to the progression of GBA-PD. The gradual changes in the associated parameters suggested a complex interplay between insulin resistance, altered energy metabolism, and increased cellular death. Furthermore, the experimental knockdown of *FOXO1* by ASOs increased the amount of dopaminergic neurons and dopamine and attenuated cell death in GBA-PD midbrain organoids. These results provide further evidence for the role of *FOXO1* in GBA-PD phenotype modulation.

There is a growing amount of studies and clinical trials addressing the usage of anti-diabetic medication in PD therapy. In this study, we chose to explore metformin's and pioglitazone's effects on GBA-PD. Metformin is described as a mitochondrial complex I inhibitor,[52] pioglitazone increases the activity of insulin signaling by selectively stimulating nuclear receptor peroxisome proliferator-activated receptor γ.[53] Both anti-diabetic medications led to reduced number of apoptotic events in GBA-PD midbrain organoids. However, considering the multicellular composition of midbrain organoids,[54,55] this effect may be cell type-specific and requires further investigation. Metformin appeared inefficient in reversing GBA-PD-associated TH-positive dopaminergic neuron loss in the organoids, suggesting that its ability to reduce apoptosis might be attributed to other neuronal types or glia instead of dopaminergic neurons. Moreover, our results support pioglitazone's beneficial effects in GBA-PD, demonstrating its ability to restore amounts of dopaminergic neurons and dopamine levels in GBA-PD-treated organoids, which indicates underlying insulin signaling dysfunction in GBA-PD. Although the clinical trial for pioglitazone was not extended for phase 3, with the conclusion that it is unlikely to modify PD progression,[26] multiple studies, including meta-analysis, have reported that pioglitazone is associated with reduced risk of PD in subgroups of patients.[23,28] This suggests the high importance of therapeutic specificity and a need for a well-stratified approach in PD therapeutics development and treatment.

Altogether our results show that insulin signaling dysregulation in GBA-PD aggravates dopaminergic neuron loss via *FOXO1* overexpression, whereas high levels of insulin alter lipid metabolism and promote cellular death, highlighting insulin signaling as a relevant target to address PD-associated neurodegeneration. ■

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Data Availability Statement

The data that support the findings of this study are openly available in R3 pages at https://r3.lcsb.uni.lu/frozen/10.17881/d22s-x010.

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