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Transcription Factor EB: The Next Target for Autophagy in Parkinson's Disease

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Abstract

As the prevalence of Parkinson's disease rises, there is a greater need to understand its pathology. The accumulation of intracellular α-synuclein in dopaminergic neurons in the substantia nigra in the basal ganglia region causes neurodegeneration and the development of motor and non-motor symptoms. Pathways that lead to neurodegeneration, such as mitochondrial activity, synaptic communication, neuroinflammation, and the autophagy-lysosome system, are not well understood. Particularly, autophagy is a vital process in which misfolded proteins are recycled, and it appears that this process is disrupted in Parkinson's disease. This review highlights how Transcription Factor EB (TFEB) contributes to the induction of autophagy and lysosomal activity. TFEB is a protective protein that promotes the expression of autophagy-related genes upon nuclear translocation to ultimately clear α-synuclein aggregates. In recent years, multiple studies have identified targets that prevent TFEB activity and compounds that upregulate TFEB, such as curcumin, myriocin, and trehalose. Additionally, it appears that TFEB can be modified through the sphingomyelin/ceramide axis. A greater understanding of Parkinson's disease pathology and pathways will help future drug development, as current treatments only alleviate associated symptoms. Ultimately, there needs also to be a focus on whether α-synuclein clearance through pharmaceutical interventions is effective in stopping the progression of Parkinson's disease.

Keywords

Neuroscience, Parkinson's disease, TFEB, autophagy, α-synuclein, sphingomyelin, ceramide, neurodegeneration, curcumin, trehalose, myriocin

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Introduction

Prevalence and Etiology

As the population ages in the United States, there is a significant concern about the rise in age-related ailments, such as Parkinson's disease (PD), which is currently the fastest-growing neurodegenerative disease. A global meta-analysis found that PD was present in up to 1,903 persons for every 100,000 persons over 80 years (1). PD prevalence is quickly escalating, so it is vital to understand the genetic and environmental factors that may influence disease development.

PD is categorized into two subtypes: sporadic PD and familial PD. While familial PD accounts for up to 10% of PD cases, sporadic PD makes up the majority of PD cases (2). Studying familial cases provides insight into the pathology of PD. Familial mutations include A53T α-synuclein (also known as SNCA), Leucine-rich repeat kinase 2 (LRRK2) (3), DJ-1 (4), and ATPase cation transporting 13A2 (ATP13A2) (5). Although rare, A53T (6), A30P (7) (7), and E46K (8) mutations were identified as autosomal dominant α-synuclein missense mutations. In regards to how the environment influences the development of PD, several toxins, such as 6-Hydroxydopamine (6-OHDA), have been confirmed as toxic to dopaminergic neurons (DAn) (9). Additionally, the compound 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is particularly toxic to DAn since once it is taken up, complex 1, which controls the mitochondrial electron transport chain (ETC), is blocked. The inhibition of complex 1 causes loss of ATP, oxidative stress, and eventually, degeneration of dopaminergic neurons (10-11). N-Methyl-4-phenylpyridine (MPP+) is another toxin that is a metabolite of MPTP and has been shown to cause loss of DAn in the nigrostriatal pathway (12). Genetics and several toxic compounds contribute to the onset of PD.

Targeting α-synuclein through autophagy

An early study from 1988 found that in PD brains, there is a 60% loss of DAn in the SNpc (13). Furthermore, many of the symptoms of PD are caused by the loss of dopaminergic neurons (DAn) in the substantia nigra pars compacta (SNpc) and striatum (14), and further exploration is needed to identify how to prevent cell death. While DAn are also vulnerable to neuronal death due to mitochondrial dysfunction (15), one hypothesis is that protein aggregate inclusions, made up of α-synuclein, are the cause of these inclusions, as they were found in these regions in PD patients (16). Higher expression levels of α-synuclein are toxic, thus resulting in cell death (17). In addition, in rats, α-synuclein expression increases due to the extensive length of DAn axons (14, 18), α-synuclein is, therefore, an essential biomarker in PD, and inclusions of these proteins, termed Lewy Bodies (LBs), are found in the soma and neurites of neurons (16). α-synuclein is present in blood, cerebral spinal fluid (CSF), saliva, serum, plasma, and peripheral tissues. α-synuclein can take different forms as oligomers and fibrils due to oxidative stress (19), and post-translational changes may additionally alter its size, charge, structure, and conformation (20). α-synuclein oligomers cause neuronal death (21), and higher levels induce toxic protein aggregation (22-23). Hence, much of PD research focuses on targeting and clearing α-synuclein due to its toxicity.

One way the cell eliminates and recycles proteins is through autophagy. In fact, impaired autophagy is linked to the progression of PD (24), suggesting that

excess α-synuclein inclusions contribute to disease severity. There are three kinds of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). The name autophagy generally refers to macroautophagy which is the most well-understood of the three types. In macroautophagy, cytosolic components are collected in a phagophore (25). As the cargo is collected and the phagophore expands, it becomes an autophagosome (26). After an autophagosome is formed, the outer layer of the autophagosome then fuses to the lysosomal membrane, arranging an autolysosome (26-27). The autolysosome contents are broken down by hydrolase enzymes in the lysosome and recycled (26, 28). Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a protein, and LC-I is a type of cytosolic LC3. While the autophagosome is being formed, LC3-I is joined to phosphatidylethanolamine and conjugated to LC3-phosphatidylethanolamine (LC3-II). LC3-II is degraded by the lysosomes, making it an important biomarker of autophagosome membranes and autophagic activity (29-31). Another significant biomarker is the autophagosome cargo protein, Sequestosome-1 (SQSTM1), or ubiquitin-binding protein p62 that transports ubiquitinated cargo for selective autophagy (32-33) This protein also binds to LC3-II to promote autophagy (34). A similar process to macroautophagy occurs in microautophagy, although lysosomal invaginations take the cargo (26, 35). In chaperone-mediated autophagy, proteins containing the KFERQ sequence are targeted by the heat shock 70 kDa protein 8 (HSPA8/HSC70), and HSC70 brings the protein to the lysosomal membrane for protein unfolding (26, 36-38). The protein binds to the lysosomal-associated membrane protein 2A (LAMP2A) receptor and translocates to the lysosome with the help of chaperone protein HSC70 (26, 39). Lysosomal-associated membrane protein 1 (LAMP1) and LAMP2A are indicators of lysosomal activity due to their role in maintaining pH, catabolic activity, and structural support of the lysosomal membrane (31). Since autophagy is necessary for the cell, it is essential to understand its regulators, including Transcription Factor EB (TFEB).

TFEB-Induced Autophagy

TFEB plays a specific role in the mammalian target of rapamycin (mTOR) kinase pathway (39). mTOR consists of two complexes, the mammalian target of rapamycin complexes 1 and 2 (mTORC1 and mTORC2) (41-43). In basal conditions, mTORC1 is brought to the lysosomal surface and inhibits autophagy. However, under starvation conditions, mTORC1 is deactivated, autophagy is induced, and TFEB is dephosphorylated. As a result, TFEB relocates to the nucleus from the cytoplasm, affects gene expression, and, therefore, induces autophagy (40, 43-44). Under nutrient-rich conditions, TFEB is phosphorylated by mTOR on S211, recruited to the lysosomal surface, and used as a binding site, held in the cytosol (44-45). TFEB overexpression causes lysosomal biogenesis in HeLa cells (46) and may contribute to the fabrication of autophagosomes (40). TFEB is a crucial target as its recent developments in autophagy are promising for PD pathology.

Lysosome Pathway

The lysosome is intimately connected with autophagy through the autophagy-lysosome pathway (ALP). For many neurodegenerative diseases such as Alzheimer's disease (AD) and PD, lysosomal activity is disrupted (47). In the lysosome, hydrolases are enzymes

responsible for degrading proteins and rely on highly acidic pH levels of 4.5-5.0. These pH levels are maintained using the v-ATPase pump, which uses energy from ATP hydrolysis to pump protons into the lysosomal lumen. The difference in voltage created between the lysosomal lumen and cytoplasm prevents additional protons from being pumped in (48-50). The lysosomal pathway should be explored as a possible target for PD as studies have shown that dysfunctional hydrolase trafficking decreases the lysosomal degradation capacity, leading to the accumulation of α-synuclein (50-51).

Cathepsin Modifiers

Since impaired lysosomal activity can be heavily implicated in neurodegenerative diseases, it is necessary to explore the role of lysosomal cathepsins, specific hydrolase enzymes involved in the chemical degradation of proteins (50). Specific cathepsins, such as cathepsin D, may prevent disease progression. Moreover, recent studies have shown a deficiency of cathepsin D in the plasma of PD patients (52). This confirms that lysosome function may be disrupted, and these biomarkers could be used to determine if a patient should be eligible for a drug that targets the lysosomes. In Huntington's disease (HD), lysosomal activity is also impaired as cathepsin D is ineffective at eliminating mutant Huntingtin (HTT) (53). A study also found that restored cathepsin D protects against stroke-induced neuronal cell death and abnormal lysosomal function in mouse cortical neurons (54). Ultimately, cathepsin D plays an integrative role in inducing autophagy and preventing the development of protein accumulation and cell death, two major characteristics of neurodegenerative diseases. On another note, there is evidence that cathepsin B is involved in neurodegenerative disorders such as AD and traumatic brain injury, and symptomatic outcomes can be alleviated by knocking the gene *cathepsin B (CTSB)* out. This is due to the theory that lysosomal leakage of cathepsin B into the cytosol leads to cell death due to the enzyme's toxicity. Furthermore, cathepsin B can be inhibited by using the compound CA-074, a derivative of E64 (55-56). CA-074 also protects neurons from cell death in the primate hippocampus by reducing enzymatic activity (56-57). Cathepsins, particularly cathepsin D and cathepsin B, can be extremely useful in preventing abnormal lysosomal activity, thus preventing the development of neurodegenerative diseases.

Lysosomal Acidifiers

Lysosomal acidification is essential in maintaining the functionality of the organelle in degrading proteins. For example, a study found that Presenilin 1 (PS1) results in early-onset AD by disrupting lysosomal acidity and preventing cathepsins' secretion (47, 50, 58). Several methods have been developed to restore lysosomal acidity, such as using acidic nanoparticles. Poly(DL-lactide-co-glycolide) (PLGA) acidic nanoparticles (aNP) protect against chloroquine (CQ) toxicity and lower lysosomal pH. PLGA-aNP restored lysosomal function by increasing cathepsin D levels in L3292 fibroblasts. Furthermore, PLGA-aNP protects against neurodegeneration in MPTP-induced parkinsonism in mice (50, 59). Additionally, a study identified progranulin (PGRN) as a facilitator of lysosomal acidification. PGRN increased lysosomal acidification in HeLa, HEK293T, and SH-SY5Y cells (60-61). Targeting the lysosome through cathepsin modulators or altering acidification can help restore the ALP pathway.

Carcinogenic and Autoimmune Pathways of TFEB

While TFEB is heavily involved in neurodegenerative diseases, it can ameliorate autoimmune diseases by triggering autophagy. In autoimmune diseases, glucocorticoids are involved in the immune system by preventing inflammatory cytokine responses. In mice with arthritis, autophagy was inhibited by using CQ, Bafilomycin A1 (BafA1), or *TFEB* KD. As a result, lysosomal activity decreased, and glucocorticoid signaling increased. Therefore, inhibition of TFEB replicates the impaired lysosomal activity that CQ causes (62). TFEB also interacts with the serine/threonine kinase MAP4K (GLK) as MAP4K phosphorylates TFEB, thus preventing TFEB's nuclear translocation. Consequently, a MAP4K inhibitor proved efficacious at reducing the severity of autoimmune diseases in mice models (63). Finally, the gene *TMEM39A* is associated with risks of autoimmune diseases such as multiple sclerosis and lupus. In *C. elegans* models, when *tmem-39* is mutated, the mTOR pathway is disrupted, and the TFEB ortholog, HLH-30, is activated. Thus, *TMEM39A* regulates lysosome activity and, when disrupted, may cause autoimmune diseases (64). Autophagy mediated by TFEB may help protect from inflammatory responses and improve symptoms in autoimmune diseases.

There is evidence suggesting that TFEB has a role in cancer as well. The dewormer agent flubendazole activates TFEB and not only induces autophagy-related genes but also induces cell apoptosis in CRC cells (65). Additionally, TFEB-mediated autophagy has also been shown to protect against the proliferation of tumor-associated macrophages in breast cancer (66). Interestingly, TFEB was found at high levels in prostate cancer and induced the ATP-binding cassette transporter A2 (ABCA2), leading to increased lysosomal biogenesis. In this case, it is suggested that TFEB promotes tumor growth (67). Therefore, TFEB can have both protective and carcinogenic effects depending on the type of cancer.

When it comes to the α-synuclein hypothesis, one must be aware that the goal in related research is to eliminate the inclusions found in SNpc and striatum. This review will specifically focus on eliminating α-synuclein through autophagy by the regulation of TFEB.

Literature Review

Poly (ADP-ribose) polymerase 1

One important target is Poly (ADP-ribose) polymerase 1 (PARP1) due to its role in TFEB regulation and, thus, autophagy. When DNA damage occurs, PARP1 is recruited and induces Poly (ADP-ribosyl)-ation (PARylation), which creates poly-ADP-ribose (PAR), a facilitator of gene expression (68-70). PARP1 is involved with many molecular mechanisms, including α-synuclein oligomer toxicity and mitochondrial dysfunction (70-71). With this context in mind, a study from 2019 explored the effects of PARP1 inhibitors on α-synuclein accumulation. Mice and nigral dopaminergic cells (SN4741) were transfected with the Adenovirus *α-synuclein* A53T mutation, and immunoblotting and immunohistochemistry confirmed that α-synuclein was overexpressed. As a result, autophagic flux was dysfunctional, exhibited by decreased TFEB nuclear translocation. Upon using the PARP1 inhibitor, Veliparib (siPARP1), the authors found that in *α-synuclein* overexpressing SN4741 cells, the

lysosomal activity improved with decreased SQSTM1/p62 and LC3B-II and amplified LAMP1 levels. Notably, the PARP1 inhibitor effectively increased TFEB nuclear translocation, as indicated by immunofluorescence in transgenic A53T α-synuclein mice. One can conclude that PARP1 may play a role in disrupting autophagy and, when blocked, can upregulate TFEB, promoting autophagy.

Mucolipin 1

Another potential target for TFEB is the calcium channel Mucolipin 1 (MCOLN1), which triggers TFEB translocation. MCOLN1 is involved in the autophagy-lysosomal pathway (ALP) and indirectly activates TFEB. Under reactive oxidative stress (ROS), MCOLN1 releases calcium into the cytosol (72-74). When this occurs, calcineurin is induced and binds and dephosphorylates TFEB, causing nuclear translocation (74). As clarified before, TFEB promotes the expression of autophagy-related genes. This mechanism could be potentially manipulated in a way that could promote TFEB activity so that autophagy could be activated.

Curcumin

Curcumin is a compound that stimulates autophagy by blocking the phosphoinositide 3-kinase-AKT-mTOR signaling pathway (75-77). The curcumin derivative, E4, effectively activates TFEB and promotes autophagy-related genes. The authors determined that E4 raised autophagosome production and improved autophagy flux. The authors used small interfering RNA (siRNA) to generate *TFEB* knockdown (KD) N2a cells and *TFEB* knockout (KO) HeLa cells. TFEB KD cells treated without E4 had lower levels of LAMP1 and CTSD compared to those administered E4. The LC3B-II levels remained indistinguishable from the controls and *TFEB* KO cells treated with E4 (78). This study used the toxin MPP+ in PC12 cells (78-79), and an Alamar Blue Assay indicated that cells treated with E4 survived. When a plasmid containing A53T *α-synuclein* was inserted in N2a cells, E4 blocked α-synuclein accumulation. E4 promoted autophagy flux and neuroprotection through TFEB (78). Therefore, E4 demonstrated that TFEB is a necessary target to investigate as the results indicated that TFEB provided neuroprotection.

This study supported findings that curcumin is not only an efficacious autophagy stimulator in E4 as a curcumin derivative but also as a curcumin analog (CA). CA binds to the N-terminus of TFEB and promotes nuclear translocation (80-81). However, the limitations of curcumin, including its lack of bioavailability, make it more feasible to use a curcumin derivative (76, 82) The CA nanoformula (NanoCA) was recently developed from polyethylene glycol and CA. NanoCA is administered intranasally and releases CA after degradation in the lysosomes, causing TFEB translocation and induction of autophagy-related genes. One significant advantage is that NanoCA and CA specifically do not alter mTOR as disruption to this pathway may affect other functions, such as cellular growth and metabolism (80, 82).

Akin to Zhuang *et al.*, the study measured intracellular calcium levels using confocal laser scanning microscopy (CLSM) and flow cytometry, this time in inducible PC12 (iPC12) cells stained with Fluo-4 and NanoCA. The increased intracellular calcium levels demonstrated lysosomal exocytosis, which has been shown to regulate cell

storage and morphology and improve the clearance of proteins (80, 83). There were also amplified levels of released exosomes in iPC12 cells, as demonstrated by LysoGreen staining under CLSM. Exosome secretion has been associated with toxic protein clearance (80, 84), implying that NanoCA promotes the clearance of proteins, such as α-synuclein. This was demonstrated when iPC12 cells with the A53T mutation displayed clearance of α-synuclein by autophagy upon administration of NanoCA. Additionally, treatment with NanoCA led to raised ALP activity as TFEB translocation in HeLa cells increased. The study also confirmed that NanoCA prevented the accumulation of α-synuclein and provided protection from the neurotoxic agent MPP+ in mice cortical neurons. NanoCA was more neuroprotective than CA as the cell viability assay revealed a 10% increased survival rate, whereas CA demonstrated decreased cell survival. The study conducted a behavioral assessment on mice by comparing the functioning of mice treated with only MPTP and both MPTP and NanoCA. Mice co-treated with NanoCA and MPTP displayed improved gait dynamics. The amplified levels of intracellular calcium, exosomes, and preserved neurons, along with α-synuclein clearance and ameliorated gait dynamics, revealed NanoCA as a potential effective TFEB agonist (80).

NanoCA is not the only curcumin analog that has been used to increase autophagy and improve behavioral outcomes. A study from 2020 determined that the TFEB enhancer, Curcumin (C1), is efficacious, along with Torin1, an mTOR inhibitor. The authors first ensured that the PD was properly modeled by using 6-OHDA to induce oxidative damage (85-87) and ascorbic acid (AA) (87-88). Through propidium iodide (PI) staining and flow cytometry, the study identified a decrease in neuronal cell death in FLAG-TFEB-overexpressing SH-SY5Y cells. When cells were introduced to the autophagy repressor CQ for 6 hours, a cell counting kit-8 (CCK8) assay revealed an increase in cell death. TFEB was further confirmed as neuroprotective when cells were treated with C1 and Torin1, which increased TFEB nuclear translocation and LC3B-II levels. With the promising results of TFEB enhancers in SH-SY5Y cells, the authors explored their use in induced Pluripotent stem cells (iPSCs), which are valuable models as they may replicate mechanisms (especially autophagy) that are difficult to completely understand in traditional models. Human UC-12 iPSCs were differentiated into DAn, and the phase-contrast microscope CCK8 assay revealed that the DAn were rescued. In addition, when TFEB expression was knocked down using siRNA, Torin1 and C1 were no longer effective. Finally, when a mouse model was used, immunostaining of the SNpc and striatum revealed rescued DAn levels of up to 90% restoration upon treatment with TFEB enhancers. Meanwhile, with only 6-OHDA/AA-induced parkinsonism, Tyrosine Hydroxylase (TH)-positive staining neurons were reduced by 65%. The study also administered a behavioral assessment to model PD symptoms that revealed an increase in rotations in mice treated with apomorphine hydrochloride, a standard treatment for PD, while the mice treated with TFEB enhancers displayed decreased rotations, quantified by turns per minute. Additionally, the authors investigated the role of mitophagy in TFEB by measuring the marker, mitochondrial import inner membrane translocase subunit (TIM23). The study found that mitophagy was improved in cells treated with both 6-OHDA/AA and Torin1/C1 compared with

SH-SY5Y cells subject only to 6-OHDA/AA toxicity. The preservation of DAn, along with improved autophagy, mitophagy, and behavioral functioning in mice, supports that curcumin is highly effective in triggering TFEB-mediated autophagy (87).

TFEB Splice Variant

Another method of targeting TFEB may be through targeting its splice variant, which may decrease TFEB activity. A study published in 2021 investigated small TFEB (TFEB-S), a splicing variant of the full-length TFEB (TFEB-L). TFEB-S is characterized by the absence of HLH and LZ motifs. Immunoblotting revealed that TFEB-S is expressed in the human brain, though at lower levels than its full-length counterpart. Importantly, gene expression, as indicated by qRT-PCR, revealed that the ALP-related genes such as *p62*/*SQSTM1*, *Autophagy Related 9B* (*ATG9b)*, *heme-oxygenase-1 (HO-1)*, and *MCOLN1* exhibited reduced expression in human neuroglioma (H4) cells expressing TFEB-S compared to those expressing TFEB-L. Co-immunoprecipitation also revealed that TFEB-S co-localized with TFEB-L at the nucleus when it was treated with Torin1. However, in the absence of the mTORC1 inhibitors, Torin1 and fisetin, TFEB-S failed to accompany TFEB-L's translocation into the nucleus. This suggests that TFEB-S reduces TFEB-L. Furthermore, TFEB-S prevents fisetin from inducing autophagy. The cells exhibited increased levels of α-synuclein compared to cells only treated with fisetin (89). Therefore, the splice variant, TFEB-S, may be a significant therapeutic target in the future as it plays a role in preventing TFEB from inducing autophagy.

Sphingomyelin/Ceramide Axis

The sphingomyelin/ceramide axis has been shown to influence the endosomal/lysosomal system. Sphingomyelin belongs to a class of sphingolipids and makes up the phospholipid cell membrane. The enzyme acid sphingomyelinase (ASM) activates sphingomyelin hydrolysis, which yields products of ceramide and phosphorylcholine (90). Sphingomyelin synthase facilitates the transfer of a phosphocholine moiety to a ceramide molecule, further synthesizing sphingomyelin and diacylglycerol (91). Previous studies have suggested that the accumulation of ceramide and sphingomyelin may lead to negative consequences such as cell death in neurodegenerative diseases (92) and dysfunctional endosomal/lysosomal activity in neurons (93). Therefore, it is necessary to investigate sphingolipid metabolism as a potential target, especially as it has been shown to alter the activity of TFEB, linking it back to autophagy.

Early-onset preeclampsia (E-PE) is linked to increased ceramide accumulation (94), and a study recently discovered its implications for TFEB activity. In a recent study, immunoblotting revealed that TFEB and LAMP-1 expression was increased in E-PE placentae compared to term and preterm control placentae. Similar results were found in choriocarcinoma JEG3 cells treated with Ceramide 16 (C16) and 2-oleoylethanolamine (2-OE), a ceramide inducer, and in the placentae of pregnant mice subject to Ceranib-2, an acid ceramide inhibitor which upregulates ceramide. In JEG3 cells treated with C16, ceramide contributed to TFEB activity, leading to increased autophagy, lysosomal biogenesis, nuclear translocation, and lysosomal exocytosis (95). In turn, increased ALP activity leads to the cell death of trophoblasts

in the placenta. The ceramide accumulation in E-PE induces autophagy through TFEB.

In cystic fibrosis (CF), myriocin, an immunosuppressant and catalyst of sphingolipid synthesis (96), effectively reduces sphingolipid accumulation. In bronchial epithelial cells derived from a CF patient, there was an increased accumulation of lipids and levels of sphingolipids compared to controls. These levels were decreased upon myriocin treatment as myriocin activated TFEB-mediated autophagy and depleted p62, leading to oxidative stress and release of Nuclear Factor Erythroid 2 (NRF2), which plays a role in the degradation of lipids. Additionally, myriocin increased the gene expression of genes involved with lipid formation, consumption, and transport (97). Myriocin enhances TFEB, which helps ameliorate the accumulation of sphingolipids in CF.

Myriocin is not only helpful in reducing sphingolipid accumulation in CF but also in murine embryonic fibroblasts in *wobbler* mice with the *Vps54* L967Q mutation. This mutation causes impaired sorting of lysosomal enzymes to the Golgi apparatus, including those catalyzing sphingolipid breakdown. This may lead to the accumulation of sphingolipids, such as ceramide. Furthermore, the authors investigated how myriocin could affect the neurodegeneration of *wobbler* mice. The behavioral outcomes for *wobbler* mice administered with myriocin were more successful compared to untreated *wobbler* mice. *Wobbler* mice treated with myriocin had increased locomotor movement, wellness index, and grip strength than those treated without myriocin. Pathologically, neurodegeneration slowed. The authors measured astrogliosis, which increases astrocytes due to the loss of neurons in the central nervous system.
Immunohistochemical staining using the astrocyte marker glial fibrillary acidic protein (GFAP) revealed that astrogliosis decreased as astrocytes were lower in *wobbler* mice treated with myriocin compared to controls. The mortality rate also increased for *wobbler* mice treated with myriocin, as 90% of the sample survived within 100 days compared to 50%. Altogether, the results suggest that the accumulation of sphingolipids, including ceramide, may lead to abnormal lysosomal/endosomal activity connected to the progression of diseases, especially those relating to neurodegeneration (98). Myriocin, which induces TFEB activity, can reduce lipid accumulation, leading to positive symptomatic outcomes.

Another method of targeting sphingolipid accumulation can be through an acid sphingomyelinase (ASM) inhibitor. One such is KARI 201, and a recent study determined its effects in mice displaying AD symptoms. KARI 201 prevents sphingomyelin from binding to the active site of ASM. Transgenic mice overexpressing hAPP695swe and presenilin-1M146V mutations (APP/PS1) were administered KARI 201. Neurons co-treated with KARI 201 and ASM displayed increased TFEB and LAMP1 expression and induction of autophagy, resulting in a decrease of amyloid-β plaques in the brain, improved synapse loss, reduced neuroinflammation, and increased microglial activation compared to the controls. In an iPSC-derived neuronal model with the PS1 mutation, KARI 201 decreased ceramide levels, although there was no change in sphingomyelin levels. Furthermore, this study supports that ASM inhibitors inhibit ceramide accumulation, which is beneficial in restoring autophagy and encompasses

other protective effects against neurodegeneration, particularly in AD models (99).

Antiparasitic Drugs Modulating TFEB

Although the evidence is limited, a small number of antiparasitic drugs have been shown to modify autophagy through the upregulation of TFEB. As mentioned previously, the antiparasitic agent flubendazole, a benzimidazole derivative, has been shown to induce autophagy in cancer. Flubendazole displays an antitumor effect by effectively preventing the activation of the STAT3 pathway, thus releasing Beclin1 from the BCL2-Beclin1 complex. As a result, autophagy is induced (65). In a recent study, when human colorectal cancer cell lines were treated with flubendazole, immunoblotting revealed that mTOR and p62 were inactivated, and LC3-I and LC3-II increased (65, 100). These results are supported by *Chauhan et al.*, a study that found that flubendazole disrupts the microtubules, preventing mTOR from binding to the lysosome. As a result, TFEB translocates to the nucleus and induces autophagy-related genes (100-101).

Flubendazole is not the only antiparasitic agent that triggers autophagy through TFEB. A study from 2017 investigated the novel antimalarial artemisinin derivative, SM1044, which induces autophagy in diffuse large B-cell lymphoma (DLBCL). This was indicated by increased LC3 punctae in SU-DHL-4 cells transfected with GFP-LC3, subject first to the pretreatment of CQ and then SM1044. The authors used high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) which indicated that there were higher C24 and C26 ceramide levels 3 hours after SM1044 was added to SU-DHL-4 cells compared to controls. Additionally, it was suggested that ceramide induces the AMP-activated protein kinase (AMPK) autophagy pathway. SU-DHL-4 cells were administered S1P, a ceramide inhibitor, and SM1044, and western blot indicated decreased AMPK levels after (102). Alluding to the previous section, ceramide, which is indirectly produced by SM1044, helps induce autophagy. It should be clarified that while ceramide accumulation is harmful as it leads to dysfunctional autophagy activity, SM1044 functions differently in that it synthesizes ceramide de novo. Therefore, the efficacy of SM1044 in modulating TFEB demonstrates a possible therapeutic target, especially in cancer models.

While antiparasitic can be used to enhance TFEB in carcinogenic cells, there are few studies regarding its implications for neurodegenerative diseases. Furthermore, there needs to be more research to confirm whether flubendazole and SM1044 are possible therapeutic candidates for reducing protein accumulation through TFEB-mediated autophagy.

Liquid Liquid Phase Separation

Liquid Liquid Phase Separation (LLPS) joins together compartments created by liquid phase condensation, a process that synthesizes a "liquid-like compartment" without a membrane, containing transcription factors (including TFEB), RNA, and other proteins (103-104). LLPS occurs when these compartments are unintentionally separated in an unhomogenized fashion (104-105).

Inositol Polyphosphate Multikinase (IPMK) prevents the liquid liquid separation of TFEB. A recent study demonstrated that inhibition of *IPMK* promotes autophagy through TFEB. In *IPMK* KO and KD HeLa cells (generated through CRISPR/Cas-9),

LC3 puncta and LC3-II levels increased under basal and starvation conditions and CQ administration, respectively. Additionally, *IPMK* depleted cells displayed increased levels of LC3 puncta using the RFP-GFP-LC3 fluorescent marker, indicating that there were higher levels of mature autophagosomes and autolysosomes. The lysosomal activity also increased as levels of LysoTracker in *IPMK* KO cells compared to controls. Using the luciferase assay, the authors determined that TFEB activity increased in *IPMK* depleted cells compared to the controls. However, there was no evidence that turning off *IPMK* influenced the nuclear translocation of TFEB as levels of nuclear TFEB remained unchanged in *IPMK* depleted cells compared to cells under starvation conditions which triggered autophagy. Phosphorylation of TFEB was also unaffected by *IPMK* depletion. The study also used LLPS assays to find that IPMK prevented LLPS of TFEB and shrunk the preformed TFEB droplets. Transcription of autophagy-related genes is enhanced when nuclear condensates are formed, mediated by the inhibition of IPMK (104).

Seeing how IPMK inhibition may successfully promote TFEB, one may investigate the effects of IPMK inhibitors. A previous study uncovered the antidepressant Vilazodone as an IPMK inhibitor. The authors concluded that this inhibitor has selective inhibition abilities as it does not disrupt inositol phosphate (IP) metabolism. The administration of an IPMK inhibitor on NIH3T3-L1 fibroblasts also mirrors the IP metabolism of *IPMK* -depleted mice and *IPMK*-deleted stem cells (106).

An editorial article published in 2021 suggested that ATP might be advantageous in dissolving the condensates of LLPS, including protein accumulation such as amyloid-β in AD or α-synuclein in PD. However, ATP levels decrease with age, so the author proposes using trehalose, a molecule with similar biochemical properties to ATP, to target the removal of misfolded protein aggregation (107).

TFEB activation independent of mTOR

Trehalose

Trehalose is a disaccharide that has been shown to induce autophagy independent of the mTOR pathway, thereby clearing A53T and A30T mutant α-synuclein in PC12 cells. (108-109). Trehalose is thought to induce TFEB-mediated autophagy by enlarging the lysosomes and releasing calcium which activates protein phosphatase 3 catalytic subunit (PPP3CB). PPP3CB further dephosphorylates TFEB, causing nuclear translocation and expression of autophagy-related genes. Using confocal immunofluorescence microscopy, the study found that trehalose induces TFEB nuclear translocation in the immortalized motoneuronal cell line, NSC34. RT-qPCR revealed that trehalose increased gene expression for several ALP-related genes and increased expression for proteins involved in chaperone-assisted selective autophagy (CASA), which effectively clears misfolded aggregates (110). The author confirmed that trehalose lost its effect when TFEB was silenced through siRNA as increased gene expression depended on TFEB. Trehalose also eliminated the accumulation of TAR DNA binding protein (TARDBP/TDP-43), often found in amyotrophic lateral sclerosis (ALS) and fronto-lateral temporal dementia. Additionally, the trehalose analogs melibiose and lactulose were proven efficacious (111). Therefore, trehalose and its analogs have been highly effective in increasing the gene

expression of autophagy-related genes and clearance of misfolded proteins through TFEB. In addition to curcumin, the compound trehalose could be used as a possible inducer of TFEB-mediated autophagy. However, there needs to be more studies confirming effect of trehalose on other misfolded proteins.

SC75741

Like trehalose, which is particularly advantageous in activating TFEB independent of mTORC1, the novel drug SC75741 has also been shown to stimulate autophagy. A recent study from 2021 determined that the accumulation of mutant TDP-43, which is found in ALS patients, can be reduced through SC75741. SC75741 inhibits c-Abl, an oncogene and non-receptor tyrosine kinase. C-Abl has been shown to inhibit TFEB nuclear translocation independent of mTORC1 (112), and when inhibited, α-synuclein aggregates are cleared (113). The study's authors found through immunoblotting that in *p62* KO HEK293T cells, TDP-25 increased upon the administration of SC75741. SC75741 mediates the increase of p62 and LC3C expression, resulting in a decrease in TDP-25 (114). Therefore SC75741 could be used to induce autophagy, but one must consider that the degradation effects of SC75741 rely on p62, which needs to be further investigated should SC75741 be used as a therapeutic intervention.

Given that many of the compounds discussed above directly involve the mTOR pathway, Both trehalose and SC75741 are promising avenues that do not involve mTOR. This is particularly advantageous, considering that there may be unknown downstream effects that compounds deactivating mTOR may have.

Conclusion

The latest findings of TFEB in promoting autophagy have been summarized throughout this review. TFEB is an important target as autophagy-related genes are activated upon translocation to the nucleus (40, 43). Indirect targeting of TFEB through PARP1 inhibition proved effective as it promoted TFEB translocation (70). MCOLN1 was also identified as a protein that emits calcium signals under ROS to ultimately turn on TFEB (74). Additionally, compounds derived from curcumin, such as C1, E4, and NanoCA, greatly improved autophagic flux, mitophagy, and, ultimately, TFEB-enhanced cellular survival (80, 82, 87). The TFEB splice variant has been shown to inhibit TFEB activity and is a possible target for future development (89). The sphingomyelin/ceramide axis modulates TFEB activity in E-PE, CF, and neurodegenerative diseases (95, 97-99). Harmful ceramide accumulation can be ameliorated through the compound myriocin, effectively eliminating sphingolipid accumulation through increasing TFEB (97-98). Targeting ASM, which synthesizes ceramide, through ASM inhibition helps increase TFEB-mediated autophagy (99).

Antiparasitic drugs such as flubendazole and SM1044 induce TFEB and autophagy activity in cancer models (65, 100-101). IPMK inhibits LLPS of TFEB, so when IPMK is inhibited, TFEB activity can increase, as shown through the antidepressant Vilazodone (104, 106). Moreover, the compound trehalose can dissolve TFEB condensates synthesized from LLPS (107). Trehalose has been shown to eliminate TDP-43 protein accumulation (111). Another method of targeting TFEB independent of mTOR is through SC75741, which inhibits c-

Abl, promoting TFEB nuclear translocation (114). Altogether, the latest literature on TFEB has provided much insight, which will hopefully help researchers better understand PD and other neurodegenerative diseases in the future.

Many different autophagy manipulations that attempt to achieve the same goal have been highlighted throughout this review. In PD pathology, the α-synuclein hypothesis has led researchers to target the ALP with the assumption that the reduction of protein accumulation will block PD progression. All of the targets discussed in this paper contribute to the regulation of TFEB, and drug development depends on targeting it to clear α-synuclein aggregates. Many may suggest that there needs to be more studies to verify whether the α-synuclein hypothesis is viable, particularly behavioral studies to measure if symptoms improve for PD patients. This is a valid concern, and recent studies mentioned in this review have confirmed that targeting the TFEB pathway can improve PD symptoms in mice. Furthermore, additional research would be beneficial, especially in more robust models, to determine the efficacy of agents that promote autophagy. This will help determine if such targets are worth continuing to pursue.

Multiple targets discussed above are possible precursors to pharmaceutical interventions targeting TFEB activity. The most promising methods of inducing TFEB appear to be using compounds derived from curcumin and trehalose. When investigating ways to target TFEB, it is very favorable when a specific mechanism minimally disrupts other pathways. Curcumin and trehalose offer this benefit by not disrupting the mTOR pathway, so this should be a consideration when approaching TFEB in other models. Additionally, there has also been extensive research demonstrating that curcumin effectively reduces symptoms in neurodegenerative models.

TFEB is extremely promising as it has much potential for therapeutic intervention. To date, there are few TFEB enhancers in clinical trials. TFEB has shown its potential in a preclinical trial for Alzheimer's disease (82, 115). Resveratrol for PD, a TFEB agonist, is in current clinical trials (115-117). Additionally, a clinical trial for rapamycin (Sirolimus), specific to the treatment of connective tissue disease-related thrombocytopenia (CTD-DP), was recently completed, and it was determined that it was effective and safe in treating CTD-DP (115, 118). TFEB enhancers need more attention as the latest studies, particularly those focused on curcumin, have shown that TFEB is highly efficacious in improving autophagy by inducing autophagy-related genes (78, 80, 87). Additionally, as of March 2022, a recent pilot study was launched by the pharmaceutical company Seelos for Trehalose (SLS-005). The study will confirm the safety of the intravenous drug and will be used with patients of various neurodegenerative diseases, including AD, ALS, HD, and Machado-Joseph disease (119). Although many hold hopeful prospects for this new drug, the study has yet to produce the results. There is a great deal of avenues for TFEB as a protective therapeutic objective, and more studies are needed to justify and confirm its potential in inducing autophagy in order for drug development to occur.

On the grounds of the α-synuclein hypothesis, autophagy is an essential element of PD pathology. With the lack of treatment

available to stop PD progression, it is necessary to understand the molecular mechanisms surrounding α-synuclein inclusion clearance. Owing to the fact that PD has been termed a "pandemic" (120), treatments that moderate disease development are exigently needed. TFEB is a promising therapeutic target.

Abbreviations

AD - Alzheimer's disease
ASM - Acid sphingomyelinase
ALP - Autophagy-lysosomal pathway
ALS - Amyotrophic lateral sclerosis
CA - Curcumin analog
CF - Cystic fibrosis
DAn - Dopaminergic neurons
E-PE - Early-onset preeclampsia
HSPA8/HSC70 - Heat shock 70 kDa protein 8
IPMK - Inositol Polyphosphate Multikinase
LAMP1 - Lysosomal associated membrane protein 1
LAMP2A - Lysosomal-associated membrane protein 2A
LC3 - Microtubule-associated protein 1A/1B-light chain 3
LLPS - Liquid liquid phase separation
MCOLN1 - Calcium channel Mucolipin 1
MPP+ - N-Methyl-4-phenylpyridine
MPTP - 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR - Mammalian target of rapamycin
SQSTM1/p62 - Sequestosome-1/ubiquitin-binding protein p62
PARP1 - Poly (ADP-ribose) polymerase 1
PD - Parkinson's disease
SNpc - Substantia nigra pars compacta
TFEB - Transcription Factor EB
TARDBP/TDP-43 - TAR DNA binding protein
6-OHDA - 6-Hydroxydopamine

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