METABOLISM IN ALZHEIMER'S DISEASE

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METABOLISM IN ALZHEIMER'S DISEASE

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EDITORIAL
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Editorial: Metabolism in Alzheimer's Disease

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Editorial on the Research Topic

Metabolism in Alzheimer's Disease

Alzheimer's disease (AD) pathology begins decades before clinical onset of dementia. Amyloid beta (Aβ) generally accumulates first in cognitively normal (CN) individuals, with tau and cognitive abnormalities following (Jack et al., 2013). AD pathologies have been found to correlate and interact with metabolic outcomes in studies spanning numerous experimental paradigms (Mosconi et al., 2009, 2010a,b,c; Mosconi, 2013; Morris et al., 2014a; Wilkins et al., 2014; Swerdlow et al., 2017; Weidling et al., 2020; Wilkins and Swerdlow, 2021).

Metabolic changes are prominent in AD. Fluorodeoxyglucose positron emission tomography (FDG-PET) comparing AD and CN individuals reveals lower glucose levels in the brains of AD patients (Herholz et al., 2002; Mosconi et al., 2010a; Marcus et al., 2014; Suppiah et al., 2019). These findings have led to overwhelming evidence of metabolic deficiencies in AD. Beyond reductions in brain glucose metabolism, mitochondrial dysfunction is observed not only within the brain but also systemically (Parker, 1991; Kish et al., 1992; Cardoso et al., 2004a,b; Morris et al., 2014b; Fisar et al., 2016; Guo et al., 2017; Swerdlow, 2018; Baloyannis, 2019; Chakravorty et al., 2019). More recent genome wide association studies (GWAS) identified risk-associated single nucleotide polymorphisms (SNPs) in genes which function in mitochondrial and metabolic pathways (Lakatos et al., 2010; Swerdlow et al., 2020; Harwood et al., 2021; Wightman et al., 2021). Apolipoprotein E (*APOE*), the strongest genetic risk factor for sporadic AD, is both central to lipid metabolism and has been found to interact with inherited mitochondrial genes to amplify risk for AD (Carrieri et al., 2001; Andrews et al., 2020; Swerdlow et al., 2020). Moreover, molecular studies of AD brain show an overall reduction in the number of intact mitochondria and mitochondrial DNA (Swerdlow, 2018; Wilkins and Swerdlow, 2021). Thus, mitochondrial function/dysfunction plays a role in protein aggregation, inflammation, and cell death; all events observed in AD. Overall, metabolism and mitochondrial function/dysfunction are strongly associated with AD.

The goal of this Research Topic was to further understand topics in the AD field that broadly focus on metabolic changes in AD and the interaction between metabolism, AD risk factors, and pathologies. These include: the role of genetic risk factors for sporadic AD (such as *APOE*) in non-cell autonomous functions, the intersection between metabolism and inflammation, the role of metabolism in protein aggregation, how current therapies target metabolism, inflammation, and protein aggregation, the role of novel metabolism/mitochondrial genes identified by GWAS in pathological mechanisms, the role of metabolism in the communication between neurons and glia

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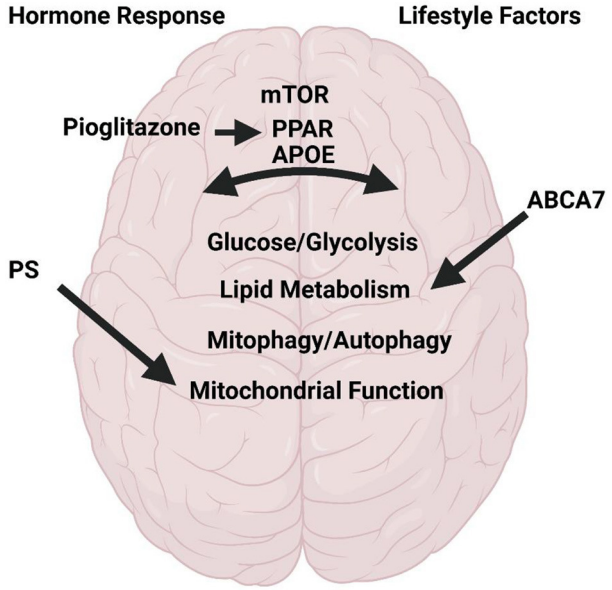

FIGURE 1 | Metabolism in Alzheimer's Disease. Research and review articles discuss the role of hormone response to meals, lifestyle factors on brain function with respect to mTOR, PPAR, PS1, ABAC7, and APOE. All of which regulate glycolysis/glucose and lipid metabolism, mitophagy/autophagy, and mitochondrial function. Created with BioRender.com.

in AD, how we can leverage existing model systems and develop better models to address questions of brain metabolism in the context of AD.

The articles of this Research Topic highlight a variety of reviews and original research which discuss and address topics of metabolism in AD (**Figure 1**). Several articles focus on potential AD therapeutics. These include a thorough review on the use of pioglitazone for AD treatment. Pioglitazone is a peroxisome proliferator-activated receptor gamma and alpha (PPAR-γ; PPAR-α) activator. PPARs are transcription factors critical for regulation of many pathways implicated in AD including insulin and glucose metabolism, lipid homeostasis, inflammation, tau and Aβ homeostasis, and mitochondrial function. The review by Saunders et al. discusses pre-clinical and clinical data with longitudinal observational studies revealing a positive impact of pioglitazone in AD and dementia onset in those at risk. The authors also discuss the dose-dependent effects and the caveats revealing future needs for further study into discrepancies found with placebo controlled blinded studies. Norowitz and Querfurth discusses mTOR regulation and drug targeting in AD. The authors focus on nuances for targeting mTOR in therapies including specificity for disease/region/and timing, pleiotropy, personalized therapy with relation to the effects of genetic factors, and the role of lifestyle factors and interventions.

Several other articles discuss the role of specific metabolic pathways in AD. Zhang et al. reviewed the role of glycolytic metabolism in brain resilience in AD. The authors highlight the correlation between glycolytic flux, Aβ, and tau accumulation in humans, where decreased glycolytic function is associated with higher pathologies. In a separate review article, Kyrtata et al. discuss glucose transport in AD with particular focus on glucose transporter (GLUT) deficiencies in AD. The authors discuss the timing of changes to GLUT expression and glucose uptake in brain through rodent studies and how this relates to the timing of onset of Aβ pathology.

An additional review presents the effect of sialometabolism on brain health and AD. Rawal and Zhao discuss the role of sialic acids in brain function and neuroinflammation. The novelty of this pathway in AD is the identification of sialic acid binding Ig-like lectin 3 (CD33) as a genetic risk factor for AD through GWAS.

A separate AD genetic risk factor, ATP binding cassette subfamily A member 7 (ABCA7) was examined. Aikawa et al. used mice with haplodeficiency of ABCA7 to determine the response to immune modulation with lipopolysaccharide (LPS). The authors report that mice deficient in ABCA7 had activated lipid metabolism pathways. This study again highlights the relationship between metabolism and neuroinflammation. Morris et al. describes the role of meal stimulated hormone response through the incretin pathway in cognitive function and brain volume. The authors report that in human AD subjects, a higher meal-stimulated response of insulin, glucose, and peptide tyrosine was observed. Brain volume significantly correlated negatively with insulin, C-peptide, and glucose-dependent insulinotropic polypeptide (GIP). These articles highlight the role of diverse metabolic pathways in brain health, aging, and AD.

A focus on genetic risk factors and metabolism was discussed through a review of *APOE* in AD by Husain et al. The authors focused on the role of *APOE* in lipid transport and interactions with AD pathologies (such as tau and Aβ). Other genetic components of AD include mutations in presenilin (PS) in familial AD, and PS has a role in mitochondrial function. Contino et al. examined the role of PS deficiency on neurons and astrocytes derived from mice. Their prior studies showed mitochondrial deficits in mouse embryonic fibroblasts, but in the current study no effects were observed on similar endpoints. This study highlights the importance of model systems used for study.

Mitophagy and autophagy are implicated in AD and are the focus of many therapeutic initiatives. Tran and Reddy discuss deficiencies in autophagy and mitophagy in AD. The authors focus on metabolic drivers of autophagy/mitophagy deficiencies, the influence of aging, and how these pathways influence AD pathologies.

Collectively, the articles in this Research Topic emphasize that the field of brain metabolism in AD is emerging and generating large interest from a therapeutic standpoint. Progress in filling our gap in knowledge on the role of metabolism in AD will advance new therapeutic avenues for this devastating disease.

AUTHOR CONTRIBUTIONS

JM, LW, and HW contributed to the recruitment and editing of the special Research Topic. All authors contributed to writing and editing the editorial.

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mTOR Mysteries: Nuances and Questions About the Mechanistic Target of Rapamycin in Neurodegeneration

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The mechanistic target of rapamycin protein complex, mTORC1, has received attention in recent years for its role in aging and neurodegenerative diseases, such as Alzheimer's disease. Numerous excellent reviews have been written on the pathways and drug targeting of this keystone regulator of metabolism. However, none have specifically highlighted several important nuances of mTOR regulation as relates to neurodegeneration. Herein, we focus on six such nuances/open questions: (1) "Antagonistic pleiotropy" – Should we weigh the beneficial anabolic functions of mTORC1 against its harmful inhibition of autophagy? (2) "Early/late-stage specificity" – Does the relative importance of these neuroprotective/neurotoxic actions change as a disease progresses? (3) "Regional specificity" – Does mTOR signaling respond differently to the same interventions in different brain regions? (4) "Disease specificity" – Could the same intervention to inhibit mTORC1 help in one disease and cause harm in another disease? (5) "Personalized therapy" – Might genetically-informed personalized therapies that inhibit particular nodes in the mTORC1 regulatory network be more effective than generalized therapies? (6) "Lifestyle interventions" – Could specific diets, micronutrients, or exercise alter mTORC1 signaling to prevent or improve the progression neurodegenerative diseases? This manuscript is devoted to discussing recent research findings that offer insights into these gaps in the literature, with the aim of inspiring further inquiry.

Keywords: Alzheimer's disease, antagonistic pleiotropy, autophagy, insulin/Akt, mTOR, Parkinson's disease

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease, Akt, protein kinase B; ALS, Amyotrophic Lateral Sclerosis; AMPK, AMP-activated protein kinase; APN, adiponectin; BAD, Bcl2 associated agonist of cell death; CREB, cAMP response element-binding protein; *C9orf72*; chromosome 9 open reading frame 72; Deptor, domain-containing mTOR-interacting protein; FTD, frontotemporal dementia; HD, Huntington's disease; IRS, insulin receptor substrate; mGluR5, metabotropic glutamate receptor type 5; mLST8, mammalian lethal with SEC13 protein 8; MPP+, 1-methyl-4-phenylpyridinium; MS, multiple sclerosis; mTOR, mechanistic target of rapamycin; PD, Parkinson's disease; PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; PPAR; peroxisome proliferator-activated receptor; PRAS40, proline-rich Akt substrate of 40 kDa; PTEN, phosphatase and tensin homolog; p70S6K1, p70 ribosomal S6 protein kinase 1; Raptor, regulatory-associated protein of mTOR; Rheb, Ras homolog enriched in brain protein; SMCR8, Smith-Magenis syndrome chromosome region 8; TFEB, transcription factor EB; TSC1/2, Tuberous sclerosis protein-complex; *UBQLN2/4*, ubiquilin genes; ULK1, Unc-51-like kinase 1; 4E-BP1, 4E-binding protein-1; 6-OHDA, 6-hydroxydopamine.

INTRODUCTION

Neurodegenerative diseases are an accelerating pandemic. The burden of Alzheimer disease (AD) alone is staggering and climbing at a precipitous rate. 5.8 million Americans over the age of 65 suffer from AD, a number that is expected to triple to 13.8 million by 2050 (Alzheimer's Association, 2020). AD is not alone in its ascent. Parkinson's disease (PD), the second most common form of neurodegeneration, is increasing in prevalence at a similarly alarming rate (Rocca, 2018). As there are currently no effective long-term treatments for these diseases, new therapies are desperately needed. One potential molecular target of such therapies is the mechanistic target of rapamycin complex 1 (mTORC1), a nutrient sensor and metabolic regulator heavily implicated in the process of aging (Sharp and Strong, 2010; Papadopoli et al., 2019; Heras-Sandoval et al., 2020).

While this manuscript will be primarily devoted to discussing and gaps in the literature surrounding mTORC1, a succinct overview of mTOR signaling and regulation is warranted as a preface to this discussion and is depicted in **Figure 1** [For a more comprehensive overview, Heras-Sandoval et al. (2020) recently published an excellent review on mTOR signaling, regulation, and drug-targeting]. mTORC1 is composed of the proteins mTOR kinase and its regulator protein, Raptor, as well as mLST8, PRAS40, and Deptor. Its primary function is to sense intracellular nutrient status and extracellular trophic factors [including, but not exclusive to insulin, shown in **Figure 1** as an example], integrate these signals, and ultimately regulate the balance between cells' anabolic and catabolic processes. Specifically, mTORC1 is a positive regulator of protein synthesis and negative regulator of autophagy.

mTORC1 itself is regulated positively by insulin-signaling and negatively by AMPK. Insulin/Akt signaling inhibits the protein complex, TSC1/2, which itself prevents the conversion of the mTORC1 activator, Rheb, into its active GTP-bound form (Inoki et al., 2002; Hers et al., 2011). Insulin/Akt signaling turns off TSC1/2, thereby activating Rheb and mTORC1. By contrast, AMP-activated protein kinase (AMPK) activates TSC1/2 (Inoki et al., 2003) and directly inhibits mTORC1 by phosphorylating Raptor (Gwinn et al., 2008). In brief, the respective growth and preservation functions of insulin and AMPK align with their respective stimulatory and inhibitory regulations of mTORC1.

mTORC1 promotes protein synthesis by phosphorylating and activating the downstream targets, 4E-BP1 and p70S6K1, which directly promote the initiation and elongation phases of translation (Graber et al., 2013). Critically, mTORC1-mediated anabolic signaling promotes the development of neuronal synapses (Dwyer and Duman, 2013, in part, by responding to established neuronal growth factors like BDNF; Takei et al., 2004) and inhibits apoptosis (Chen et al., 2010; Chong et al., 2013). Through these two mechanisms, mTORC1 activity has the potential to promote learning and memory and protect against neurodegeneration. Correspondingly, excessive inhibition of mTORC1 can impair learning and memory and permit neuronal death (Blundell et al., 2008; Belelovsky et al., 2009; Gafford et al., 2011; Jobim et al., 2012; Graber et al., 2013).

Despite these potentially positive functions of mTORC1 signaling in the brain, far more attention has been paid to its negative regulation of autophagy, an intracellular recycling process essential to maintaining neuronal integrity and protecting against neurodegenerative diseases (Oddo, 2012; Sarkar, 2013; Heras-Sandoval et al., 2020). mTORC1 inhibits autophagy at multiple levels, including the inhibitory phosphorylation of ULK1 and transcription factor EB (TFEB), which respectively initiate autophagy and promote the lysosomal biogenesis required to break down the contents of autophagosomes (Kim et al., 2011; Napolitano et al., 2018).

Importantly, multiple independent human post-mortem studies confirm levels of phosphorylated mTOR and its downstream targets are elevated in the AD brain as compared to those of controls (An et al., 2003; Li et al., 2004, 2005; Griffin et al., 2005). Dysregulated autophagy is also a hallmark of multiple neurodegenerative conditions (Fujikake et al., 2018), which is not surprising because autophagy is required to prevent the accumulation of toxic intracellular protein aggregates that contribute to neurodegenerative diseases, such as amyloid-β (Aβ) (Nixon, 2007; Nilsson et al., 2013; Yang et al., 2014), phospho-tau (Hamano et al., 2008; Kruger et al., 2012; Wang and Mandelkow, 2012), α-synuclein (Webb et al., 2003; Lee et al., 2004; Xilouri et al., 2016), and mutant huntingtin (Martin et al., 2015). Autophagy is also required to recycle mitochondria and prevent mitochondrial dysfunction (Chakravorty et al., 2019; Li et al., 2019a), another hallmark of neurodegenerative diseases, and one which can further lead to the pathologies of oxidative stress and inflammation (Lopez-Armada et al., 2013; Norwitz et al., 2019a,b). Given these data and the clinical burden of neurodegenerative disease, it's reasonable that translational research generally focuses on the inhibition of mTOR (and promotion of autophagy), rather than on its activation.

ANTAGONISTIC PLEIOTROPY

"Antagonistic pleiotropy" is a term typically used to refer to an evolutionary tradeoff between fitness in an organism's early life at the expense of health later in life (Schmeisser and Parker, 2019). An example of antagonistic pleiotropy is the *ApoE4* allele, the leading genetic risk factor of AD (Yamazaki et al., 2019). This allele sensitizes the immune system and protected ancestral humans from infections that compromised reproductive fitness and cognition (Vasunilashorn et al., 2011; Trumble et al., 2017). Further relevant to modern contexts, *ApoE4* is associated with accelerated neurodevelopment (Wright et al., 2003) and improved memory during youth (Mondadori et al., 2007).

Another example of possible antagonistic pleiotropy in neurodegenerative disease is that of adiponectin (APN), a hormone secreted by adipose tissue. APN has broad beneficial functions on metabolism, including stimulating neurogenesis, and is generally thought to be neuroprotective (Zhang et al., 2011, 2016). On the other hand, APN can induce astrocyte mediated neuroinflammation (Wan et al., 2014), oxidative stress (Fujimoto et al., 2010), and plasma levels of APN are correlated with the severity of cognitive decline and Aβ

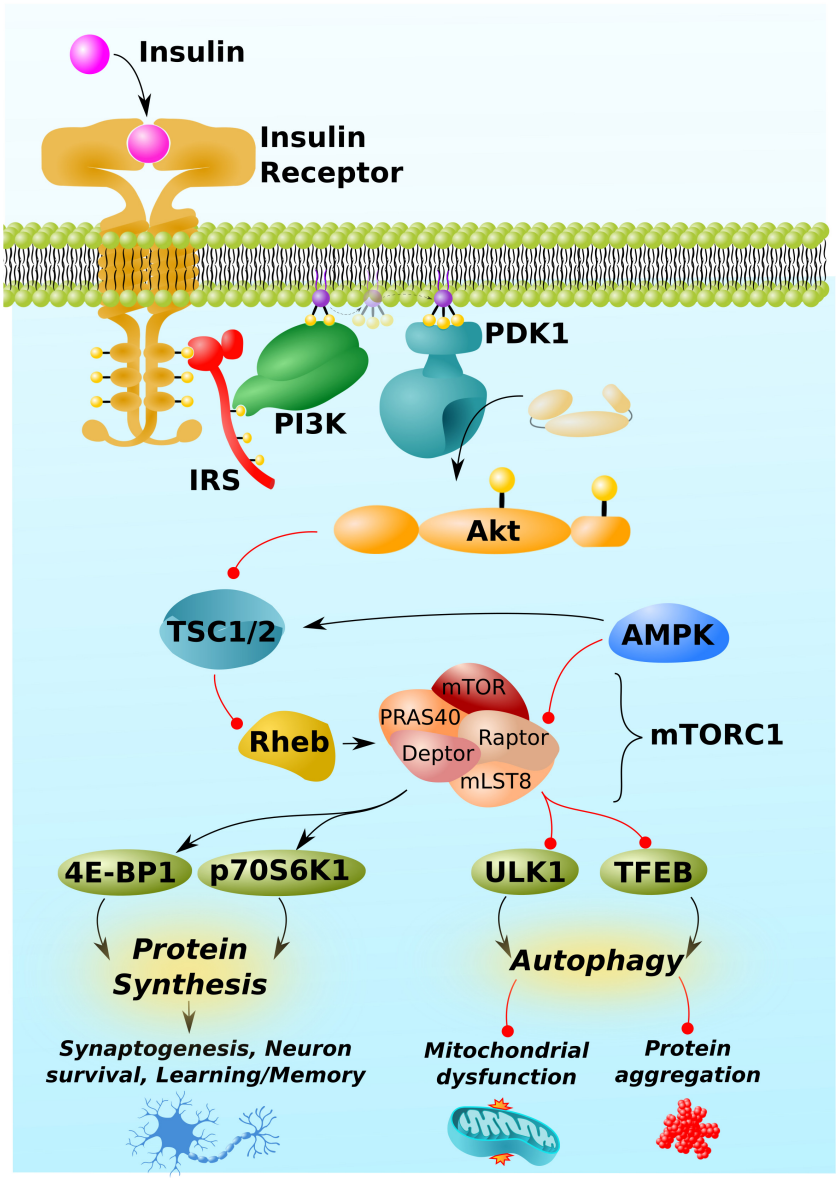

FIGURE 1 | mTORC1 pathway and regulation. mTORC1 is activated by insulin. Insulin/Akt signaling inhibits TSC1/2, thereby permitting the activation of the GTP-binding protein, Rheb. Rheb is the proximal activator of mTORC1. AMPK inhibits mTORC1 activity through indirect and direct mechanisms, phosphorylating TSC1/2 and the Raptor regulatory component of mTORC1. (Other trophic factors and pathways beyond insulin/Akt and AMPK, not shown for simplicity, also regulate mTORC1). mTORC1 downstream targets include proteins involved the mRNA translation, 4E-BP1 and p70S6K1, and those involved in autophagy, such as the initiator of autophagy, ULK1, and the master regulator of lysosomal biogenesis, TFEB. By regulating the activity of these and other proteins, mTORC1 promotes protein synthesis, which is required for synaptogenesis, learning, and memory, but can also impair autophagy, leading to mitochondrial dysfunction and neurotoxic protein aggregation (Aβ, phospho-tau, α-synuclein, etc.). Black arrows and red lines respectively represent positive and negative regulation.

accumulation (Wennberg et al., 2016). [Waragai et al. (2020) provide a comprehensive overview of antagonistic pleiotropy with regards to APN].

Hashimoto et al. (2018) have even proposed that amyloidogenic proteins, including Aβ in AD and α-synuclein in PD, might exhibit antagonistic pleiotropy. They hypothesize that the heterogeneity of amyloidogenic aggregates reflects the heterogeneity of metabolic stressors to which the human brain is exposed, and that specific amyloidogenic aggregates may serve to "precondition" the brain against future toxic exposures (Hashimoto et al., 2018). In effect, Aβ and α-synuclein could serve, in youth, as adaptive hormetic stressors. [As an aside, the Aβ/α-synuclein antagonistic pleiotropy hypothesis is intertwined with the "evolvability hypothesis" of amyloidogenic proteins, which is beyond the scope of this piece and reviewed by Hashimoto et al. (2018)].

The moral of these examples – *ApoE4*, APN, and Aβ/α-synuclein – is that the trade of better health and

cognition during youth, at the expense of cognition during non-reproductive years, was evolutionarily judicious. Furthermore, each these examples would not have been specifically mentioned if they did not plausibly involve mTORC1. With respect to *ApoE4*, mTORC1 activates pathways that promote synaptogenesis and neuronal development, which would benefit cognition during youth at the expense of decreased autophagy and increased risk of accumulating mitochondrial damage and neurotoxic protein aggregates over time, as in the case of *ApoE4* (Wright et al., 2003; Mondadori et al., 2007). Indeed, the *ApoE4* genotype is associated with elevated mTOR signaling (Li et al., 2019b). APN has been shown to induce oxidative stress in an mTORC1-dependent manner by modulating both insulin and AMPK signaling (Fujimoto et al., 2010; **Figure 1**). And, of course, mTORC1 activity is assumed to be culpable for dysfunctional autophagy and accumulation of neurotoxic protein aggregates in neurodegenerative diseases, as noted in the introduction. Thus, mTORC1 may be a keystone player of antagonistic pleiotropy in neurodegenerative disease.

Consideration of antagonistic pleiotropy is important for evaluating the preventative value of inhibiting mTORC1 prior to the development of symptoms. No doubt, it's important to prevent the development of the pathologies underlying neurodegenerative diseases, which are established decades before symptoms develop (Braak et al., 2003; Dickson et al., 2010; Hoglund et al., 2017). But when and by how much? During mid-life, should one strive for mTORC1 inhibition, or value activating mTORC1 in a cyclic manner in order to build neural networks and increase her/his cognitive reserve, thus protecting against cognitive decline later in life? These are open questions.

EARLY/LATE-STAGE SPECIFICITY

Although inhibiting mTORC1 to increase autophagy (and therefore clear damaged mitochondria and protein aggregates) may seem like a prudent intervention for neurodegenerative diseases, that may not be universally true. What if a disease progresses past a threshold beyond which the pathology is too well-established to be meaningfully improved by an upregulation in autophagy? For instance, the mTORC1 inhibitor, rapamycin, does not reverse pathology or benefit cognition in late-stage AD models (Majumder et al., 2011). More importantly, because mTORC1 can inhibit apoptosis by activating p70S6K, which itself inhibits the pro-apoptotic protein BAD (Harada et al., 2001; Castedo et al., 2002), what if inhibiting mTORC1 beyond this hypothetical threshold increases apoptotic cell death?

Evidence consistent with this hypothesis is provided by multiple independent cell and rodent models of PD. These models of established late-stage disease suggest that increasing, rather that decreasing, mTOR activity could be beneficial under certain circumstances. In MPP^+-treated SH-SY5Y cells, activation of mTOR with cannabidiol led to protection against MPP^+-induced cell death (Gugliandolo et al., 2020). In genetic and pharmacologic mouse models, upregulation of mTOR signaling (through PTEN ablation) is likewise associated with less cell death and improved symptomology (Domanskyi et al., 2011). A limitation of these early PD studies is that they do not involve α-synuclein accumulation, which may better recapitulate the human form of the disease and relative importance of autophagy therein. Nevertheless, given the knowledge that mTORC1 can inhibit apoptosis, and distinct possibility that there may be a point past which activation of autophagy is insufficient to improve disease course (Majumder et al., 2011), it's worth questioning whether mTORC1 inhibition could actually be harmful in late-stage neurodegenerative disease.

REGIONAL SPECIFICITY

In addition to considering the temporal dimension (early/late-stage disease), it's important to consider the spatial dimension. As the brain is partitioned into networks, nuclei, and cell types, a given intervention may impact one region differently than another. For example, Ramalingam et al. (2019) discovered that rotenone injections (used to generate murine models of PD) oppositely impact mTORC1 activity in different regions of mouse brains, increasing activity in the midbrain and decreasing activity in the striatum. Lifestyle interventions (more on this below), such as exercise, may also alter mTOR activity in a region-specific manner. In mice, wheel running regulates mTORC1 signaling most strongly in the nucleus accumbens and hippocampus, as compared to other brain regions (Lloyd et al., 2017). This is notable because atrophy of nucleus accumbens and hippocampus is most strongly associated with AD (Nie et al., 2017).

The data are nascent but sufficient to issue caution. What if a PD patient suffering from substantia nigra atrophy were treated with an mTORC1 inhibitor based on a rationale from data collected from hippocampal pathology? What if a frontotemporal dementia (FTD) patient suffering from primarily temporal lobe pathologies was treated with an mTORC1-targeting drug based on frontal lobe data? As there is limited evidence to support that mTORC1 responds consistently to a wide range of interventions across brain regions, and some evidence to the contrary, it's responsible to not overgeneralize and assume globalized impact on the brain. More research needs to be conducted on the region-specific impacts of different mTORC1-directed interventions.

DISEASE SPECIFICITY

While many neurodegenerative diseases share several key core pathologies, including mitochondrial dysfunction, protein aggregation, oxidative stress, and inflammation, it's also important to consider disease-specific aspects of neurometabolim that could interact with mTORC1. For example, Zhuang et al. (2020) recently discovered that TFEB activity (which stimulates lysosomal biogenesis and promotes autophagy) is increased in a 6-OHDA-treated SH-5YSY model of PD, as well as in dopaminergic neurons, and that TFEB activity is calcium/calcineurin-dependent. This is important because PD is characterized by loss of substantia nigra pars compacta neurons, which exhibit a unique form of calcium pacemaking activity

not seen in most other neurons. This suggests that regulation of autophagy may be different in the brain region most affected by PD as compared to brain regions impacted in other diseases.

Another example is Amyotrophic Lateral Sclerosis (ALS), which can be caused by loss-of-function mutations in the *UBQLN2/4* genes. While the products of these genes, ubiquilin proteins, are known best as components of the ubiquitin-proteasome system, they are also important in autophagy. Specifically, ubiquilins are required to maintain the vacuolar H^+-ATPase function that acidifies lysosomes (Senturk et al., 2019). In a scenario in which mTORC1 were inhibited to induce autophagy in ALS, induction of autophagy and lysosomal biogenesis may be increased (**Figure 1**), but if lysosomes are not sufficiently acidic to destroy the contents of the autophagosome, the contents could accumulate and exacerbate cellular stress. Therefore, inhibiting mTORC1 to upregulate autophagy could impair autophagic flux, leading to a back-up of components, and be harmful in such genetic cases of ALS.

PERSONALIZED THERAPY

There is a need for informed, disease-specific interventions. In this section, we provide three hypothetical examples of personalized interventions involving mTORC1. These will include glutamatergic antagonism for Huntington's disease (HD) (Abd-Elrahman and Ferguson, 2019), metformin treatment for multiple sclerosis (MS) (Sanadgol et al., 2019), and SMCR8-centered therapy for ALS and FTD (Lan et al., 2019).

Glutamate hyperactivity plays a prominent role in HD (Andre et al., 2010) and can activate mTORC1 via the mGluR5-PDK1-Akt-mTORC1 pathway (Abd-Elrahman and Ferguson, 2019). Correspondingly, Abd-Elrahman and Ferguson (2019) recently demonstrated, in a mouse model of HD, that antagonism of the mGluR5 metabotropic glutamate receptor can correct overactive mTORC1 signaling and, consequently, increase autophagic clearance of mutant huntingtin protein. The authors of this paper also point out that huntingtin aggregates sequester the transcription factor, CREB, leading to a down regulation of neuroprotective BDNF. They show that mGluR5 inhibitors not only clear pathological aggregates, but also increase BDNF expression (Abd-Elrahman and Ferguson, 2019). Therefore, mGluR5 antagonism, by inhibiting hyperactive mTORC1, could simultaneously promote the clearance of pathological huntingtin aggregates and increase neurotrophic factor signaling.

MS is characterized by demyelination of nerve cell axons. As oligodendrocytes are responsible for building myelin sheaths within the central nervous system, a goal of MS treatments is to boost oligodendrocyte renewal and remyelination. In a cuprizone-challenge mouse model of MS, Sanadgol et al. (2019) recently reported that the diabetes drug, metformin, did precisely that: it increased oligodendrocyte renewal and remyelination. These beneficial effects were mediated by a direct stimulatory interaction between metformin and AMPK, and subsequent inhibition of mTORC1 (Sanadgol et al., 2019; **Figure 1**). Thus, metformin is a candidate for an mTORC1-targeting therapy for MS.

Mutations in the *C9orf72* gene are the leading cause of inherited ALS and FTD. Only recently was it discovered that another protein, SMCR8, complexes with the C9orf72 protein to form a heterodimer that negatively regulates mTORC1 activity (Lan et al., 2019). Furthermore, a *SMCR8*-deficient mouse model recapitulates the *C9orf72*-deficient phenotype, leads to a decrease in C9orf72 protein, and is associated with upregulation of mTORC1 activity and decreased autophagy (Lan et al., 2019). Future treatments for genetic causes of ALS and FTD might consider SMCR8 therapy or other interventions that target the SMCR8-mTORC1-autophagy axis.

These examples were chosen because HD, MS, ALS, and FTD are lesser studied than AD and PD. However, the same personalization principle applies to all conditions in which mTORC1 plays a role. In PD, for example, levodopa-induced dyskinesia is thought to be induced by D1-receptor-mediated phosphorylation of mTORC1, a hypothesis supported by the fact that genetic variability in mTOR pathway components is associated with PD dyskinesia (Zhu et al., 2019). The development of useful future interventions for neurodegenerative disorders would benefit from a deeper consideration of the interactions between mTORC1 signaling and disease/patient-specific mechanisms.

LIFESTYLE INTERVENTIONS

Two reasons most neurodegenerative diseases are refractory to treatment are that interventions may be initiated too late in the disease process and/or are too specific. These limitations are a function of the pharmacologic approach to neurodegenerative disease in which symptomatic patients, who have usually been afflicted by the underlying disease for years to decades, are prescribed drugs not available for prevention during the preclinical stage. Certainly, drugs have their place. But to quell the neurodegenerative disease pandemic will require universally accessible preventative measures based on safe lifestyle interventions, including diet and exercise. Evidence suggests such interventions could operate, in part, through mTORC1-mediated mechanisms.

Turmeric is the best-studied nutraceutical for neurodegenerative diseases. In a genetic mouse model of AD, turmeric's active component, curcumin, inhibited mTORC1 to increase autophagy and prevent Aβ accumulation (Wang et al., 2014a). Correspondingly, curcumin-induced inhibition of mTORC1 protected against memory impairments in this model (Wang et al., 2014a). A more specific dietary example would be the mineral manganese in HD. As manganese deficiency might contribute to the pathogenesis of HD by affecting the insulin/Akt/mTORC1 pathway, correcting a simple micronutrient deficiency could be protective in some cases of HD (Bryan and Bowman, 2017). A third example is that of PPARs, a family of transcription factors that can inhibit mTORC1 and promote autophagy to protect against neurodegenerative disease (San et al., 2015; Heras-Sandoval et al., 2020). Many nutrients and their derivates activate PPARs, including oleoylethanolamide derived from oleic acid in olive

oil (Rodriguez de Fonseca et al., 2001; Fu et al., 2005) and the monoterpenes carvacrol and thymol found in mint family plants (basil, mint, rosemary, sage) (Hotta et al., 2010; Rigano et al., 2017). Curcumin, manganese, and dietary PPAR activators are just three examples of nutraceuticals from different classes that, when combined in a well-formulated diet and with other dietary mTOR regulators (Wang et al., 2014b; Rigano et al., 2017), could have a meaningful impact on cognitive longevity.

In addition to nutraceuticals and micronutrients, shifts in macronutrient intake can also impact mTORC1 activity. The most evident examples are intermittent fasting and high-fat, low-carbohydrate ketogenic diets, which can modulate mTORC1 activity through at least three mechanisms. First, fasting and ketogenic diets diminish insulin-mediated mTORC1 activation. Second, they activate AMPK (by altering the AMP/ATP ratio and causing glycogen depletion) to inhibit mTORC1 and induce autophagy (Alirezaei et al., 2010; Miller et al., 2018). Third, fasting and ketogenic diets share the common feature of stimulating hepatic production of the ketone body, β-hydroxybutyrate, which itself is a signaling molecule that regulates mTORC1 (Li et al., 2017; Newman and Verdin, 2017; Norwitz et al., 2019a). Interestingly, it has recently been demonstrated that both short-term ketogenic diets and acute administration of exogenous β-hydroxybutyrate improve a marker of brain aging called "brain network stability," in contrast to standard Western diets and sugar which decrease network stability (Mujica-Parodi et al., 2020). Long-term prospective studies will need to be conducted to determine whether fasting and ketogenic diets are truly neuroprotective in humans. Nevertheless, these mechanisms and data coincide with the growing popularity of intermittent fasting and ketogenic diets as prevention or treatment strategies for neurodegenerative conditions (Roberts et al., 2017; Zhang et al., 2017; Shin et al., 2018; Sohn, 2018; Taylor et al., 2018, 2019; Broom et al., 2019; Norwitz et al., 2019a; Wlodarek, 2019; Mujica-Parodi et al., 2020; Soto-Mota et al., 2020).

Exercise is another lifestyle intervention that benefits brain health. Prospective cohort and randomized controlled studies have found that exercise reduces the risk of developing dementia by as much as 38% (Larson et al., 2006) and improves cognitive function in those already living with AD (Groot et al., 2016; Jia et al., 2019). Kou et al. (2019) recently published a compelling review arguing that the benefits of exercise on cognitive function and AD may be mediated by mTORC1 regulation. Even a cursory consideration of this hypothesis suggests it has merit. Exercise alters nutrient flux, trophic factor signaling, and can activate AMPK. Exercise can also correct overactive mTORC1 signaling to increase autophagy by correcting dysfunctional microRNA expression in a mouse model of AD (Kou et al., 2017; Chen et al., 2019). These particular studies focus on microRNA-34a, but there is reason to believe that exercise can influence mTORC1, autophagy, and cognitive aging by regulating a wide network of microRNAs (Kou et al., 2019). In another rodent model of AD, treadmill exercise decreased phospho-mTOR levels [Ser-2,448, Akt target residue (Nave et al., 1999)], increased autophagy, and completely rescued cognitive function on the Morris water maze test (Kang and Cho, 2015).

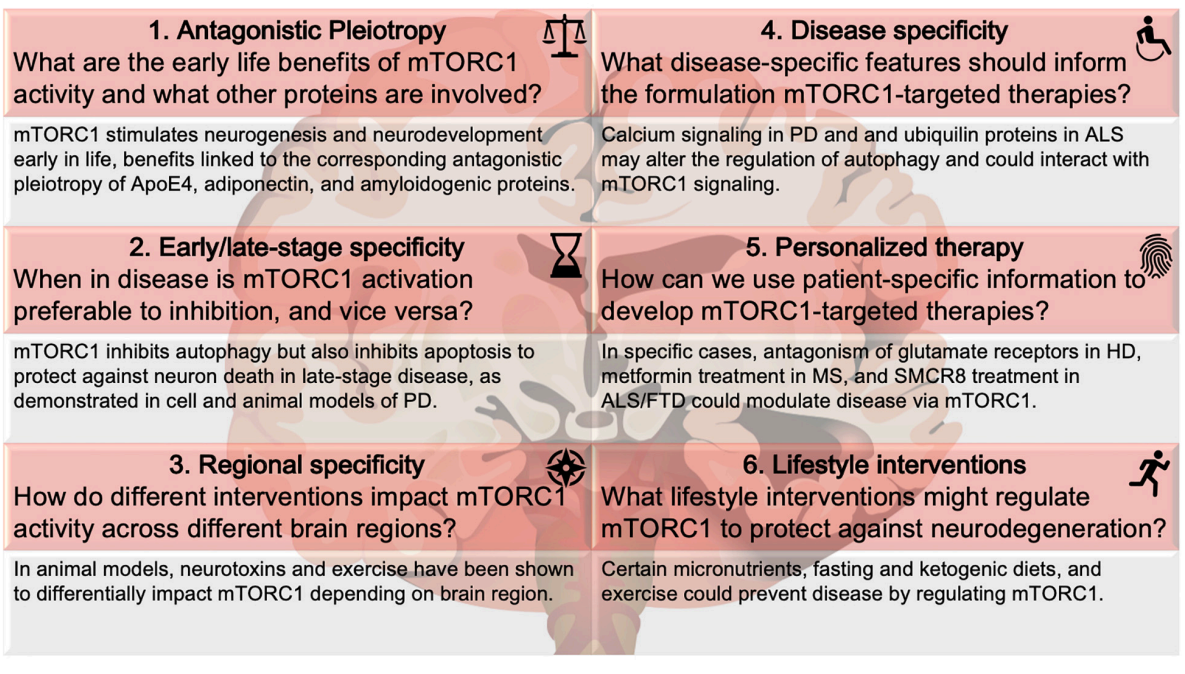

FIGURE 2 | mTORC1 mysteries. Six nuances regarding mTORC1 in neurodegenerative disease. The questions and examples below each topic are illustrative, not comprehensive, of the literature covered in this review. Disease abbreviations: ALS, Amyotrophic Lateral Sclerosis; FTD, frontotemporal dementia; HD, Huntington's disease; MS, multiple sclerosis; PD, Parkinson's disease.

Dietary micronutrients, fasting and ketogenic diets, and exercise are but a few illustrative examples of lifestyle interventions that may interact with mTORC1 to modulate the course of neurodegenerative diseases. Additional therapies include probiotics to modulate the gut-brain axis, which has been heavily implicated in the development of neurodegenerative diseases (Sampson et al., 2016; Sochocka et al., 2019), and heat therapy to induce chaperone heat shock proteins [whose expression is at least partially mediated by mTORC1 (Sun et al., 2011)] that could promote the proper folding of amyloidogenic proteins (Singh et al., 2006, 2010; Laukkanen et al., 2017). At the present time, clinical studies examining the impact of lifestyle interventions on mTORC1 signaling for cognitive decline are few (Halikas and Gibas, 2018; Kou et al., 2019) and more research needs to be conducted in this area to inform holistic and universally available best practices for the treatment and prevention of neurodegenerative disease.

CONCLUSION

While references to the most pressing open questions are scattered throughout the abundant literature on mTOR and neurodegenerative disease, herein, we have consolidated these gaps in the literature (**Figure 2**). How do we balance the beneficial effects of mTORC1 against its negative effects? How does this balance shift with disease progression or brain region? How can we use knowledge of biochemical pathways, specific to diseases and even individual cases, to inform personalized therapy? And what universally available lifestyle interventions might help in the prevention of neurodegeneration? Consideration of these mTOR mysteries will inform future research.

AUTHOR CONTRIBUTIONS

Both authors contributed to this work.

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Characterization of the Meal-Stimulated Incretin Response and Relationship With Structural Brain Outcomes in Aging and Alzheimer's Disease

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Background: Individuals with Alzheimer's Disease (AD) are often characterized by systemic markers of insulin resistance; however, the broader effects of AD on other relevant metabolic hormones, such as incretins that affect insulin secretion and food intake, remains less clear.

Methods: Here, we leveraged a physiologically relevant meal tolerance test to assess diagnostic differences in these metabolic responses in cognitively healthy older adults (CH; n = 32) and AD (n = 23) participants. All individuals also underwent a comprehensive clinical examination, cognitive evaluation, and structural magnetic resonance imaging.

Results: The meal-stimulated response of glucose, insulin, and peptide tyrosine tyrosine (PYY) was significantly greater in individuals with AD as compared to CH. Voxel-based morphometry revealed negative relationships between brain volume and the meal-stimulated response of insulin, C-Peptide, and glucose-dependent insulinotropic polypeptide (GIP) in primarily parietal brain regions.

Conclusion: Our findings are consistent with prior work that shows differences in metabolic regulation in AD and relationships with cognition and brain structure.

Keywords: insulin, PYY, Alzheimer's disease, glucose, insulin resistance, neuroimaging, MRI, voxel based morphometry

INTRODUCTION

Insulin resistance and Type 2 Diabetes (T2D) increase with age, and over 60% of older adults (> 65 years) in the United States exhibit impaired fasting glucose or T2D (Cowie et al., 2006). These conditions are also known risk factors for Alzheimer's Disease (AD) (Leibson et al., 1997; Ott et al., 1999; Stewart and Liolitsa, 1999; Peila et al., 2002; Arvanitakis et al., 2004; Janson et al., 2004;

Yaffe et al., 2004; van der Heide et al., 2006a; Luchsinger et al., 2007; Xu et al., 2009; Profenno et al., 2010; Cheng et al., 2011). However, insulin resistance is also related to dysfunction in a broader, integrated network of metabolic hormones beyond insulin, including peptide tyrosine tyrosine (PYY), glucagon like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP). These peptides are released by the gastrointestinal tract to stimulate the insulin response and control blood glucose regulation. Their effects are critically important because high glucose levels and impaired glucose regulation are associated with increased AD clinical progression and markers of AD neuropathology (Morris et al., 2014a,b; Macauley et al., 2015).

Although PYY, GLP-1 and GIP are secreted peripherally from the gastrointestinal tract, they cross the blood-brain barrier (Banks and Kastin, 1998; Kastin et al., 2002; Nonaka et al., 2003; Dogrukol-Ak et al., 2004) and have receptors in many brain regions, including those involved in the metabolic response and affected in AD, such as the hypothalamus, temporal and parietal cortex, and hippocampus (Martel et al., 1990a; Usdin et al., 1993; Dumont et al., 1996; Jhamandas et al., 2011).Change in peripheral metabolic hormone secretion thus has the potential to modulate both central nervous system (CNS) and peripheral metabolic function. However, meal-stimulated incretin response has not been compared between cognitively healthy (CH) older adults and those diagnosed with AD.

Older adults with T2D have decreased cross-sectional brain volume (Callisaya et al., 2019), and brain atrophy in T2D individuals may begin as early as midlife (Fang et al., 2018). This suggests that factors related to insulin resistance may be related to brain structure. Given that metabolic hormones are released with each meal and penetrate the brain, it is important to understand these responses in CH older adult and AD populations. The relationship of these responses to brain-relevant outcomes, such as brain structure and cognitive performance, has also never been examined. Thus, the goal of this project was twofold; to characterize the physiological metabolic response to a small mixed meal in cognitively healthy aging and AD, and to determine if these responses track with brain structure and cognition. We also characterized bioenergetic outcomes in platelet mitochondria obtained from these individuals, to further examine differences in energy metabolism. We present here a novel comparison of the incretin response to a mixed meal in CH older adults and individuals with AD, and the relationship of these important metabolic hormones with disease-relevant brain outcomes.

MATERIALS AND METHODS

Participants

All participants in this study provided informed consent according to institutional guidelines and in accordance with the Declaration of Helsinki. Fifty-five participants (n = 32 CH, n = 23 AD) were recruited by the KU Alzheimer's Disease Center (KU ADC) recruitment division as previously described (Vidoni et al., 2018). For this study, all enrolled individuals were part of the KU ADC Clinical Cohort and received a comprehensive cognitive and diagnostic evaluation. All participants were evaluated with the Clinical Dementia Rating (CDR) (Hughes et al., 1982; Morris, 1993) and a standard physical and neurological examination using UDS 3.0 Forms and Scales. The UDS 3.0 neuropsychological test battery was then administered.

A weekly diagnostic consensus conference attended by KU ADC clinicians, nurses, neuropsychologists, and psychometricians was held to classify individuals as cognitively healthy (CH; CDR = 0 without clinically significant cognitive impairment evident on testing or evidence of functional decline), Mild Cognitive Impairment (MCI), or AD by standard criteria (Albert et al., 2011; McKhann et al., 2011). Individuals with MCI were further assigned an etiologic diagnosis (i.e., probable or possible AD, etc.). First, CDR impairment and severity staging was reviewed and finalized by consensus review (without reference to cognitive testing). Available cognitive testing results were then reviewed and additional clinical information considered to arrive at consensus on the classification (CH, MCI, AD) and etiologic diagnosis. For this study, all participants met criteria for either etiologic diagnosis of probable AD (any age, CDR 0.5 or 1) or were CH (60 years and older). Exclusion criteria were neurological disease or condition other than AD that may affect cognition (e.g., stroke, major depression, etc.), history of cancer within the last 5 years (except for non-metastatic basal or squamous cell carcinoma), history of drug/alcohol abuse (DSM-IV criteria) within the last 2 years, diagnosed diabetes, and visual or auditory limitations that will interfere with cognitive assessment. Our data flow process has been previously reported (Graves et al., 2015). This study was approved by the University of Kansas Medical Center's Institutional Review Board (IRB # 03492).

Neuropsychometric Assessment

All participants received a cognitive examination consisting of the Uniform Data Set (UDS) version 2.0 (Weintraub et al., 2009). Tests were administered by a trained psychometrician in the non-fasting state within 2 months of their metabolic visit date. We used the UDS 3.0 normative calculator (Weintraub et al., 2018) to compute global normative values for each participant. In addition to the UDS, participants were also evaluated using the Mini Mental State Examination (MMSE).

Anthropometric Measures and Genotyping

Individuals reported for Visit 1 following an overnight fast. Vital signs were measured after a 5 min rest. We measured height to the nearest whole cm and total body mass using a digital scale accurate to 0.1kg (Seca Platform Scale, model 707) and from these values computed body mass index (BMI). Whole blood was collected for Apolipoprotein epsilon 4 (*APOE4)* genotyping. To determine *APOE* genotypes, frozen

whole blood was assessed using a Taqman single nucleotide polymorphism (SNP) allelic discrimination assay (Thermo Fisher Scientific). *APOE* ε*2*, ε*3*, and ε*4* alleles were distinguished using Taqman probes to the two *APOE*-defining SNPs, rs429358 (C_3084793_20) and rs7412 (C_904973_10). The term "Carrier" is used to describe the presence of 1 or 2 APOE ε4 alleles.

Meal Tolerance Testing

Following an overnight fast, subjects consumed 1 bottle of Ensure (220 calories, 33g carbohydrates) within 5 min. Blood was collected at 0, 15, 30, 45, 60, 90, and 120 min post-meal into tubes containing EDTA (for glucose, insulin, and C-peptide analyses) or DPP-IV inhibitors (p800 tubes, BD Biosciences) for incretins (GIP, GLP-1, PYY). Plasma glucose was measured using a glucose analyzer (YSI 2300, Yellow Springs Instruments). Plasma insulin and C-Peptide (ALPCO) as well as GIP (IBL) were measured using ELISA. Both GLP-1 and PYY were analyzed using a multi-plex electrochemiluminescent (ECL) assay per previously established methods with commercially available kits (Meso Scale Discovery, MSD) (O'Bryant et al., 2014, 2016). ECL utilizes a label that emits light when electronically stimulated, thus improving sensitivity of detection even at low concentrations. The coefficient of variation (CV) and lowest level of detection (LLOD) are reported for the following MSD assays: GLP-1 (CV = 3.75; LLOD = 0.06 pg/mL) and PPY (CV = 3.39; LLOD = 5.79 pg/mL).

Platelet Mitochondrial Enzyme Activity Measures

For a subset of subjects [n = 40 (n = 20 HC and n = 20 AD)], platelet mitochondria were isolated from fasted fresh whole blood into acid citrate dextrose tubes at fasting and re-suspended into MSHE buffer. Cytochrome oxidase (COX) and citrate synthase (CS) Vmax activities were assessed spectrophotometrically. For the COX Vmax, we followed the conversion of reduced cytochrome C to oxidized cytochrome c and calculated the pseudo-first order rate constant (ms^{-1}). For the CS Vmax, we followed the formation of 5-thio-2-nitrobenzoate (nmol/min). Both rates were normalized to mg total protein (BCA assay).

Neuroimaging Measures

T1-weighted MPRAGE anatomic images (TR/TE = 2,000/3.06 ms, flip angle = 8°, FOV = 192 × 100 mm, matrix = 192 x 192) were collected on 55 subjects using a 3T Skyra Siemens scanner. Every scan was checked for image and motion artifacts and gross anatomical abnormalities, resulting in the removal of 1 subject, as well as 1 subject who did not have PYY data for analysis, leaving a VBM sample of 53 subjects (31 CH subjects and 22 AD subjects).

For voxel-based morphometry (VBM) analyses and pre-processing of T1-weighted images we used the Computational Anatomical Toolbox 12 (CAT12 Version 12.6, C. Gaser, Structural Brain Mapping Group, Jena University Hospital, Jena, Germany)[1] through Statistical Parametric Mapping version 12 (SPM12; Wellcome Trust Centre for Neuroimaging, London, United Kingdom)[2] that operate under Matlab (R2019b) (the Mathworks, Natick, MA) on Mac. T1 images were corrected for bias-field inhomogeneities, registered using linear (12-parameter affine) and non-linear transformations, spatially normalized using the high-dimensional DARTEL algorithm into MNI space (O'Bryant et al., 2016), and segmented into gray matter (GM), white matter (WM), cerebrospinal fluid (CSF) and white matter hyperintensity (WMH). We calculated total intracranial volume (TICV) using total gray, white, and CSF volumes. The amount of volume changes were scaled, in order to retain the original local volumes (modulating the segmentations) (O'Bryant et al., 2016). The modulated gray matter segmentations were smoothed using a 10 × 10 × 10 mm full-width at half-maximum Gaussian kernel prior to group level analysis uses and images from the internet).

Statistical Analyses

Metabolic, Anthropometric, and Cognitive Outcomes

Diagnostic differences were assessed using ANOVA (for continuous variables) or chi square analyses (for categorical variables). Given prior work showing relationships to both metabolic function and AD, all measures were controlled for age, sex, body mass index (BMI), and APOE4 (carrier status). Results were considered significant at $p < 0.05$.

Neuroimaging

We used a General Linear Model full factorial analysis with *post-hoc* t-tests to assess the main effect of diagnosis on smoothed normalized gray matter images, including the same covariates as metabolic outcomes, in addition to TICV. In each separate analysis we included the metabolic biomarker response to a mixed-meal measured as area under the curve (AUC) [glucose, insulin, PYY, GIP, GLP1, C-Peptide, and platelet mitochondrial enzyme function (Citrate synthase Vmax)] as a covariate for interaction with diagnosis (see **Table 2** for variable details). *Post-hoc* tests also included a combined-group (ND and AD combined) regression of the metabolic biomarkers across gray matter volume with the same covariates as the initial analysis.

For all analyses, voxels are reported with reference to the MNI standard space within SPM12. To avoid possible edge effects at the border between GM and WM and to include only relatively homogeneous voxels, we used an absolute threshold masking of 0.10 for each analysis. Results for f-tests and t-tests were considered significant at $p < 0.05$ after correction for multiple comparisons (family-wise error, FWE), and results at $p < .001$ for t-tests are shown in **Supplementary Table 1**, with a minimum cluster size of 100 voxels ($k > 100$) for all analyses. After whole brain unmasked analysis, we used a small volume correction (SVC) to test for associations between the overall meal response (AUC) variables and two anatomical mask regions, one encompassing regions involved in eating and reward behavior (Batterham et al., 2007; Neary and Batterham, 2010; Weise et al., 2012), and a second combining regions involved in the default-mode network (DMN), where we have previously observed relationships between glucose metabolism and amyloid

[1]http://dbm.neuro.uni-jena.de/cat/

[2]http://www.fil.ion.ucl.ac.uk/spm/software/spm12/

neuropathology (Morris et al., 2016b; Taylor et al., 2017). The mask for food regions included the caudate nucleus, globus pallidus, thalamus, prefrontal regions, anterior cingulate gyrus and the cerebellum and was created by combining these regions into a single anatomical masks using the WFU pickatlas tool (Maldjian et al., 2003) and the integrated automatic anatomic labeling (AAL) tool (Tzourio-Mazoyer et al., 2002). The mask for the DMN network included the hippocampus, parahippocampal gyrus, amygdala, anterior cingulate gyrus, superior medial frontal cortex, precuneus, inferior parietal lobe, superior parietal lobe, and the posterior cingulate (Buckner et al., 2008).

RESULTS

Subject Characteristics

CH and AD diagnosis groups did not differ by age, sex, BMI, body weight, APOE4 carrier status, education or blood pressure (**Table 1**). As expected, individuals with AD were characterized by lower MMSE scores and higher CDR-Sum of Boxes compared to CH older adults.

Meal Stimulated Response

Area-Under-the-Curve (AUC)

We characterized diagnostic differences in the mixed-meal stimulated responses by calculating the AUC for key metabolic hormones. AD subjects exhibited a higher AUC compared to CH individuals in response to a mixed meal for PYY ($p = 0.001$; **Figure 1**, insulin ($p = 0.036$; **Figure 2**), and glucose ($p = 0.035$; **Figure 2**), with no diagnostic differences observed in the response of GLP-1, GIP, or C-Peptide.

Early Meal Response

The difference between the fasting and 30 min timepoint of glucose tolerance tests has been used to determine the early meal response (Cozma et al., 2005). This early meal response tracks well with first phase insulin secretion, which is especially important for control of glucose production by the liver (Luzi and DeFronzo, 1989). We calculated the early meal response (Δ0–30) values for these same metabolic biomarkers and identified diagnostic differences for PYY ($p < 0.001$) and GLP-1 ($p = 0.026$), with higher responses in AD individuals (**Table 2**).

TABLE 1 | Subject characteristics.

Measure	CH ($n = 32$)	AD ($n = 23$)	p-value
Age (year)	74.0 (5.4)	76.3 (6.3)	0.161
Sex (#, % male)	14 (42.4)	12 (52.2)	0.470
APOE4 (#, % carrier)	12 (36.4)	12 (54.5)	0.180
BMI	28.1 (4.3)	27.7 (5.4)	0.746
Education (y)	15.9 (2.8)	15.6 (3.0)	0.719
Systolic BP (mm/Hg)	135.7 (16.3)	130.6 (12.2)	0.173
Diastolic BP (mm/Hg)	76.2 (8.3)	73.6 (6.3)	0.136
Global cognition (z-score)	0.186 (0.55)	−1.57 (1.1)	**<0.01***
Weight (kg)	76.8 (14.1)	77.8 (21.3)	0.740
CDR-SB	0 (0)	4.26 (2.4)	**<0.01***
MMSE	29.1 (0.87)	22.7 (5.1)	**<0.01***

*Groups were well matched based on participant characteristics. As expected, AD subjects had lower cognitive scores. CH, Cognitively Healthy; AD, Alzheimer's Disease; APOE4, Apolipoprotein epsilon 4; BMI, body mass index; BP, blood pressure; CDR-SB, Clinical Dementia Rating Sum of Boxes; MMSE, mini mental state examination. *p < 0.05. Bold values indicate significant differences.*

Neuroimaging

In the overall cohort there was a significant difference in gray matter volume between diagnosis groups, the AD group had decreased gray matter volume compared to the ND group in a large cluster encompassing the left middle temporal gyrus and right post-central gyrus (**Table 3**). We identified that Insulin AUC, GIP AUC and C-Peptide AUC all had significant negative relationships with gray matter volume in CH and AD subjects, primarily in the parietal cortices (**Figure 3**). At a whole brain level, C-Peptide AUC negatively correlated with the left cuneus ($p < 0.001$, $Z = 5.51$, −12, −87, 8) and another cluster in the left parietal lobe seen using the DMN network SVC ($p < 0.05$, $Z = 4.17$, −58, −46, 46) (**Table 3**). Also at a whole brain level, GIP AUC negatively correlated with the left precuneus ($p < 0.05$, $Z = 4.57$, −3, −50, 62), which also presented significant in the DMN network SVC. In the DMN network SVC Insulin AUC was negatively correlated with the Left Inferior Parietal Lobe ($p < 0.05$, $Z = 4.27$, −42, −72, 40). There were no significant positive relationships with gray matter volume with the metabolic hormones.

There were no significant interactions of diagnosis with any of the metabolic hormones at a whole brain level or in the food region or DMN SVC, however, there were several interactive effects that reached a trend level of significance ($p < 0.001$ uncorrected, $k > 100$) (**Supplementary Table 1**). There was an interactive relationship between PYY and diagnosis such that CH individuals had a more negative relationship between PYY and right anterior cingulate and inferior frontal gyrus than individuals with AD (**Figure 4**). There were also interactive relationships between Insulin AUC and diagnosis, and similarly in GLP1 and diagnosis, in superior temporal gyrus as well as frontal regions (**Supplementary Table 1**), as well as a negative relationship between GLP1 AUC and gray matter volume in the right superior temporal gyrus in both diagnosis groups. Finally, there was a positive interactive relationship (greater gray matter volume alongside larger hormone AUC in the AD group compared to CH) between C-Peptide AUC and diagnosis in the right angular gyrus and right inferior parietal lobe.

Cognitive Performance

All individuals in this study were administered a cognitive battery consisting of the Uniform Data Set Version 2.0 (Weintraub et al., 2009). We computed normative z-scores for each UDS cognitive test as previously described (Weintraub et al., 2018) and well as a z-score for global cognition, which is a mean of all z-scores in the battery (**Table 1**). In the overall cohort, we observed a significant negative linear relationship between gAUC and the global cognition z-score ($\beta = -0.391$, $p = 0.008$). Global cognition did not track with other metabolic biomarkers.

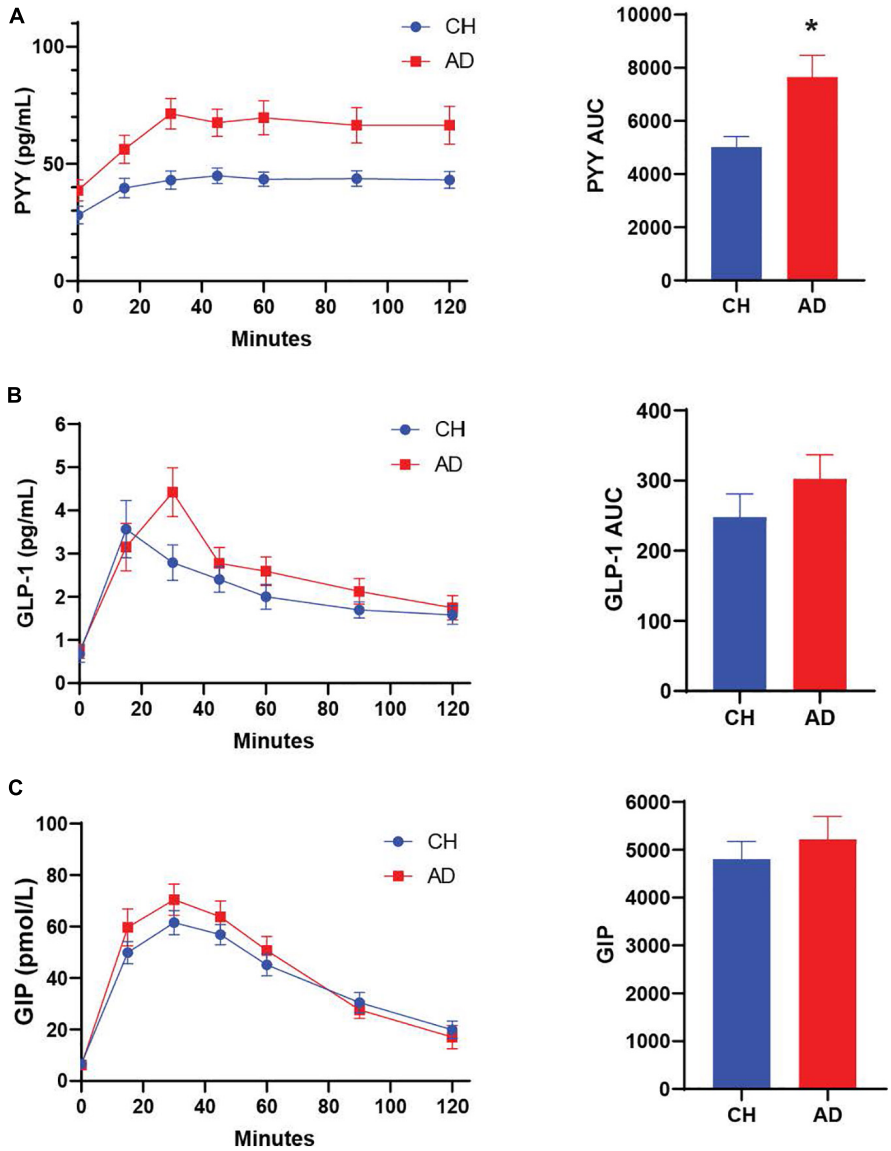

FIGURE 1 | (A) PYY response to a mixed meal (AUC 0-120) is significantly higher in AD subjects compared to CH older adults. No significant differences are observed for GLP-1 and GIP **(B,C)** *$p < 0.05$.

Platelet Mitochondrial Function

Generation of ATP in mitochondria is coupled to insulin exocytosis, (Maechler and Wollheim, 2001) and insulin secretory granules are in close proximity to mitochondria to promote coupling of metabolism and insulin secretion (Wollheim, 2000). Given the potential impact of mitochondrial dysfunction on insulin dysregulation, we performed functional assessments to characterize the activity of key mitochondrial enzymes in blood platelets to evaluate potential diagnostic differences (**Table 1**). Cytochrome oxidase activity (maximal velocity; Vmax) was measured and did not differ between diagnosis groups ($p = 0.583$). Citrate synthase (CS) Vmax was also characterized and did not differ between groups in this study ($p = 0.277$). Because the relationship of platelet mitochondrial function and brain structure has never been examined, we also characterized the relationship between these measures and brain structure using VBM. Across diagnosis groups, we observed a positive relationship between CS Vmax and brain volume in the left and right frontal gyrus, as well as the left precuneus (**Supplementary Table 1**). However, these findings did not hold up to multiple comparisons corrections.

DISCUSSION

The hormone insulin has been implicated in neurotransmission and cell survival (Wan et al., 1997; Skeberdis et al., 2001; Uemura and Greenlee, 2006; van der Heide et al., 2006b;

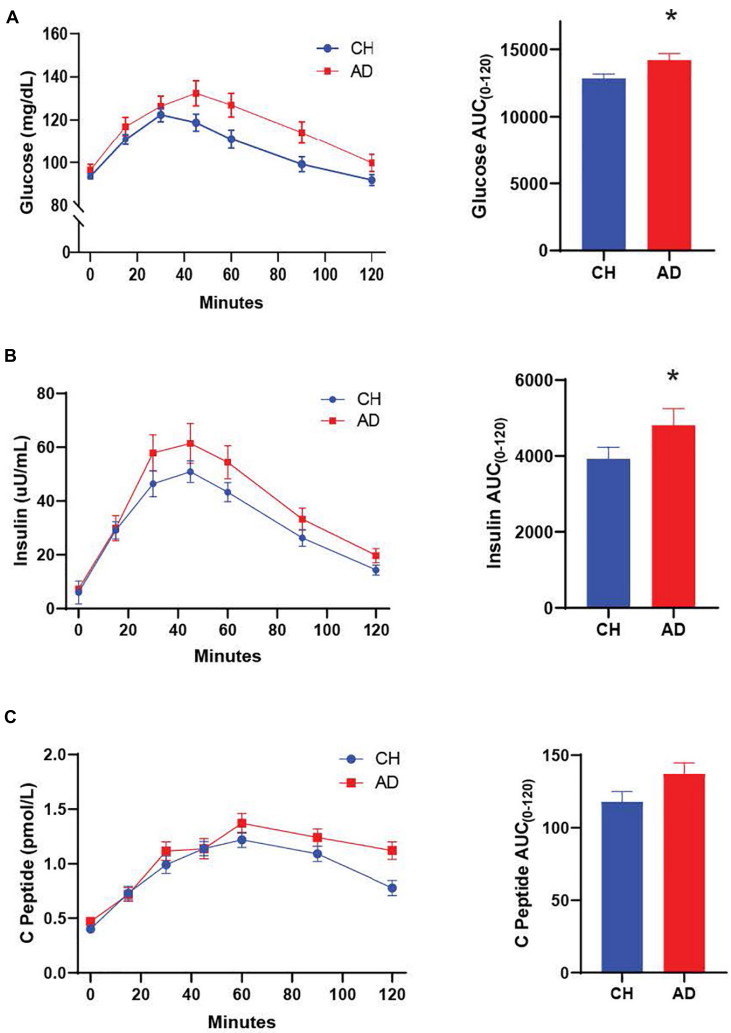

FIGURE 2 | Both glucose **(A)** and insulin **(B)** responses to a mixed meal were elevated in AD subjects. **(C)** The C-Peptide response did not differ between diagnosis groups. *$p < 0.05$.

Jin et al., 2011), associated with better cognition and less brain atrophy in AD (Burns et al., 2007), and been shown to improve memory in AD when administered intranasally (Reger et al., 2008a,b; Craft et al., 2012). However, insulin-sensitizing agents have not shown cognitive benefit or improved brain glucose metabolism (Gold et al., 2010; Tzimopoulou et al., 2010; Harrington et al., 2011). This may be due in part to inefficient transport of these compounds across the blood-brain barrier, but also suggests that additional mechanisms associated with production of insulin rather than just sensitization may be important. Here, we characterized diagnostic differences in metabolic biomarkers following a mixed meal and identified relationships of these biomarkers with brain structure. We focused on metabolites directly involved in or affected by insulin secretion, including insulin, GIP, GLP-1, PYY, C-peptide, and glucose. This is the first study to examine the response of Peptide YY and revealed striking elevations in AD participants compared to CH subjects. Elevations in the glucose and insulin AUC, which have been previously described as related to structural brain outcomes in other studies of AD subjects (Burns et al., 2007; Burns et al., 2012), were also observed. Although no group differences in the meal-stimulated AUC response were observed

TABLE 2 | Early response to a mixed meal.

Measure	Cognitively healthy (n = 32)	AD (n = 23)	p-value
Glucose (mg/dL)	29.1 (16.0)	35.7 (14.1)	0.250
Insulin (μU/mL)	40.4 (25.3)	50.6 (29.6)	0.147
C-Peptide (ng/mL)	0.59 (0.33)	0.686 (0.33)	0.347
GIP (pmol/L)	54.9 (26.4)	64.3 (27.9)	0.220
Peptide YY	14.9 (15.2)	32.8 (19.3)	**<0.001***
GLP-1	2.11 (1.9)	3.68 (2.7)	**0.026***

*Early response (Δ0–30) values for bioenergetic outcomes following a mixed meal. GIP, Gastric inhibitory polypeptide; Peptide YY, Peptide Tyrosine Tyrosine; GLP-1, GLP-1, Glucagon-like peptide 1. *$p < 0.05$. Bold values indicate significant differences.*

TABLE 3 | Regional imaging relationships.

F-test main effect of diagnosis	Peak F	*Z*	Cluster (k)	Peak *p*(FWE-corr)	Peak p(unc)	x,y,z (mm)	Regions
Whole brain	**47.27**	**5.52**	**2052**	**0.001**	**0.000**	**−63, −46, -9**	**Left middle temporal gyrus**
	33.82	**4.85**	**101**	**0.012**	**0.000**	**62, −22. −32**	**Right post-central gyrus**
T-test direction specific effects	Peak T	Z	cluster (k)	Peak p (FWE-corr)	Peak p(unc)	x,y,z (mm)	Regions
Negative regression of insulin AUC and GMV (ND and AD together)							
DMN network SVC	**4.80**	**4.27**	**326**	**0.026**	**0.000**	**−42, −72, 40**	**Left inferior parietal lobe**
	3.97	3.65	286	0.207	0.000	45, −74, 42	Right angular gyrus
	3.75	3.46	276	0.336	0.000	12, −51, 30	c
Negative regression of GIP AUC and GMV (ND and AD together)							
Whole brain	**5.23**	**4.57**	**663**	**0.035**	**0.000**	**−3, −50, 62**	**Left precuneus**
	4.99	4.41	385	0.066	0.000	−52, −80, 10	Left middle temporal gyrus
	4.08	3.73	229	0.512	0.000	−54, −40, 51	Left Inferior Parietal Lobe
DMN network SVC	**5.21**	**4.56**	**250**	**0.008**	**0.000**	**−3, −51, 62**	**Left precuneus**
	4.08	3.73	136	0.158	0.000	−54, −40, 51	Left Inferior Parietal Lobe
Negative regression of C-peptide AUC and GMV (ND and AD together)							
Whole brain	**6.73**	**5.51**	**6581**	**0.001**	**0.000**	**−12, −87, 8**	**Left cuneus**
					0.000	**−22, −96, 0**	**Right cuneus (calcarine)**
					0.000	**−38, −84, 0**	**Left middle occipital gyrus**
	4.68	4.17	259	0.155	0.000	−58, −46, 46	Left Inferior Parietal Lobe
	4.52	4.06	642	0.224	0.000	−39, 46, −2	Left middle frontal gyrus
	4.50	4.04	1604	0.236	0.000	26, −76, 26	Right occipital gyrus
	4.37	3.95	172	0.307	0.000	40, 20, 15	Right inferior frontal gyrus
	4.22	3.83	487	0.416	0.000	64, −33, 3	Right superior temporal gyrus
	4.07	3.71	152	0.545	0.000	40, −42, −14	Right fusiform gyrus
	3.94	3.62	130	0.65	0.000	−4, −4, 52	Left superior motor area
	3.89	3.57	187	0.698	0.000	−15, 36, 34	Left superior motor area
	3.79	3.49	102	0.778	0.000	0, −14, 75	Medial frontal gyrus
	3.76	3.47	116	0.802	0.000	40, −63, −4	Right middle temporal gyrus
DMN network SVC	**4.68**	**4.17**	**168**	**0.037**	**0.000**	**−58, −46, 46**	**Left parietal lobe (BA 40)**

Bold values indicate significant differences.

for either GIP or C-peptide, significant negative relationships between brain volume and AUC for these hormones, as well as insulin, were observed in highly metabolic brain regions such as the precuneus and parietal lobe across groups. Taken together, our data shows that a variety of diagnostic differences and relationships with structural outcomes are evident with the metabolic hormone response in response to a small (220 calorie) mixed meal. The effects of these peripheral hormone response differences reach far beyond the tissues of origin (**Figure 5**).

The observed increase in the early meal response of Peptide YY and GLP-1 suggests that in AD subjects, compensatory responses exist to increase insulin levels and maintain normoglycemia. Although both PYY and GLP-1 are stored within enteroendocrine cells, they exist within discrete compartments that allow for differential release (Cho et al., 2014). This may explain the sustained elevation of PYY beyond that of GLP-1. PYY is also produced in pancreatic islet cells, where the full-length peptide can locally inhibit insulin secretion through effects on Y1 receptors, but cleaved PYY activates Y2 receptors and is linked to increased circulating insulin levels, potentially due to simultaneous release of GLP-1 (Persaud and Bewick, 2014). This is worth noting as we did observe early increases in GLP-1 release in AD subjects, although again, this was not sustained throughout the MTT. It is known

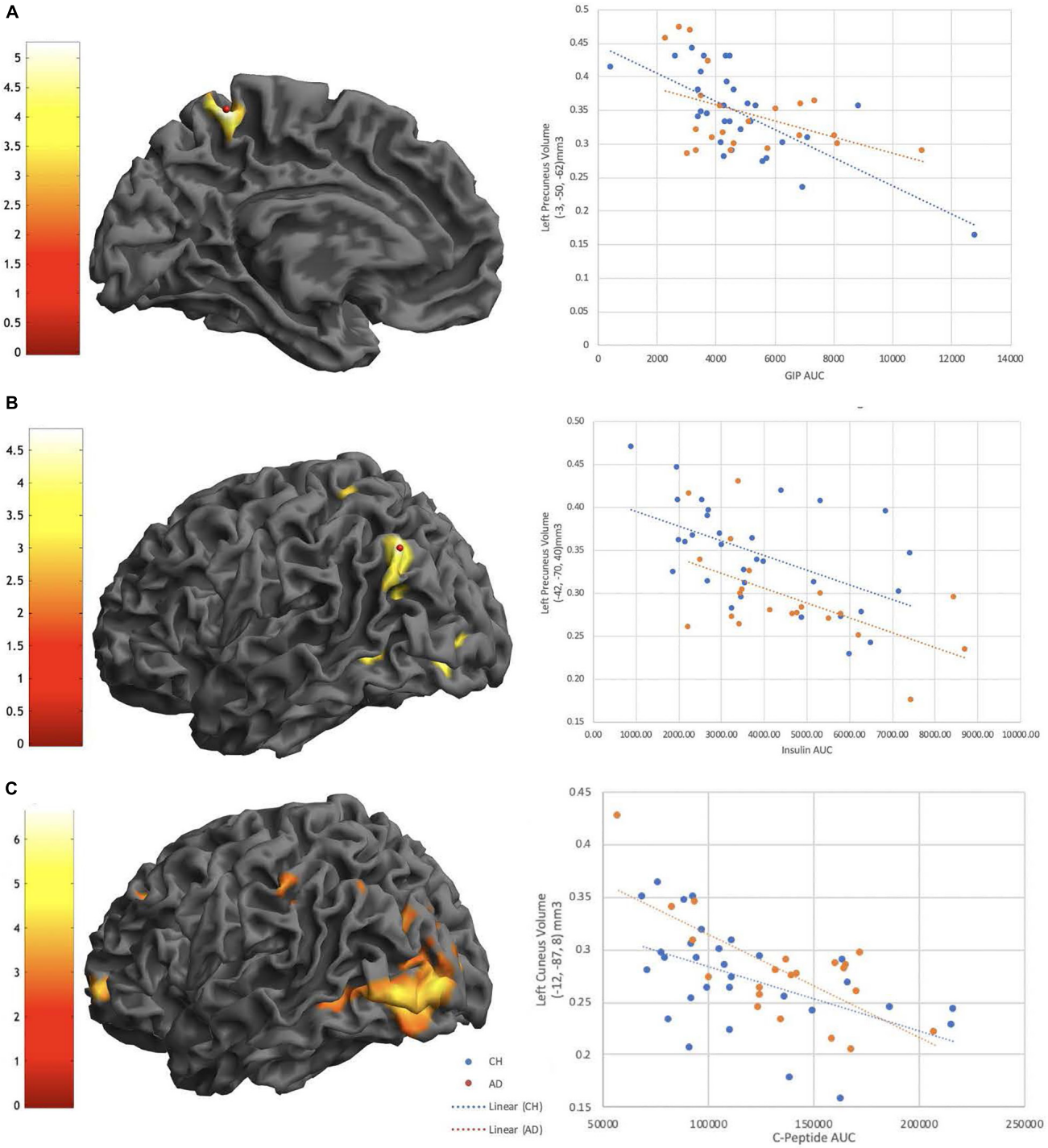

FIGURE 3 | GIP AUC **(A)**, Insulin AUC **(B)**, and C-Peptide AUC **(C)** are significantly negatively correlated with interior left precuneus **(A)**, Left precuneus **(B)**, and Left Cuneus volume **(C)** all in the parietal cortex. Extracted volumes plotted *post-hoc* against hormone AUC in plots on the right.

that plasma PYY levels rise in response to a meal (Adrian et al., 1985), and PYY can freely cross the blood-brain barrier (Batterham and Bloom, 2003). PYY inhibits food intake through actions in the arcuate nucleus of the hypothalamus, but also acts on receptors present in the temporal cortex and hippocampus (Martel et al., 1990b; Roder et al., 1996). While PYY receptor affinity is not altered between AD and CH individuals, PYY receptor density is lower in the hippocampus in AD (Martel et al., 1990a). Thus, it is possible that the increase in PYY release we observed in response to a mixed meal could be a

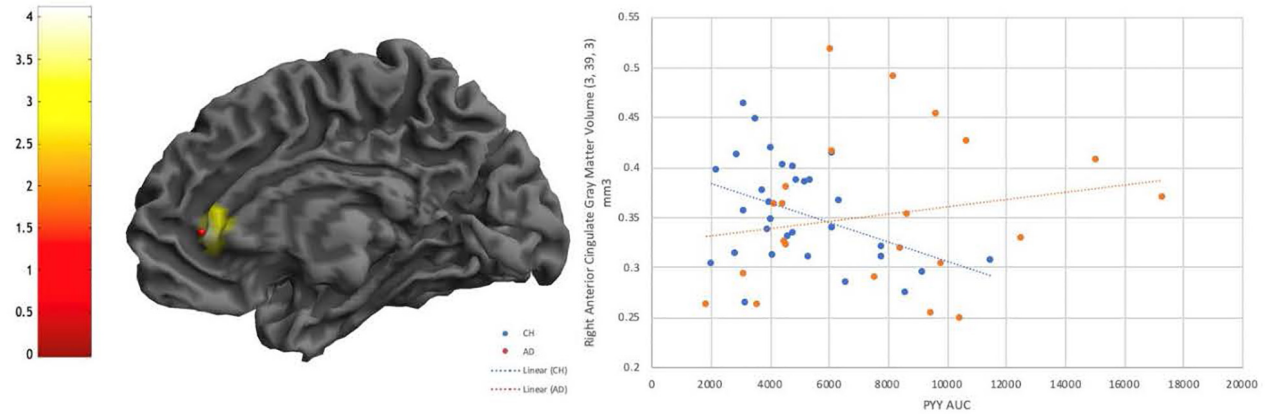

FIGURE 4 | Diagnostic interaction effect is evident for PYY in the Anterior Cingulate, with a negative relationship visible in CH individuals that is not evident in AD. $P < 0.001$ uncorrected.

compensatory response due to decreased receptor density in key brain regions. We also observed diagnostic elevations in the glucose and insulin meal responses. Our observation of an elevated glucose response in response to increased insulin and PYY compensation is insufficient to normalize glucose tolerance in these individuals.

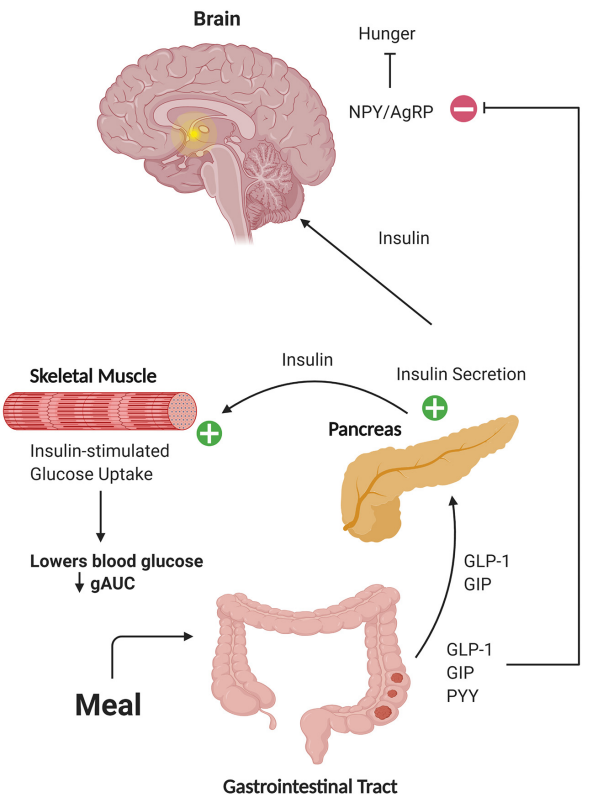

FIGURE 5 | Schematic representation of hormone action on target tissues. Gut-secreted hormones can travel through the circulation to the pancreas and brain. In these tissues, these hormones can potentiate secretion of insulin and affect regulation of blood glucose levels, as well as activate neuronal populations involved in responses such as satiety. Figure created with BioRender.

We then investigated whether the meal response of these metabolically active peptides (AUC value) tracked with brain structure using VBM. The strongest effects were consistently in the negative direction across both groups, occurred in the parietal cortex (**Figure 3**), and withstood multiple comparisons correction. Specifically, both insulin and C-Peptide AUC tracked negatively with brain volume in the left inferior parietal cortex (**Table 3**), while GIP and C-peptide AUC tracked negatively with brain volume in the precuneus and cuneus, respectively (**Table 3**). Given the role of GIP as an incretin hormone and C-peptide as an insulin cleavage product, these relationships in known highly metabolic brain regions underscore important relationships between insulin and brain structure. Insulin resistance is linked to increased atrophy (Benedict et al., 2012; Willette et al., 2013), and we have shown that prediabetes is related to increased rate of overall brain atrophy over two years in AD (Morris et al., 2016b). We have also shown a relationship between insulin resistance and decreased medial temporal, frontal and occipital cortical volumes (Morris et al., 2014b). These are consistent with our finding that increases in GIP, insulin and C-Peptide were related to decreased volume in regions associated with AD disease pathology, and a growing amount of data are linking insulin and insulin-like growth factor, type 1 (IGF-1) deficiencies to the pathogenesis of AD (de la Monte et al., 2018). It is worth noting that consistent negative relationships with brain volume were also observed with the AUC response of glucose, insulin, GIP, and C-peptide in temporal regions, and although these did not withstand FWE correction (**Supplementary Table 1**), they were in metabolically sensitive regions consistent with our previous work and will need to be investigated with a larger sample or a larger meal stimulus.

PYY was the only hormone that showed an interaction effect between diagnosis groups (**Figure 4**), which occurred in the right anterior cingulate and inferior frontal gyrus. In CH older adults, increased PYY was associated with decreased brain volume, but

this was not observed in AD subjects. Our findings in CH individuals correspond with results from a previous study on PYY concentrations and brain volume in non-diabetic young adults, where the authors found a relationship with PYY response and anterior cingulate volumes among others (Weise et al., 2012). The anterior cingulate bridges brain regions involved in autonomic function, cognition, and reward processing (Stevens et al., 2011), and many studies have shown decreased functional connectivity with this region in AD compared to healthy controls. This suggests that cognitively healthy individuals who have a compensatory increase in PYY (potentially due to very early stage IR) have lower anterior cingulate brain volume. It is possible that the lack of relationship in AD individuals is due to more heterogeneity in brain volume in this group, which prior work has shown to vary based upon the presence of neuropsychiatric symptoms in AD subjects (Tascone et al., 2017).

Despite the lack of neuroimaging relationships that withstood multiple comparisons corrections compared to the other biomarkers examined, gAUC was the only biomarker that tracked significantly with cognitive performance (global cognition). This suggests that the effect of insulin and related hormones and brain volume was stronger, or that the effect of glucose may be more readily detected using a larger meal stimulus. This finding also underscores the important relationship between glucose regulation and cognitive function previously demonstrated in larger epidemiological studies (Altschul et al., 2018; Zheng et al., 2018), an effect may occur independently of large changes in brain structure.

Our findings build upon prior work from our group and others that suggest insulin dysregulation exists peripherally and centrally in AD (Burns et al., 2007; Craft et al., 2012; Talbot et al., 2012; Morris et al., 2016a), and extends these findings to include additional metabolic-related hormones. Strengths of this study include the robust clinical characterization of our diagnosis groups, which were also well-matched in terms of age, sex, and BMI. An important additional strength is the careful pre-processing of plasma samples for incretin analysis, which were collected in tubes containing dipeptidyl peptidase 4 (DPP-4) inhibitor prior to processing and storage. This is critical for accurate incretin measurement. A limitation of the study is the sample size, which may have limited our ability to detect smaller diagnostic differences in responses, as well as the cross-sectional nature of the study. Additional considerations should include the fasting time, caloric content, and type the meal stimulus, which likely affect the hormone response, as well as potential effects of APOE4 genotype and sex, which should be explored in future studies. It is also important to note that it is unclear how closely our measures of the peripheral hormone response reflect hormone levels in brain. Nonetheless, we provide evidence for diagnostic differences in the response of emerging gut hormones to a small mixed meal. Given that multiple meals and snacks are consumed throughout the day, these differences may have important physiological consequences that become evident over time. Future studies should examine the longitudinal effects of these changes on brain outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Kansas Medical Center Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JM and JB: study design. JM, CJ, HW, and XW: conducting experiments. JM, CJ, HW, XW, and SO'B: acquiring data. JM, ZG, AK, EV, and RH: analyzing data. JM, CJ, ZG, HW, EV, RS, MP, RS, and JB: manuscript drafting.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.608862/full#supplementary-material

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REVIEW
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Defective Autophagy and Mitophagy in Aging and Alzheimer's Disease

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Aging is the time-dependent process that all living organisms go through characterized by declining physiological function due to alterations in metabolic and molecular pathways. Many decades of research have been devoted to uncovering the cellular changes and progression of aging and have revealed that not all organisms with the same chronological age exhibit the same age-related declines in physiological function. In assessing biological age, factors such as epigenetic changes, telomere length, oxidative damage, and mitochondrial dysfunction in rescue mechanisms such as autophagy all play major roles. Recent studies have focused on autophagy dysfunction in aging, particularly on mitophagy due to its major role in energy generation and reactive oxidative species generation of mitochondria. Mitophagy has been implicated in playing a role in the pathogenesis of many age-related diseases, including Alzheimer's disease (AD), Parkinson's, Huntington's, and amyotrophic lateral sclerosis. The purpose of our article is to highlight the mechanisms of autophagy and mitophagy and how defects in these pathways contribute to the physiological markers of aging and AD. This article also discusses how mitochondrial dysfunction, abnormal mitochondrial dynamics, impaired biogenesis, and defective mitophagy are related to aging and AD progression. This article highlights recent studies of amyloid beta and phosphorylated tau in relation to autophagy and mitophagy in AD.

Keywords: Alzheimer's disease, mitochondria reactive oxygen species, mitophagy, autophagy, aging

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Abbreviations: Aβ, Amyloid beta; AD, Alzheimer's disease; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; Drp1, Dynamin-1-like protein; EF, Helix-loop-helix structural domain; ERMCS, ER mitochondria encounter structures; ETC, Electron transport chain; Fis1, Fission 1 protein; FUNDC1, FUN14 domain containing 1; LC3, Microtubule-associated protein 1A/1B-light chain 3; LRRK2, Leucine-rich repeat kinase 2; mAPP, Mutant amyloid beta precursor protein; Mff, Mitochondrial fission factor; Mfn1, Mitofusin-1; Mfn2, Mitofusin-2; Mid49, Mitochondrial dynamics protein MID49; Mid51, Mitochondrial dynamics protein MID51; Miro, Mitochondrial Rho GTPase; mtDNA, Mitochondrial DNA; NRF1, Nuclear respiratory factor 1; NRF2, Nuclear respiratory factor 2; NSF, N-ethylmaleimide sensitive factor; OMM, Outer membrane of mitochondria; Opa1, optic atrophy 1; OPTN, Optineurin; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PINK1, PTEN-induced kinase 1; ROS, Reactive Oxygen Species; SIAH1, E3 ubiquitin-protein ligase SIAH1; TBK1, TANK-binding kinase 1; TEM, Transmission electron microscopy; TFAM, transcription factor A, mitochondrial.

INTRODUCTION

Aging is generally thought of as the time-dependent accumulation of cellular damage and decline in physiological function. Although many events can lead to cellular dysfunction, several factors have been identified as the defining characteristics of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, cellular senescence, stem cell exhaustion, altered intercellular communication, and mitochondrial dysfunction (**Figure 1**; Lopez-Otin et al., 2013). Accumulation of genetic damage is one of the hallmarks of aging, and the integrity of an organism's genome is constantly being challenged (Moskalev et al., 2013). Exogenous threats include physical, chemical, and biological agents, whereas endogenous threats include DNA replication errors, spontaneous mutations, and alterations due to reactive oxygen species (ROS). To help protect from these threats, most organisms have evolved multiple forms of protective DNA mechanisms that collectively help minimize damage and maintain genomic stability.

One such mechanism is the existence of telomeres on the end of chromosomal DNA, which are DNA repeats that prevent erosion of coding segments of DNA during replication. Most DNA polymerases cannot completely replicate the terminal ends of linear DNA, with telomerase being the only specialized form capable of doing so. However, most mammalian cells do not express telomerase, which ultimately leads to the progressive loss of the protective factor of telomeres (Blackburn et al., 2006). Telomere shortening limits the proliferative capacity of all cells, and telomere depletion leads to cell senescence, which arrests cell cycle progress and halts replication. Epigenetic modifications are a form of genetic alteration that can be signs of aging. Epigenetic changes are involved in alterations in the methylation patterns of DNA, post-translational modification of histones, and chromatin remodeling, which can alter protein expression (Fraga and Esteller, 2007). Dysregulation of the epigenetic machinery is related to aging in invertebrates (Greer et al., 2010; Maegawa et al., 2010).

The purpose of this article is to critically examine the role of age-related factors in autophagy and mitophagy in AD pathogenesis. This article will also highlight how mitochondrial dysfunction, abnormal mitochondrial dynamics, and impaired biogenesis are related to aging and AD pathogenesis.

AGING AND CELLULAR SENESCENCE

Aging is a major risk factor for a large number of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's, and amyotrophic lateral sclerosis (ALS) (Reddy and Reddy, 2011). Aging occurs at different rates in different species, and interindividual variations exist within a species and in the different tissues of an individual. Aging is a progressive deterioration that leads to cell senescence and an increased risk of developing many diseases.

Cellular senescence is a series of cellular states after an initial growth arrest in which cells go through phenotypic alterations. Cellular senescence and aging are two distinct phenomena; aging is the progressive decline with time, whereas senescence occurs throughout the lifespan, including during embryogenesis. The number of senescent cells increases with age, but senescence also plays an important role during development. These events are directly associated with autophagy and mitophagy.

Hayflick and Moorhead first characterized senescence as the phenomenon of irreversible growth arrest linked to telomere attrition (Hayflick and Moorhead, 1961). Senescence helps guard against the continual replication of damaged cells and plays a role in embryonic development, wound healing, tissue repair, and aging. As cells age out, new cells must take their place, and eventually, the regenerative potential of tissue decreases as stem cells become exhausted. Hematopoiesis has been shown to decline with age resulting in reduced production of adaptive immune cells and anemia (Shaw et al., 2010). Furthermore, studies on aged mice have shown that hematopoietic stem cells go through decreased cell-cycle activity and division compared with younger mice (Rossi et al., 2007). These decreases in divisions have been correlated to the accumulation of DNA damage resulting in overexpression of cell-cycle inhibitory proteins such as $P16^{INK4a}$, which is a known inducer of cell senescence.

As cells age, DNA damage accumulates not only in chromosomal DNA but also in mitochondrial DNA leading to mitochondrial dysfunction. Mitochondria play a key role in respiratory oxidation for ATP generation, and dysfunction leads to electron leakage and energy deficits (Green et al., 2011). Turnover of dysfunctional and damaged mitochondria is paramount to maintaining healthy cell populations, and impairment of turnover can lead to greater cell death. The effects of aging in mitochondrial dysfunction are discussed later in this paper. Although many factors lead to biological aging within an organism, one common trend is the accumulation of defects over time.

AGE-RELATED FACTORS IN DEFECTIVE AUTOPHAGY

Several lines of research revealed that multiple age-related factors are implicated in defective autophagy (Cheon et al., 2019). In the last decade, several groups studied autophagy and mitophagy in aging and age-related diseases of different species such as flies, worms, humans, rats, and mice. Studies have also been done on rodent models of human disease and postmortem brains of healthy humans and humans with neurodegenerative disorders (Palikaras et al., 2015, 2018; Schiavi et al., 2015; Harper et al., 2018; Reddy et al., 2018; Manczak et al., 2018; Andreux et al., 2019; Castellazzi et al., 2019; Fang et al., 2019a,b; Hou et al., 2019; Martín-Maestro et al., 2019; Reddy and Oliver, 2019; Oliver and Reddy, 2019a,b; Wang et al., 2019; Li et al., 2020; Lou et al., 2020; Chen et al., 2020; Bakula and Scheibye-Knudsen, 2020; Liang and Gustafsson, 2020; Varghese et al., 2020; Luo et al., 2020; Markaki and Tavernarakis, 2020; Babbar et al., 2020; Aman et al., 2020; Cai and Jeong, 2020; Pakpian et al., 2020; Oh et al., 2020; Yang et al., 2020; Han et al., 2020; Pradeepkiran and Reddy, 2020). These articles (original and high impact review articles) have provided a

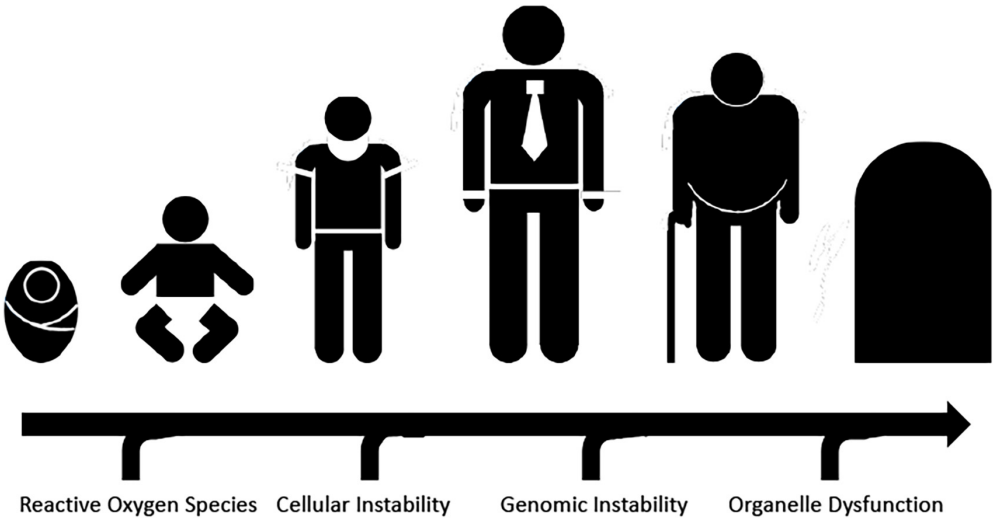

FIGURE 1 | Normal physiological aging accelerated by factors leading to cellular dysfunction, including reactive oxygen species, organelle dysfunction, and genomic damage.

large body of useful information about autophagy and mitophagy in different species of vertebrates and non-vertebrates.

Age-related factors, including oxidative stress, DNA damage, and telomere shortening, are involved in defective autophagy (Cheon et al., 2019) in both vertebrates and non-vertebrates. We briefly discuss these factors below:

Oxidative Stress and Defective Autophagy

Mitochondria are the powerhouses of cells, providing energy for several cellular functions, including intracellular calcium regulation, ATP production, the release of proteins that activate the caspase family of proteases, and the alteration of the reduction–oxidation potential of cells and free radical scavenging. Cellular aging induces mitochondrial ROS production and disrupts the electron transport chain (ETC). Disruption of the ETC has been recognized as an early feature of apoptotic cell death. The ETC involves the reduction of hydrogen peroxide (H2O2) to H2O and O2 by superoxide dismutase, catalase, peroxidase, and glutathione accepting electrons donated by NADH and FADH2, which yields the energy for the generation of ATP from adenosine diphosphate and inorganic phosphate (Reddy, 2006, 2008). Mitochondrial superoxide radical (O●- 2) production occurs primarily at discrete points in the ETC at complexes 1 and 3 and in components of tricarboxylic acid, including α-ketoglutarate dehydrogenase. In addition, mitochondrial O●- 2 are generated in the outer mitochondrial membrane. Monoamine oxidase, localized on the outer mitochondrial membrane, catalyzes the oxidative deamination of primary aromatic amines (Reddy, 2006). This deamination produces a large amount of H2O2 that contributes to an increase in the steady-state concentrations of ROS within the mitochondrial matrix and the cytosol. These released H2O2 and O●- 2 are carried to the cytoplasm via voltage-dependent anion channels and ultimately lead to the oxidation of cytoplasmic proteins.

The age-related chronic exposure cells to ROS can result in oxidative damage to mitochondrial proteins, cellular proteins, lipids, and nucleic acids, whereas the acute exposure to ROS can inactivate the tricarboxylic acid-cycle aconitase and the iron–sulfur centers of ETC at complexes 1, 2, and 3, resulting in a shutdown of mitochondrial energy production (Reddy, 2008). Therefore, mitochondria undergo morphological and functional changes with age, including declines in ETC function, mitochondrial integrity, and mitochondrial quality, which results in impairments of cellular energy production and activity.

Autophagy plays a key role in the clearance of damaged cellular organelles, including mitochondria. However, age-related impairments of autophagy lead to the accumulation of abnormal mitochondria, which increases oxidative stress. Based on these studies, it is proposed that mitochondria-targeted antioxidants, such as MitoQ, and SS31 can be potential drugs that reduce free radicals, maintain mitochondrial quality and function, boost autophagy and mitophagy, and clear damaged mitochondria from cells.

DNA Damage and Defective Autophagy

Oxidative stress, which is caused by an imbalance between the production of free radicals and the presence of endogenous antioxidants within a cell, is the major cause of damage to DNA (Reddy, 2006, 2008). During aging and age-related conditions, free radicals' increased production occurs, and this increase of free radicals damages both nuclear and mitochondrial DNA (Reddy and Beal, 2008; Oliver and Reddy, 2019a,b). DNA damage is distinctly different from the germline mutation, but both lead to errors in DNA. DNA damage is an abnormality in the chemical structure of DNA, whereas a mutation is a change in the sequence of standard base pairs. Damage to DNA can cause changes in the

structure of the genetic material and prevents and/or alters the replication of DNA (Cheon et al., 2019).

Age-dependent DNA damage plays a large role in defective autophagy. The generation of free radicals can occur after several cellular insults, including ultraviolet irradiation damage of DNA and redox-cycling of quinones (Cheon et al., 2019). Both mitochondrial damage and nuclear DNA damage occur in an age-dependent manner. Somatic mitochondrial changes, including single nucleotide changes and large deletions, have been extensively reported in both vertebrates and non-vertebrates (Reddy and Beal, 2005; Oliver and Reddy, 2019a,b). DNA base-pair repair is defective and increased in an age-dependent manner. As mentioned earlier, mitochondrial DNA is more vulnerable to ROS than nuclear DNA because of its lack of protective shields—histones (Reddy and Beal, 2005; Oliver and Reddy, 2019a,b). Mutations of mitochondrial DNA (mtDNA) are usually due to replication errors by mtDNA polymerase and point mutations/deletions that spontaneously accumulate during aging. Several DNA repair events are activated in response to damaged DNA, including homologous recombination repair, non-homologous end joining, mismatch repair base excision repair, and nucleotide excision repair (Reddy and Beal, 2005; Oliver and Reddy, 2019a,b).

Previous studies support that the base excision repair is mainly involved in the repair of oxidative mtDNA modification and mitigates mitochondrial impairment. Mismatch repair-dependent autophagy requires Bcl-2-interacting protein 3 in a mammalian target of rapamycin (mTOR)-dependent manner (Cheon et al., 2019). Decreased ability to repair DNA and consequent accumulation of DNA damage may contribute to cellular senescence. Also, mutations in nuclear and mitochondrial genes caused by impaired DNA repair have been associated with aging.

Overall, DNA damage plays a significant role in defective autophagy and is directly associated with aging and age-related diseases such as AD, Parkinson's, and ALS.

Telomere Shortening and Defective Autophagy

What are telomeres?—these are specific DNA–protein structures found at both ends of each chromosome that protect the genome from nucleolytic degradation, unnecessary recombination or repair, and inter-chromosomal fusion. Telomeres, therefore, play a vital role in preserving the information in our genome (Shammas, 2011). As a normal cellular process, a small portion of telomeric DNA is lost with each cell division. Telomeres become shorter with aging, influenced by environmental factors and specific genetic defects in the underlying telomere mechanisms. When telomere length reaches a critical limit, the cell undergoes senescence and/or apoptosis. Telomere length, therefore, serves as a biological clock to determine the lifespan of a cell and an organism.

Telomerase is a reverse transcriptase enzyme complex capable of adding DNA sequence repeats (TTAGGG) to the 3′ end of DNA strands in the telomere regions at the ends of eukaryotic chromosomes (Harris and Cheng, Nephron 2017). Telomerase contains two major components in its transcriptase ribonucleoprotein complex, the RNA-directed DNA polymerase, TerT, and the RNA template, TerC, which together prevent telomere shortening by adding telomeric DNA repeats to chromosome ends. However, telomeric DNA repeats become defective in aging and age-related conditions.

A gradual loss of telomeric DNA leads to defective autophagy cellular events. Earlier studies have demonstrated that shortened telomeres are associated with autophagy (Aoki et al., 2007; Ali et al., 2016; Nassour et al., 2019). In cells with shortened telomeres, autophagy-related proteins and cytoplasmic vacuoles were increased (Nassour et al., 2019). Telomeric 3′ DNA oligonucleotides can induce autophagosomes and inhibit mTOR signaling in malignant glioma cells (Aoki et al., 2007). In addition, in multiple cell lines such as HEK 293T, HepG2, and U-2 OS, TERT binds to mTORC1 kinase and suppresses its activity, inducing autophagy. On the other hand, TERT knockdown increases the components of mTORC1, resulting in autophagy impairment under basal starvation conditions (Ali et al., 2016).

Overall, the shortening of telomeres impacts autophagy in aging and age-related diseases. Based on these studies, it has been proposed that autophagy and mitophagy enhancers are potential therapeutic targets.

AUTOPHAGY

Autophagy is the lysosome-mediated self-degradative process that plays a major role in nutrient balancing and housekeeping by selectively degrading dysfunctional organelles and proteins (Deter and De Duve, 1967; Glick et al., 2010; Parzych and Klionsky, 2014; Pradeepkiran and Reddy, 2020). Autophagy is carried out by a class of proteins called autophagy-related proteins (Atg), which were discovered in yeast cells. Atg proteins have been found throughout many different types of organisms, including mammalian cells, with several analogs within mammalian cells being identified with similar mechanisms as Atg proteins within yeast cells. When the mechanisms underlying autophagy are disrupted, cells become more prone to accumulating defects leading to reduced cell viability. If the autophagic pathways are not rescued, the accumulation of cellular debris can occur and lead to an acceleration of physiological aging (Rubinsztein et al., 2011). Conversely, normal aging associated with accumulations of DNA damage due to oxidative damage can lead to dysregulation of the autophagy machinery (Vellai, 2009). This leads to a positive-feedback loop in which normal cellular damage experienced in aging leads to a decreased ability for cells to protect themselves from further insult compromising cellular viability.

Autophagy can work in both a non-selective or selective manner, which targets organelles such as peroxisomes, mitochondria, or portions of the endoplasmic reticulum for degradation. Three modes of autophagy have been characterized: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy (**Figure 2**). Although each mode of autophagy is mechanically distinct, all three ultimately lead to the delivery of cellular cargo to the lysosome or vacuole for degradation and

recycling (Parzych and Klionsky, 2014). Many factors can induce autophagy within a cell, which often center on mitochondria. Release of cytochrome c, increased ROS production, the opening of mitochondrial permeability transition pore (mPTP), and oxidative damage all lead to selective autophagy of damaged mitochondria deemed mitophagy (Quinsay et al., 2010). When mitophagic function is overwhelmed, autophagy can be initiated on a grander scale leading to cell death and apoptosis.

As discussed earlier, autophagy is defective in aging and age-related diseases, mainly due to reduced clearance of subcellular organelles/proteins, such as mitochondria, endoplasmic reticulum, and other cellular debris. We discuss the detailed events of autophagy later.

Microautophagy

Microautophagy is a process in which a lysosome or vacuole will engulf cellular material directly in response to states of starvation (Mijaljica et al., 2011; Oku and Sakai, 2018; Yoo and Jung, 2018). The mechanisms that regulate microautophagy are poorly understood, with most microautophagy research being performed primarily in a yeast cell. These studies focused primarily on the mechanism by which lysosomes engulf peroxisomes by altering the environmental carbon source from methanol to glucose (Sienko et al., 2020). Microautophagy can be carried out both in a non-selective and selective process that targets proteins and organelles. In the non-selective form of microautophagy, the mechanism of absorption is based on the size of what is being engulfed. For smaller cytosolic contents such as proteins, tubular invaginations form in the vacuolar membrane, which then pinch off into autophagic vesicles. The invaginations are formed through the action of a GTPase and can occur without the normal proteins of vacuole fusion such as SNAP receptors and N-ethylmaleimide sensitive factor.

Larger structures like organelles cannot fit in these invaginations, so the vacuole will form finger-like projections that can engulf cellular contents and fuse to absorb the contents into the vacuole. This process is partially dependent on the target of rapamycin (TOR) and exit from the rapamycin-induced growth arrest (EGO) complex. The TOR complex is also related to macroautophagy, and the intersection of these processes is counterbalanced in such a way that excessive autophagy does not occur (Oku and Sakai, 2018). Utilizing yeast mutants, studies have shown that some Atg genes that are involved in macroautophagy also play as key enzymes in microautophagy (Dunn et al., 2012). Micropexophagy, the selective autophagy of peroxisomes, is carried out in a series of steps, including initiation, target recognition, peroxisome sequestration, and terminal vacuole enclosure, and it has been shown that all of these steps are mediated by a series of Atg proteins. It has also been shown that non-selective microautophagy and other

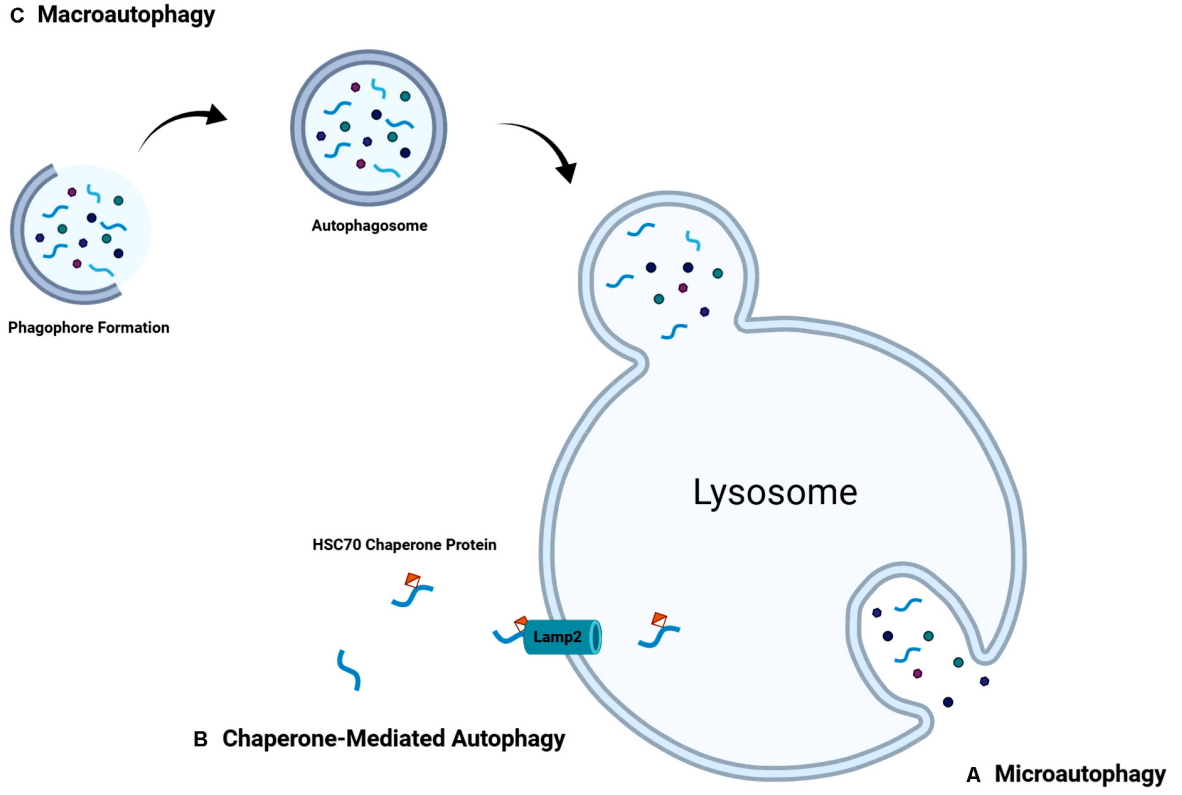

FIGURE 2 | Overview of various forms of autophagy including, **(A)** microautophagy in which lysosomal invaginations directly take up cellular substrate, **(B)** chaperone-mediated autophagy in which chaperone proteins target specific proteins to lysosomal membrane proteins, and **(C)** macroautophagy in which phagophore formation around cellular substrate occurs and is trafficked to the lysosome where it will fuse.

selective autophagy are reliant on Atg proteins as well (Sienko et al., 2020). The exact role Atg proteins plays in microautophagy, and the regulating mechanisms surrounding microautophagy are poorly understood and warrant further study. One key challenge in understanding these processes is how it is being studied; microautophagy induced *in vitro* does not accurately represent physiological states *in vivo*.

Chaperone-Mediated Autophagy

Chaperone-mediated autophagy (CMA) is a form of autophagy in which specific proteins are targeted by chaperone proteins and trafficked to lysosomes where they directly enter through the membrane (Majeski and Dice, 2004; Dice, 2007; Li et al., 2012). CMA has only been found in higher eukarya and is an important mechanism for the maintenance and regular turnover of cellular proteins to help ensure optimal function. Although CMA is also used in times of energy deficiency, activation of this pathway is slower than macroautophagy and may play a more important role in regulating the amounts of specific proteins in metabolic pathways. The constitutive chaperone, heat shock-cognate protein of 70 kDa (hsc70), targets a pentameric motif within proteins for autophagy. In some cases, the motif is not accessible by hsc70 until the protein becomes unfolded due to degradation or if post-translational modification by other proteins alters the charge state of a pentamer to the appropriate motif. Once the hsc70-protein dimer arrives at the lysosome, it interfaces with lysosome-associated membrane protein type 2A (LAMP-2A), forming a complex with other lysosomal membrane proteins to allow for the translocation of the substrate directly into the lysosomal lumen for degradation. The maximal rate of CMA is primarily dependent on the amount of LAMP-2A within the lysosomal membrane (Li et al., 2012). LAMP-2A transcription is increased in times of oxidative stress and decreased in times of prolonged starvation.

Malfunctions in the CMA pathway play a key role in the pathogenesis of many human disorders, including some neurodegenerative diseases (Dice, 2007). Decreases in the CMA pathway compromise a cell's ability to properly remove deleterious proteins leading to accumulation within cells. Such accumulations alter proteostasis and can lead to the deposition of protein aggregates leading to neuronal demise (Majeski and Dice, 2004). It has been observed that in normal physiological aging, CMA decreases. Age-related changes in CMA activity have been linked to alterations in the lipid composition of lysosomal membranes, which threatens the integrity of LAMP-2A proteins (Cuervo and Wong, 2014). CMA function is imperative in maintaining protein integrity and ensuring proper cellular function.

Macroautophagy

Macro-autophagy is the most studied and most understood mode of autophagy. The defining mechanism of macro-autophagy is the *de novo* formation of a double-membrane vesicle termed "autophagosomes," which engulfs cellular debris. Macro-autophagy follows five steps for breakdown: (1) induction of the isolation membrane, (2) elongation of membrane, (3) closure and autophagosome formation, (4) autophagosome–lysosome fusion, and (5) lysosomal degradation. In yeast, the formation of autophagosomes is initiated at the phagophore assembly site, which is a single site adjacent to the vacuole (Glick et al., 2010). In eukaryotes, the formation of autophagosomes is initiated at multiple sites throughout the endoplasmic reticulum called omegasomes. The initial formation of the double-membrane is termed the phagophore, which rapidly begins to expand into the spherical autophagosome. The autophagosome will bend and engulf its target within its double-membrane and then be translocated to the lysosome or vacuole. Once at the target site, the autophagosome will fuse with the lysosome, at which point it becomes the autolysosome. The acidic contents of the lysosomal lumen then break down the autophagosomal membrane as well as its contents, and the breakdown products are exported back into the cellular cytoplasm for reuse in biosynthetic pathways. In mammals, autophagosomes can integrate into the endocytic pathway and fuse with endosomes, which are subsequently broken down together (Deter and De Duve, 1967).

The mechanisms by which macroautophagy is regulated were first studied in yeast, and many autophagy-related genes (Atg) were identified. Many of the Atg proteins are found in other eukaryotic organisms, including mammals, but some of the Atg proteins have homologs, such as the Unc-51-like kinase family, which serves the same role as yeast Atg13 (Parzych and Klionsky, 2014). The mammalian system of macroautophagy is more complex than yeast systems and includes many additional regulatory proteins. Both yeast and mammalian autophagosomes require proteins that regulate autophagosome nucleation and elongation.

Several stressors trigger macroautophagy, such as nutrient deficiency, insulin concentrations, endoplasmic reticulum stress, and energy levels. Due to the role macroautophagy plays in cellular recycling, autophagosome formation is tightly regulated by two major pathways that are sensitive to carbon and nitrogen balance. The cAMP-dependent protein kinase A (PKA) pathway regulates macroautophagy by sensing carbon balance within both yeast and mammalian cells. PKA activation by high cAMP concentrations signals a nutrient-rich state within the cell and will inhibit further cellular recycling by autophagy. The mammalian target of rapamycin complex 1 (MTORC1) is a key protein in macroautophagy that is sensitive to amino-acid levels within a cell, which in turn is an indicator of the nitrogen balance (Kiffin et al., 2007). Some studies have shown that PKA interacts with MTORC1 and can phosphorylate and activate MTORC1, suggesting that these pathways are linked. In mammalian cells, AMP-activated protein kinase (AMPK) is a substrate of PKA and is a major energy-sensing kinase that responds to cellular AMP levels, which is a strong indicator of the energy level within a cell. Yeast has a similar mechanism in which macroautophagy is regulated by energy-sensing using the Snf1, which works similarly to AMPK. Endoplasmic reticulum stress can also precipitate autophagosome formation. Calmodulin-dependent protein kinase 2, beta (CaMKKβ) activation leads to increased cytosolic Ca^{2+} concentrations, which can induce AMPK and subsequently macroautophagy. The endoplasmic reticulum can also detect unfolded proteins, which will induce macroautophagy through similar mechanisms. As macroautophagy is the most

well-studied of autophagic pathways, we will use the term autophagy to describe it hereafter.

Proteins of Autophagy

Due to the importance of autophagy in maintaining cellular integrity and health, many proteins are involved in the induction and maintenance of these pathways. Although not an exhaustive list, we will briefly discuss some of the major proteins of autophagy in the following sections.

Mammalian Target of Rapamycin

Mammalian Target of Rapamycin is a complex of 2 protein kinases consisting of MTOR1 and MTOR2 that is sensitive to cellular nutrition levels and plays a role in regulating cell growth and survival via autophagy (Hale et al., 2012; Lett, 2016). MTOR is integrated into many cell survival pathways and utilizes nutrition levels to modulate cell growth. In nutrient-rich conditions, MTOR is activated, leading to the phosphorylation of key autophagy enzyme Unc-51-like kinase-1 (ULK1). Phosphorylation of ULK1 suppresses protein activity and prevents phagosome formation.

Unc-51-Like Kinase-1/ATG13

Unc-51 like kinase-1 is a protein associated with MTOR that will dissociate in response to nutrient-poor cellular conditions. ULK1 then acts to phosphorylate both ATG13 and RB1-inducible coiled-coil 1, which are proteins required to form phagosomes around cellular content (Khang et al., 2011). ULK1 also acts on a multitude of proteins involved in autophagy progression and regulation, including Beclin1 and Ambra1.

Beclin1 and Ambra1

Beclin1 is a key protein of autophagy activated by ULK1. Once activated, Beclin1 promotes the formation of the Vps34 complex, which consists of BCL1, Vps34/CIII PI3K, and Vps15 (Lett, 2016). The Vps34 complex is a key regulator of autophagy initiation and progression. Beclin1 contains three structural domains, a Bcl-2 homology 3 (BH3) domain, a central coiled-coil domain, and an evolutionarily conserved domain. Under normal cellular conditions, the BH3 domain interacts with Bcl-2 to inhibit autophagy. The coiled-coil domain interacts with multiple proteins that promote activation of autophagy, including Ambra1, UV radiation resistance association gene, and Atg14L (Khang et al., 2011). The evolutionarily conserved domain allows Beclin1 to modulate autophagy and inhibit tumorigenesis. As previously noted, Ambra1 is an essential activator of the Beclin1-dependent pathway of autophagy, but it also promotes stabilization of ULK1 and kinase activity (Maria Fimia et al., 2007).

Phagosome Elongation

Phagosome completion around targeted cytosolic content is accomplished by a series of ATG genes primarily through 2 ubiquitin-like systems. ATG7 is activated in an ATP-dependent manner, which then activates ATG12. ATG12 then is complexed to ATG5 by ATG10, an E2-like enzyme, and forms the first complex, ATG5-ATG12-ATG12L1. This complex works to elongate the phagophore. The second system starts with MAP1LC3, the mammalian ATG8 homolog, and ATG4B. ATG4B cleaves LC3 into LC3-I, which is conjugated to phosphatidylethanolamine via ATG3 and ATG7 forming LC3II. LC3II is integrated into the nascent phagosome membrane and acts as a marker that facilitates phagophore fusion with lysosomes (Hale et al., 2012). Together, these two systems help complete phagosome formation and target it to the lysosome for degradation.

SELECTIVE AUTOPHAGY

Autophagy in response to nutrient imbalances usually occurs as a non-specific process, but it can also be conducted in a highly specific manner for cell maintenance by targeting peroxisomes, mitochondria, and other organelles (He and Klionsky, 2010). Selective autophagy plays a role in destroying malignant cells, damaged organelles, invasive pathogens, protein aggregates, and excess peroxisomes. In selective autophagy, autophagosomes target specific cargo using the Atg-8 family proteins on the isolation membrane (Mehrpour et al., 2010). CMA also only works as a selective process, utilizing Hsc-70 chaperone proteins to traffic targeted proteins to lysosomal receptors for recycling. In mammalian cells, Atg8 analogs such as LC3 and GABARAP help to selectively sequester target substrate within autophagosomes utilizing cargo-specific receptors (Majeski and Dice, 2004; Dice, 2007; Li et al., 2012). LC3-interaction regions (LIR) have been shown to not only play a role in autophagy but also recruit other autophagosomal proteins. Ubiquitin is a well-known marker targeting proteins for degradation, and it is also used to mark cellular material for autophagy. Other pathways exist for specific autophagy independent of ubiquitin as well. Many selective autophagy pathways have been discovered and named according to the cellular target, such as mitochondria (mitophagy), ribosomes (ribophagy), endoplasmic reticulum (reticulophagy), peroxisomes (pexophagy), and many other organelle-specific mechanisms. Although selective autophagy ultimately utilizes many of the same mechanisms underlying non-selective autophagy, certain proteins and factors are associated with each organelle that will induct the autophagy machinery (He and Klionsky, 2010; Zaffagnini and Martens, 2016). Although all forms of specific autophagy play an important role in cell maintenance and health, we focus on mitophagy in this paper.

Mitophagy

As previously discussed, mitophagy is the selective autophagy of mitochondria and has been a focus of research in recent years for its potential role in many diseases (Lynch-Day and Klionsky, 2010; Youle and Narendra, 2011; Pradeepkiran and Reddy, 2020). Mechanisms underlying mitophagy are best documented in yeast studies, and several proteins are essential for mitophagy (Ding and Yin, 2012). One such protein is Uth1, a SUN-domain protein essential for mitophagy in yeast (Ashrafi and Schwarz, 2013). Ancient ubiquitous protein, a phosphatase 2C, plays a role in facilitating mitophagy in yeast in a stationary phase (Tal et al.,

2007; Youle and Narendra, 2011). Ancient ubiquitous protein is not required for non-specific autophagy. Although Atgs are utilized in all autophagy pathways, a few have been shown to play a role in the selective uptake of mitochondria. In two studies, it has been shown that Atg 32 is a mitochondrial receptor capable of inducing mitophagy in yeast. Atg32 can bind to Atg11, which acts as an adaptor to Atg8, which is thought to signal mitochondria absorption into autophagosomes (Kanki et al., 2009; Okamoto et al., 2009).

Although these proteins are essential in yeast for mitophagy, no homologs have been found in mammalian cells with their own pathways for initiating mitophagy. Although mitochondria play a pivotal role in protecting cells from oxidative stress, they themselves are not immune to the destructive effects of reactive species. Proteins involved in mitophagy that are responsible for the upkeep of healthy mitochondrial populations are encoded by nuclear DNA and so are subject to the mutative effects of oxygen species (Sun et al., 2016; D'Amico et al., 2019). Furthermore, post-translational modifications observed in aging have been associated with decreased expression of mitophagy proteins, which accelerates the aging process. Studies have demonstrated that Pumilio2 (Pumilio homolog 2 is an RNA-binding protein) regulates synaptic plasticity via translational repression of synaptic receptors and is activated in aging leading to suppression of mitochondrial fission factor. This disrupts mitochondrial dynamics, ultimately leading to decreased mitochondrial fission and inhibiting mitophagy of abnormal mitochondria. Both mitochondrial function and the ability of a cell to conduct mitophagy are expected to decrease in normal physiological aging; these effects are exacerbated in aging-related diseases such as AD, which accelerates the decay of mitochondrial function. Due to the essential role of mitophagy in maintaining cellular health, several mechanisms for its regulation exist. Although not an exhaustive list, the two most predominant pathways are discussed in the next sections.

PTEN-Induced Putative Kinase 1/Parkin Pathway

One of the most well understood and important regulators of mitophagy is the PINK1/Parkin pathway (**Figure 3**). PTEN-induced putative kinase 1 (PINK1) is a serine/threonine kinase localized in the inner mitochondrial membrane. In healthy mitochondria, PINK1 is constantly degraded by mitochondrial proteins, including matrix processing peptidases and presenilin-associated rhomboid-like (Youle and Narendra, 2011; Ding and Yin, 2012; Ashrafi and Schwarz, 2013). Fragmented PINK1 is then translocated to the cytosol and further degraded by other proteases. In damaged mitochondria, the mitochondrial membrane becomes depolarized, deactivating matrix processing peptidases and presenilin-associated rhomboid-like. PINK1 can then auto-phosphorylate, leading to activation and accumulation on the outer mitochondrial membrane, where it then can recruit cytosolic Parkin (Pickrell and Youle, 2015). Parkin, an E3 ubiquitin ligase, is phosphorylated by PINK1 and subsequently translocated into the mitochondrial membrane (Ashrafi and Schwarz, 2013). Once phosphorylated, Parkin enters the mitochondria, where it is believed to play a role in the ubiquitylation of mitochondrial proteins and substrate, marking the mitochondria for autophagy. Once protein Parkin works on, mitofusin 1, becomes activated, leading to mitochondrial fission, allowing for easier mitophagy (Pickrell and Youle, 2015). Ultimately, phosphorylation of Parkin by PINK1 leads to the initial induction of mitophagy in damaged or defective mitochondria.

BNIP3/NIX Pathway

BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 and NIX are both transmembrane proteins found in the outer mitochondrial membrane that share a similar structure and play a role in mitophagy (Zhang and Ney, 2009; Ney, 2015). These proteins were initially found to play a role in the selective exocytosis of mitochondria from nascent red blood cells, and their presence is required for the final maturation of RBCs. BNIP3 contains a BH3 domain as well as a carboxyl-terminal transmembrane domain, which works as a pro-apoptotic factor. The transmembrane of the domain of BNIP3 is required for mitochondrial targeting and subsequent pro-apoptotic activity (Ney, 2015). Expression of BNIP3 and NIX has been shown to increase in hypoxic cell conditions leading to cytochrome C release, membrane depolarization, and mitochondrial swelling. Nix has also been shown to directly interact with LC3 and GABARAP, which are inducers of the general autophagic machinery (Ding et al., 2010). Although the exact role of BNIP3 and NIX in the induction of mitophagy is not well understood, several potential mechanisms have been proposed. The role of these proteins in membrane depolarization has been shown to play a role in the induction of general autophagy mechanics, and Nix may play a role in ROS generation, which leads to the recruitment of markers for autophagy. A second theory is that BNIP3 and NIX competitively bind BCL2, liberating Beclin-1, which can then activate autophagy. The last theory is that BNIP3 inhibits Rheb, an upstream target of the mTOR, thereby inducing autophagy (Ney, 2015). Most studies suggest that BNIP3 and NIX do not act through a singular pathway and that they may work through several pathways to promote mitophagy and cell death. Although other pathways are theorized to play a role in mitochondrial autophagy, they are not well understood and believed to work in mechanisms similar to those outlined earlier.

ALZHEIMER'S DISEASE

Alzheimer's disease is an insidious neurodegenerative disease characterized by the presence of senile plaques composed of amyloid-beta (Aβ) peptides and the formation and accumulation of hyper-phosphorylated tau into neurofibrillary tangles in the brain (Selkoe, 2010; Hyman et al., 2012; Reiss et al., 2018; Shefa et al., 2019). Although the exact pathogenesis of AD is not well understood, it is believed that both Aβ and neurofibrillary tangles play a major role in the progression and symptoms present in AD. AD is one of the most common forms of dementia worldwide and is believed to be the sixth leading cause of death in the United States (Reiss et al., 2018). Increases in life expectancy have

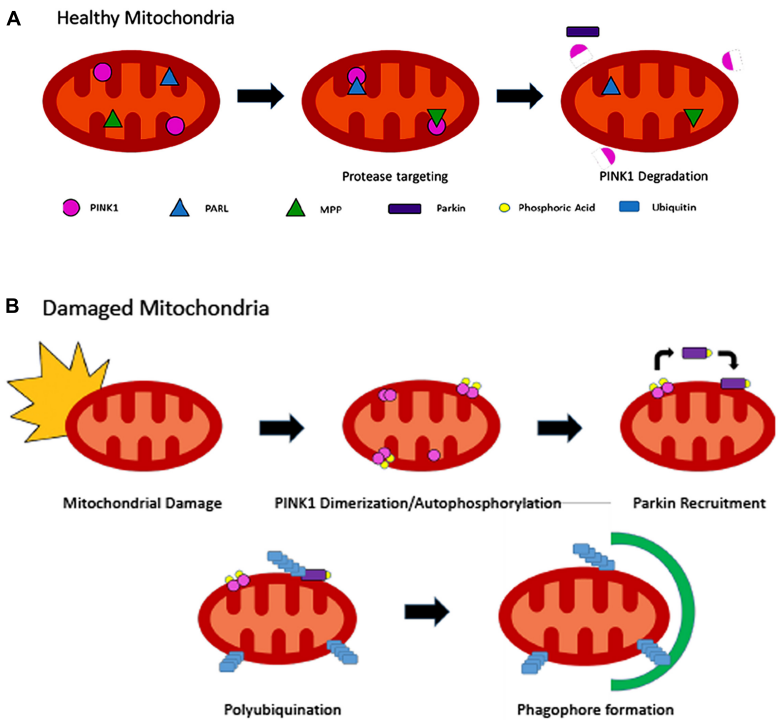

FIGURE 3 | Mechanism by which PINK1/Parkin pathway for autophagy occurs in healthy **(A)** and damaged mitochondria **(B)**.

greatly increased the number of cases of dementia, driving more research into the underlying causes and progression of aging and AD. The defining features of AD include cognitive impairment due to synaptic dysfunction, increased confusion, and issues with short-term memory loss, which progressively worsens (Reddy et al., 2017). Most cases of AD are sporadic and occur late in life, but genetic components are found in approximately 2% of cases and are termed "familial" AD with symptoms manifesting as early as 30–40 years of age. Most cases of familial AD are associated with genetic alterations in genes coding for proteins related to the processing of amyloid precursor protein or amyloid precursor protein itself. In particular, mutations in the presenilin 1 (PS1), a core component of γ-secretase, which plays a role in APP processing, is a major risk factor for developing early-onset AD (Goiran et al., 2018). Alterations in the processing proteins of APP leads to an increased amount of Aβ-42, which is believed to play a role in AD pathogenesis. Diagnosis of AD is through cognitive testing supported with imaging techniques such as magnetic resonance imaging. However, due to the normal, expected cognitive decline with age, it can be difficult to diagnose AD in the early stages of the disease.

Amyloid Beta

In AD, accumulation of Aβ into oligomers and fibrils is implicated as one of the early events in AD development and progression (Reddy, 2006; Reddy and Oliver, 2019; Murphy and Levine, 2010; Hamley, 2012). Aβ plaques accumulate in the hippocampus, amygdala, and associated neocortex, all of which play roles in memory formation. Aβ is generated as the breakdown of amyloid precursor protein (APP) by cleavage via secratases. Although the exact role of APP is not well understood, the breakdown products, including Aβ, play a role in AD (Zhang et al., 2011; Lauritzen et al., 2019). APP is processed via two different pathways, including the non-amyloidogenic pathway in which APP is cleaved by α-secretase to produce sAPPα, which is believed to play a role in neuronal survival and is further processed by γ-secretases to produce p83. The second pathway, termed the amyloidogenic pathway, involves APP processing by β-secretases and subsequent processing by γ-secretases into Aβ. Although the first pathway produces benign breakdown products of APP, the formation of Aβ as well as the intermediates of the amyloidogenic pathway are associated with AD pathogenesis. Although many lines of research have demonstrated an association between Aβ and AD, more recent lines of research have suggested that some of the other breakdown intermediates of APP, namely C9, may also play a role in AD (Laurtizen et al., 2016). Cells with impaired lysosomal function show increased levels of C99, which has been associated with AD. Furthermore, in studies with mice with increased expression of C99, neuronal populations displayed significantly reduced long-term potentiation, suggesting that C99 may play a role in AD progression. Studies with transgenic mice have demonstrated that C99 upregulation can lead to AD pathology even in the absence of Aβ (Laurtizen et al., 2016).

Overall, the accumulation of Aβ leads to mitochondrial dysfunction, synaptic damage, and defective autophagy within neuronal cells.

Abnormal Interactions of Amyloid Beta With Drp1 and Defective Mitophagy

Abnormal interactions of amyloid beta and phosphorylated tau with mitochondrial and other cellular proteins have been reported in AD (Manczak et al., 2011; Manczak and Reddy, 2012a,b; Reddy and Oliver, 2019).

Reddy Lab (Manczak et al., 2011) investigated the molecular links between increased mitochondrial fission protein Drp1 and Aβ using co-immunoprecipitation and colocalization studies. Utilizing postmortem AD brains and brain tissues from APP mice and Drp1 immunoprecipitation/immunoblotting analysis of Aβ antibodies 6E10 and A11 revealed that Drp1 interacts with Aβ monomers and oligomers in AD patients and APP mice. These abnormal interactions are increased with disease progression. Their colocalization studies using Drp1 and the Aβ antibodies revealed the colocalization of Drp1 and Aβ (Manczak et al., 2011). These findings suggest that increased production of Aβ and the interaction of Aβ with Drp1 are crucial factors in mitochondrial fragmentation, abnormal mitochondrial dynamics, and synaptic damage in AD.

Amyloid Beta Interaction With Voltage-Dependent Anion Channel 1 and Defective Mitophagy

To determine the role of mitochondrial outer membrane protein, voltage-dependent anion channel 1 protein (VDAC1) in AD, the Reddy group (Manczak and Reddy, 2012b) used brain specimens from AD patients and control subjects and 6-, 12- and 24-month-old Aβ precursor protein transgenic mice to assess VDAC1 protein levels. Furthermore, they also studied the interaction between VDAC1 and Aβ (monomers and oligomers) using cortical tissues from AD patients, control subjects, APP, APP/PS1, and 3XTg.AD mice. They also studied age- and VDAC1-linked, mutant APP/Aβ-induced mitochondrial dysfunction in APP and non-transgenic wild-type (WT) mice. Progressively increasing levels of VDAC1 in the cortical tissues from the brains of patients with AD were observed relative to control subjects, and significantly increased levels of VDAC1 were found in the cerebral cortices of 6-, 12- and 24-month-old APP transgenic mice relative to the age-matched control WT mice. Co-immunoprecipitation and co-labeling analysis of postmortem AD brains and brain tissue from APP transgenic mice revealed that VDAC1 interacted with Aβ in the brains of AD patients and APP, APP/PS1, and 3XTg.AD mice. They found progressively increased mitochondrial dysfunction in APP mice relative to control WT mice. Based on these observations, they concluded that VDAC1 interacts with Aβ and may in turn block mitochondrial pores leading to mitochondrial dysfunction in AD pathogenesis.

Based on these observations, they propose that reduced levels of VDAC1, Aβ, and phosphorylated tau may reduce the abnormal interaction between VDAC1 and APP, VDAC1 and Aβ. Reduced levels of VDAC1 and Aβ may maintain normal mitochondrial pore opening and pore closure, ultimately leading to normal mitochondrial function, allowing mitochondria to supply ATP to nerve terminals and boosting synaptic and cognitive function in AD (Manczak and Reddy, 2012b).

Phosphorylated Tau

Tau proteins normally play a role in the assembly and stabilization of microtubules and other cytoskeletal elements within neurons. However, when tau becomes hyper-phosphorylated, it loses its activity leading to disruption of the cytoskeleton, causing synaptic transmission dysfunction and neuronal death (Alonso et al., 1994; Rajmohan and Reddy, 2015). Hyper-phosphorylated tau (P-tau) will form paired helical filaments, which will then aggregate to form the neurofibrillary tangles characteristic of AD. Studies with transgenic mouse models of AD suggest that Aβ toxicity is mediated by tau. It has also been shown that Aβ plays a role in triggering the hyper-phosphorylation of tau, suggesting that generation of Aβ precedes the accumulation of P-tau (Rapoport et al., 2002). Oxidative stress from other sources, such as decreased levels of insulin-like growth factor 1, has also been implicated in the formation of P-tau, leading to decreased cell viability. Loss of cytoskeletal integrity and subsequent neuronal death leads to the symptoms associated with AD.

Phosphorylated Tau Interaction With Drp1 and Defective Mitophagy

In a previous study, the Reddy lab (Manczak and Reddy, 2012a) tested whether P-tau interacted with Drp1 and attempted to elucidate how mitochondria are damaged in the progression of AD. They also investigated GTPase Drp1 enzymatic activity, which is critical for mitochondrial division in postmortem brain tissues from patients with AD as well as brain tissues from three different lines of transgenic APP, APP/PS1, and 3XTg.AD mice. Using co-immunoprecipitation and immunofluorescence analyses, they demonstrated the physical interaction between P-tau and Drp1 for the first time. Mitochondrial fission-linked GTPase Drp1 activity was significantly elevated in the postmortem frontal cortex tissues from AD patients and cortical tissues from APP, APP/PS1, and 3XTg.AD mice. Based on these findings, they concluded that Drp1 interacts with P-tau, likely leading to excessive mitochondrial fragmentation and mitochondrial synaptic deficiencies and ultimately leading to neuronal damage and cognitive decline (Manczak and Reddy, 2012a).

Phosphorylated Tau Interaction With Voltage-Dependent Anion Channel 1 Protein and Defective Mitophagy

To determine the role of mitochondrial outer membrane protein, VDAC1 and its interaction with p-tau in AD, the Reddy group (Manczak and Reddy, 2012b) studied the interaction between VDAC1 and phosphorylated tau, using cortical tissues from AD patients, control subjects, APP, APP/PS1, and 3XTg.AD mice. They found increased levels of VDAC1 in the cortical tissues from the brains of patients with AD, relative to control subjects. Co-immunoprecipitation and co-labeling analysis of postmortem AD brains, brain tissues from tau transgenic mice revealed that VDAC1 interacted with phosphorylated tau in the brains of

AD patients and 3XTg.AD mice. They concluded that VDAC1 interacts with phosphorylated tau, which may, in turn, block mitochondrial pores, leading to mitochondrial dysfunction in AD pathogenesis.

Based on these observations, they propose that reduced levels of VDAC1 and phosphorylated tau may reduce the abnormal interaction between VDAC1 and phosphorylated tau. Reduced levels of VDAC1 and phosphorylated tau may maintain normal mitochondrial pore opening and pore closure, ultimately leading to normal mitochondrial function, mitochondria supplying ATP to nerve terminals, and boosting synaptic and cognitive function in AD (Manczak and Reddy, 2012b).

Mitochondrial Dysfunction in Alzheimer's Disease

Another common finding in AD postmortem brains is signs of oxidative damage and mitochondrial dysfunction (Reddy, 2007; Swerdlow et al., 2010). Mitochondria are responsible for the majority of ATP generation and produces ATP through the ETC, a series of complexes found within the inner mitochondrial membrane. However, many byproducts are produced during this process, including ROS, superoxide (O_2^-), hydroxyl radicals (OH.), and hydrogen peroxide (H_2O_2) (Reddy and Oliver, 2019). ROS production plays a role in the degradation of both chromosomal and mitochondrial DNA, leading to compromised production of machinery, which can lead to even further ROS production and cell death. Both Aβ and P-tau have been associated with mitochondrial dysfunction in some way as well.

In several studies, it has been shown that microtubule destabilization from overexpression of tau and hyper-phosphorylation of tau leads to disruption of cellular trafficking (Ebneth et al., 1998). Kinesin, the motor protein responsible for transport to the cell periphery, is preferentially inhibited by P-tau. In neurons where organelle transportation is important, it was observed that mitochondria would concentrate in the cell body and would not be present in neurites (Stamer et al., 2002). Other organelles would likewise be affected, including peroxisomes, which help to alleviate oxidative stress. The increased concentration of organelles in the cell body prevents further production of organelles leading to a decrease in the numbers of important organelles, including mitochondria and peroxisomes. With the deficit of these organelles, neurites are made vulnerable by the decreased production of energy and increased susceptibility to oxidative stress. Interruption of kinesin-driven transport also negatively impacts the ability of APP to be transported into axons and dendrites (Reddy et al., 2004). This leads to the accumulation of APP within cell bodies, which can then be processed into Aβ .

Many lines of research have shown that Aβ and APP play a more direct role in mitochondrial dysfunction. Research done on mice at various stages of AD showed that genes regulating mitochondrial metabolism and regulators of apoptosis were upregulated (Reddy et al., 2004). These findings suggest that energy metabolism in the presence of Aβ impairs mitochondrial energy metabolism and that the upregulation of genes is a compensatory response. The same study examined mRNA expression in patients with early AD and definite AD and found downregulation of mitochondrial genes in complex 1 of the ETC, whereas genes for complexes III and IV were upregulated in both populations. Increased expression of complexes III and IV suggests that greater demand is put on the mitochondria for energy output. Aβ also directly interfaces with mitochondrial proteins. *In vitro* studies show Aβ peptides (25-35) are capable of blocking the entry of proteins into mitochondria leading to mitochondrial dysfunction, membrane depolarization, increased ROS production, and altered mitochondrial morphology (Reddy and Beal, 2005; Moreira et al., 2007). Increased ROS production from mitochondria also activates the fission proteins Drp1 and Fis1, causing mitochondrial fragmentation (Barsoum et al., 2006). Studies have also shown that Aβ and APP can enter mitochondria, and Aβ is able to form oligomers within mitochondria (Devi et al., 2006; Manczak et al., 2006). To further reinforce the role of Aβ, genetic analysis of individuals with familial AD commonly show defects in amyloid-beta precursor protein (APP) and presenilin 1 and 2 (Martin-Maestro et al., 2016). Aberrant APP produces more Aβ-42, the isoform of Aβ that is implicated in pathology, and presenilin 1 and 2 are essential cofactors for γ-secretase, which is the final step of processing of APP into Aβ. Overall, the role of Aβ in mitochondrial disruption is multifaceted, with many different pathways in which Aβ can both, directly and indirectly, interfere with normal mitochondrial function.

DYSFUNCTIONAL MITOPHAGY IN AGING

The major role of mitochondria in energy generation and ROS regulation makes their integrity paramount to cell health. Dysfunction in the mitochondrial health checkpoints and mitophagy machinery has been implicated in the acceleration of physiological aging and neurological diseases such as AD (Magrané et al., 2014; Ye et al., 2015; Rodolfo et al., 2018; Pradeepkiran and Reddy, 2020). The unique structure of neurons and their high energy demand makes neurons particularly reliant on proper mitochondrial function, and loss of mitochondrial integrity can lead to neuronal population loss and neurodegeneration (**Figure 4**). Neurons are also a non-proliferating cell type and so will accumulate cellular and oxidative stress over long periods (Bakthavachalam and Shanmugam, 2017).

As previously discussed, impairment of mitophagy can occur in several ways, but all ultimately lead to improper turnover of damaged mitochondria, accumulation of mitochondrial debris, and ultimately cell death (**Figure 4**). Neurodegenerative diseases have a complex set of symptoms and causative events, but most have familial forms that have helped to elucidate potential mechanisms underlying pathology. Several aberrant genes identified in these diseases have been linked to autophagy, implicating the role of autophagy in symptom genesis and progression. However, due to the complexity of the autophagic pathways and their regulators, the exact steps leading to pathology have been obfuscated. Despite the complexity,

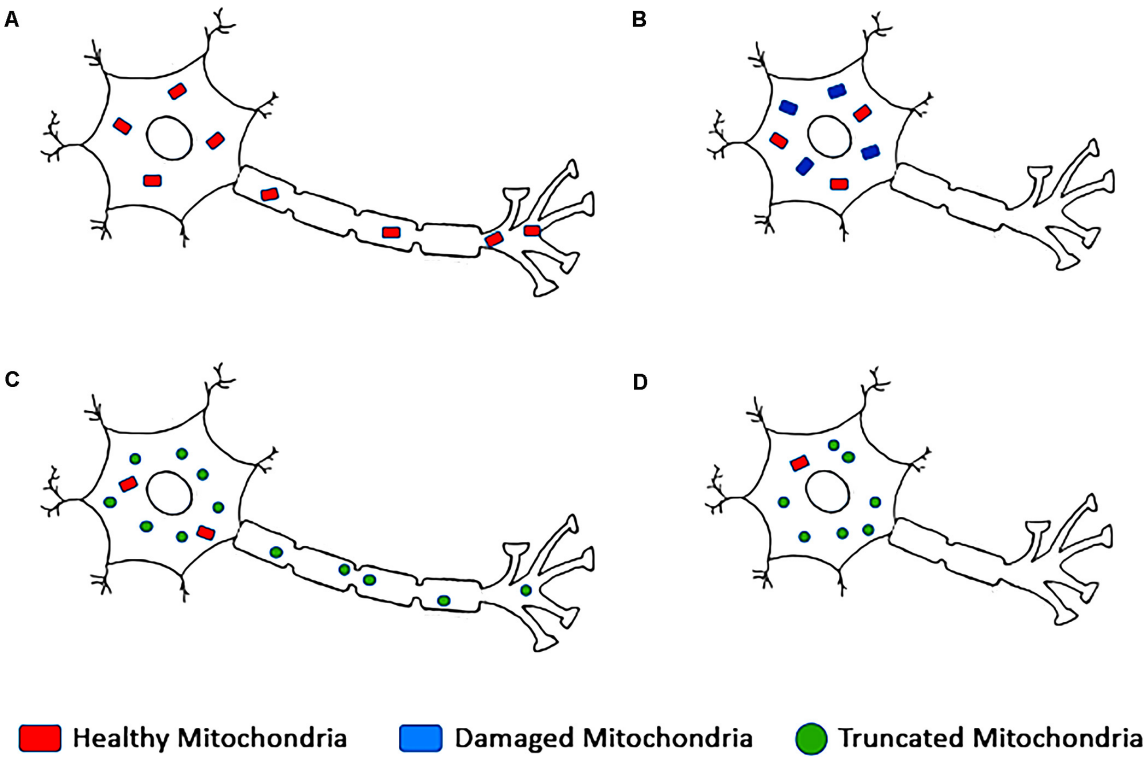

FIGURE 4 | Mitochondrial profile of neurons in **(A)** healthy neurons, **(B)** neurons with disrupted microtubule networks, **(C)** neurons with upregulated mitochondrial dynamic proteins, and **(D)** damaged neurons observed in Alzheimer's disease.

autophagy is generally broken down into a few steps: initiation, elongation, cargo recognition, and fusion with lysosomes.

DEFECTIVE MITOPHAGY IN ALZHEIMER'S DISEASE

As previously discussed, both soluble Aβ and abnormally phosphorylated tau characteristically found in AD directly interact with mitochondria and impair function. Beyond the role Aβ plays in mitochondrial function, Aβ impacts mitochondrial mRNA and protein expression in mice with increased expression of APP (Reddy et al., 2018). Mitochondrial structural genes, autophagy genes, and mitophagy-specific genes have all been shown to change in some way. Of the mitochondrial structural genes, Drp1 and Fis1, which both play a role in mitochondrial fission, have increased expression. Mfn1, Mfn2, and OPA1, proteins of mitochondrial fusion, were shown to have decreased levels of mRNA expression. Combined, these alterations in protein expression lead to increased mitochondrial fission and fragmentation making it harder for the mitophagy machinery to keep up with the cellular demand to clean out damaged mitochondria. Other genes examined in this study included genes for proteins that regulated autophagy and mitophagy, which were all downregulated. Of note, PINK1 was observed to have a 2.4-fold decrease in mRNA expression, and the PINK1/Parkin pathway has been considered one of the main pathways in which mitophagy is carried out. These data suggest that the initiation and cargo recognition component of mitophagy is greatly inhibited by Aβ. Furthermore, initial Aβ accumulation and related mitochondrial damage aggressively induce the PINK1/Parkin pathway of mitophagy (Cai and Jeong, 2020). As the disease progresses, cytosolic Parkin is depleted, leading to reduced cellular mitophagy capabilities over time. Although this phenomenon does not inhibit the mitophagy pathway, it still decreases the cell's capability to recycle damaged mitochondria leading to cellular stress. Studies have shown that basal levels of mitophagy can be restored in some cases when Parkin levels are overexpressed in mice (Martin-Maestro et al., 2016).

Early studies into the effects of abnormal tau on mitochondrial dynamics have focused primarily on the impairment of cellular trafficking. The destabilization of microtubule networks and interruption of organelle migration leads to the accumulation of damaged organelles within the neuronal soma. It has been found that tau also plays a role in inducing mitophagy by modulating membrane potential and Parkin levels (Hu et al., 2016). In individuals with increased total levels of tau and AD, increased mtDNA for mitophagy markers was observed, suggesting a mitophagy deficit within cells. The study also found that the membrane potential of mitochondria actually increases in the presence of abnormal tau, leading to decreased levels of PINK1 within mitochondria and subsequently decreased

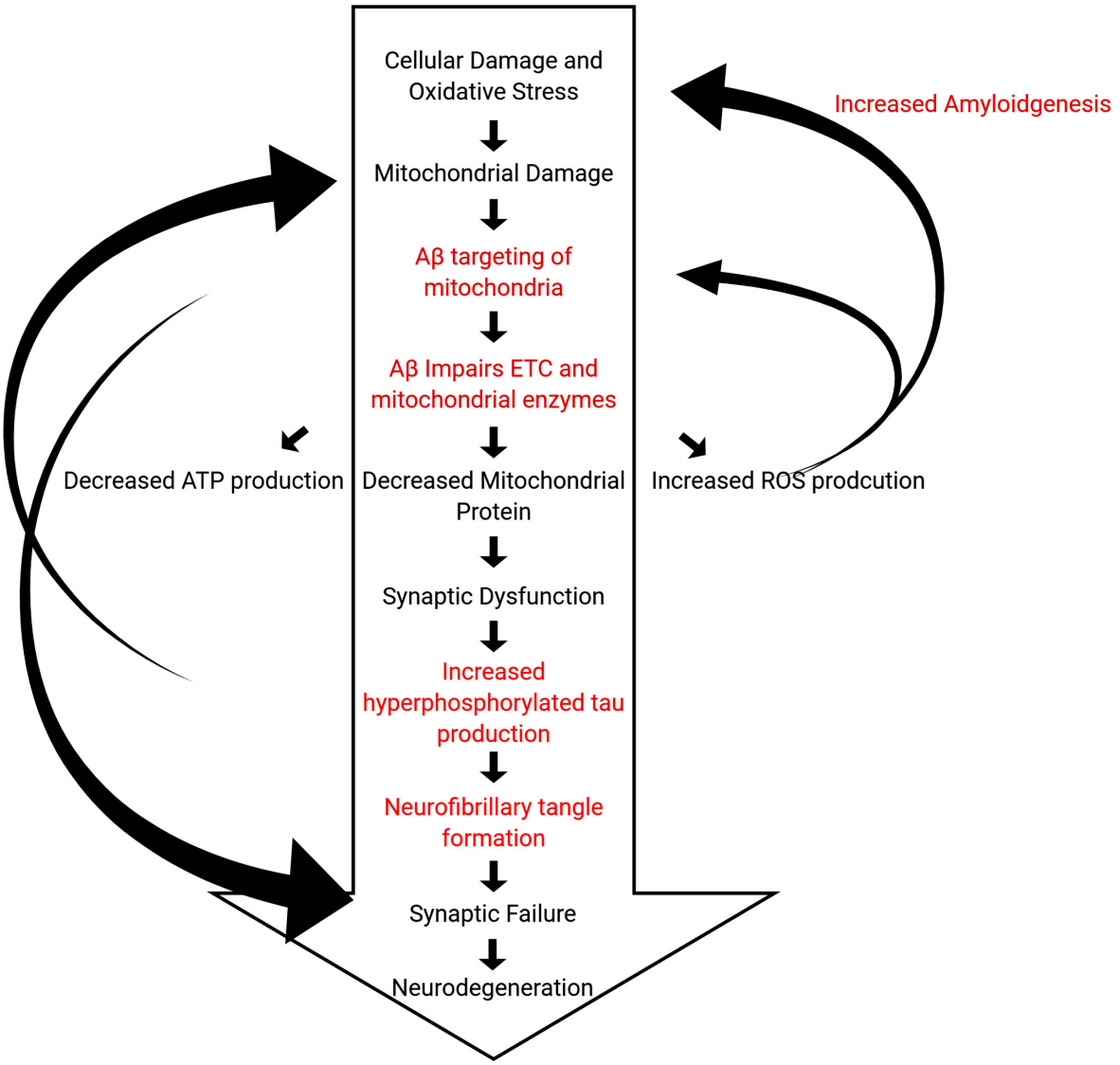

FIGURE 5 | Hypothetical outline of events of the pathogenic steps in the mitochondrial cascade hypothesis leading to death of neurons. Steps outlined in black are observed in normal physiological aging, whereas steps outlined in red are characteristics of AD that accelerate these processes.

Parkin localization to mitochondria. Tau also directly interacts with Parkin, which directly interacts with the projection domain of Tau, leading to the cytosolic sequestration of Parkin (Cummins et al., 2019). Tau has also been shown to interact with Drp1, suggesting that tau also plays a role in the excessive mitochondrial fragmentation observed in AD (Manczak and Reddy, 2012a). As noted, the effects on mitochondrial dynamics by tau are widespread and inhibit mitophagy in multiple ways.

Defective PINK1 and Parkin in Alzheimer's Disease

Recently, Ye et al. (2015) studied Parkin-mediated mitophagy using mutant hAPP neurons and AD patient brains. They found Parkin-mediated mitophagy is involved in mutant hAPP neurons and postmortem AD brains. In the absence of $\Delta\psi m$ dissipation reagents, hAPP neurons exhibit increased recruitment of cytosolic Parkin to depolarized mitochondria. Under AD-linked pathophysiological conditions, Parkin translocation predominantly occurs in the somatodendritic regions leading to decreased anterograde and increased retrograde mitochondrial axonal transport. Enhanced mitophagy was further confirmed in AD brains, accompanied by depletion of cytosolic Parkin over disease progression. Thus, aberrant accumulation of dysfunctional mitochondria in AD-affected neurons is likely attributable to inadequate mitophagy capacity and inability to clear damaged mitochondria. Altogether, these studies substantiate AD-linked chronic mitochondrial stress under *in vitro* and *in vivo* pathophysiological conditions.

Reddy et al. (2018) investigated the toxic effects of hippocampal mutant APP (mAPP) and Aβ in primary mouse hippocampal neurons (HT22) that express human APP Swedish mutation. Using quantitative reverse-transcriptase polymerase chain reaction, Western blotting and immunofluorescence, and transmission electron microscopy studies, they assessed mRNA and protein levels of synaptic, autophagy, mitophagy, mitochondrial dynamics, and biogenesis of proteins and assessed mitochondrial changes in mAPP-HT22 cells. Mitochondrial function was assessed by measuring the levels of hydrogen peroxide, lipid peroxidation, cytochrome c oxidase activity, and mitochondrial adenosine triphosphate. Increased levels of mRNA and protein levels of mitochondrial fission genes (Drp1 and Fis1) and decreased levels fusion (Mfn1, Mfn2, and Opa1) biogenesis (PGC1α, NRF1, NRF2, and TFAM), autophagy (ATG5 and LC3BI, LC3BII), mitophagy (PINK1 and TERT, BCL2 and BNIPBL), synaptic (synaptophysin and PSD95), and dendritic (MAP2) genes were found in mAPP-HT22 cells relative to WT-HT22 cells. Cell survival was significantly reduced by mAPP-HT22 cells. GTPase-Drp1 enzymatic activity was increased in mAPP-HT22 cells. Transmission electron microscopy revealed significantly increased mitochondrial numbers and reduced mitochondrial length in mAPP-HT22 cells. These findings suggest that hippocampal accumulation of mAPP and Aβ is responsible for the abnormal mitochondrial dynamics and defective biogenesis of MAP2, autophagy, mitophagy, and synaptic proteins as well as reduced dendritic spines and mitochondrial structural changes in mAPP hippocampal cells.

Reddy Lab (Manczak et al., 2018) also investigated the toxic effects of hippocampal mutant APP and Aβ in 12-month-old APP transgenic mice (Tg2576 strain). Using rotarod and Morris water maze tests, immunoblotting and immunofluorescence, Golgi-cox staining, and transmission electron microscopy, they assessed cognitive behavior, protein levels of synaptic, autophagy, mitophagy, mitochondrial dynamics, biogenesis, and dendritic protein MAP2 and also quantified dendritic spines and mitochondrial number and length in APP mice that express Swedish mutation. Mitochondrial function was assessed by measuring the levels of hydrogen peroxide, lipid peroxidation, cytochrome c oxidase activity, and mitochondrial ATP. Morris water maze and rotarod tests revealed that hippocampal and memory and motor learning and coordination were impaired in APP mice relative to WT mice. Increased levels of mitochondrial fission proteins and decreased levels of fusion, biogenesis, autophagy, mitophagy, synaptic, and dendritic proteins were found in 12-month-old APP mice relative to age-matched non-transgenic WT mice. Golgi-cox staining analysis revealed that dendritic spines were significantly reduced. Transmission electron microscopy revealed significantly increased mitochondrial numbers and reduced mitochondrial length in APP mice. These findings suggest that hippocampal accumulation of mutant APP and Aβ is responsible for abnormal mitochondrial dynamics and defective biogenesis, autophagy, mitophagy, and synaptic proteins and reduced dendritic spines and hippocampal-based learning and memory impairments in APP mice.

Fang et al. (2019b) also studied mitophagy in the progression of AD in pluripotent stem cell-derived human AD neurons, in animal AD models, and Aβ and tau *Caenorhabditis elegans* models of AD. They also found mitophagy is impaired in the hippocampus of AD patients, in induced pluripotent stem cell-derived human AD neurons, and in animal AD models. In both Aβ and tau *C. elegans* models of AD, mitophagy enhancers reversed memory impairment through PINK- 1-, Parkinson's disease-related-1; parkin-, or DAF-16/FOXO-controlled germline-tumor affecting-1-dependent pathways. Mitophagy diminishes insoluble $A\beta_{1-42}$ and $A\beta_{1-40}$ and prevents cognitive impairment in an APP/PS1 mouse model through microglial phagocytosis of Aβ and suppression of neuroinflammation. Mitophagy enhancement abolishes AD-related tau hyperphosphorylation in human neuronal cells and reverses memory impairment in transgenic tau nematodes and mice. Their findings further support the findings of previous studies of Ye et al. (2015), Reddy et al. (2018), and Manczak et al. (2018) that defective mitophagy is a major cellular change in AD progression and pathogenesis.

Overall, these studies clearly demonstrate that PINK1 and Parkin pathways are involved in AD. Both mRNA and protein levels of PINK1 and Parkin and other mitophagy and autophagy proteins are reduced in human and mouse AD cells and both APP and tau transgenic mouse models. These reductions inhibit/reduce mitochondrial function. Furthermore, the ability to clear damaged mitochondria through mitophagy is also compromised. The baseline-decrease in mitochondrial function associated with age initiates events leading to the formation and accumulation of Aβ, which exacerbates mitochondrial duress in what is termed the "mitochondrial cascade hypothesis," introduced by Swerdlow et al. (2010) in Swerdlow et al. (2010) (**Figure 5**). The intricate interplay among Aβ, tau, and mitochondrial proteins is not completely understood, and further investigations into the events triggering symptoms of AD still need to be done.

CONCLUSION AND FUTURE DIRECTIONS

Mitochondria play a key role in the production of energy and balance of ROS within cells. Mitophagy, the selective breakdown and clearance of aberrant and dead mitochondria, is a regulatory process essential to promoting cellular health and maintaining healthy mitochondrial populations. As a person age, oxidative stress and cellular damage accumulate, and autophagic pathways can become overwhelmed. This is especially true in non-actively dividing cells such as neurons, and cortical degeneration is commonly observed in aging populations.

AD, a characteristic illness of aging, is associated with cognitive deficits, including loss of memory formation and increased loss of cortical mass. Furthermore, characteristic conglomerates of Aβ and fibrillary tangles of abnormally

phosphorylated tau are observed within the brains of AD patients. Recent research also revealed that phosphorylated tau and fibrillary tangles are definitive AD features, both clinically and pathologically. Synaptic damage and defective mitophagy are early changes in disease progression, and as discussed earlier, aging plays a key role in synaptic and autophagy and mitophagy in AD progression and pathogenesis. Improved understanding of microglial activation and mitochondrial damage in neurons, particularly at synapses, is urgently needed.

In the past 20 years, the toxicity of these substrates has been studied extensively, and their role in neuronal death partially elucidated. The buildup of abnormal mitochondria is noted in AD neurons. More recently, studies have focused on the interaction between Aβ and tau on the components of mitophagy. Although some interactions between Aβ and tau and also Aβ and tau interactions with mitochondrial proteins and the components of mitophagy have been noted; the exact mechanisms and sequence of events leading to the genesis of AD have yet to be elucidated. Accumulation of damaged mitochondria, excessive mitochondrial fission, the buildup of ROS within cells, and compromised cellular health are all noted within neuronal populations in AD brains.

A major challenge in studies on the pathology of AD is identifying individuals with early-onset AD as the symptoms mimic what is normally expected in aging populations. Identification of the early events of AD within these populations can help elucidate the development of biomarkers and pathology in AD and outline the mechanisms by which symptoms occur. Further research could potentially develop mitophagy-based therapies to block or even reverse the adverse effects of AD. Further research is still needed to identify the role of mitophagy in AD.

AUTHOR CONTRIBUTIONS

PR contributed to the conceptualization and formatting of the article. MT and PR were responsible for writing, original draft preparation, and finalization of the manuscript. PR was responsible for funding acquisition. Both authors contributed to the article and approved the submitted version.

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Presenilin-Deficient Neurons and Astrocytes Display Normal Mitochondrial Phenotypes

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Presenilin 1 (PS1) and Presenilin 2 (PS2) are predominantly known as the catalytic subunits of the γ-secretase complex that generates the amyloid-β (Aβ) peptide, the major constituent of the senile plaques found in the brain of Alzheimer's disease (AD) patients. Apart from their role in γ-secretase activity, a growing number of cellular functions have been recently attributed to PSs. Notably, PSs were found to be enriched in mitochondria-associated membranes (MAMs) where mitochondria and endoplasmic reticulum (ER) interact. PS2 was more specifically reported to regulate calcium shuttling between these two organelles by controlling the formation of functional MAMs. We have previously demonstrated in mouse embryonic fibroblasts (MEF) an altered mitochondrial morphology along with reduced mitochondrial respiration and increased glycolysis in PS2-deficient cells (PS2KO). This phenotype was restored by the stable re-expression of human PS2. Still, all these results were obtained in immortalized cells, and one bottom-line question is to know whether these observations hold true in central nervous system (CNS) cells. To that end, we carried out primary cultures of PS1 knockdown (KD), PS2KO, and PS1KD/PS2KO (PSdKO) neurons and astrocytes. They were obtained from the same litter by crossing PS2 heterozygous; PS1 floxed ($PS2^{+/-}$; $PS1^{flox/flox}$) animals. Genetic downregulation of PS1 was achieved by lentiviral expression of the Cre recombinase in primary cultures. Strikingly, we did not observe any mitochondrial phenotype in PS1KD, PS2KO, or PSdKO primary cultures in basal conditions. Mitochondrial respiration and membrane potential were similar in all models, as were the glycolytic flux and NAD^+/NADH ratio. Likewise, mitochondrial morphology and content was unaltered by PS expression. We further investigated the differences between results we obtained here in primary nerve cells and those previously reported in MEF cell lines by analyzing PS2KO primary fibroblasts. We found no mitochondrial dysfunction in this model, in line with observations in PS2KO primary neurons and astrocytes. Together, our results indicate that the mitochondrial phenotype observed in immortalized PS2-deficient cell lines cannot be extrapolated to primary neurons, astrocytes, and even to primary fibroblasts. The PS-dependent mitochondrial phenotype reported so far might therefore be the consequence of a cell immortalization process and should be critically reconsidered regarding its relevance to AD.

Keywords: presenilins, OXPHOS, mitochondria, astrocyte, neuron, Alzheimer's disease

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INTRODUCTION

Alzheimer's disease (AD) is the most prevailing age-related neurodegenerative disease. Its cost and impending rise owed to societal aging makes it a major social concern and a critical public health burden. This pathology is characterized by the progressive spreading of two typical lesions in the brain: senile plaques and neurofibrillary tangles (NFTs), that are extracellular deposits of the amyloid-β peptide (Aβ) and intracellular aggregates of hyperphosphorylated tau protein, respectively (Serrano-Pozo et al., 2011). The most admitted comprehensive hypothesis for the onset and development of AD is the amyloid cascade hypothesis (ACH) (Hardy, 2006). It postulates that changes in Aβ production, accumulation, or clearance are triggering events that induce the formation of NFTs eventually leading to neurodegeneration and clinical symptoms. Many efforts have been undertaken to better understand the pathological processes responsible for the altered production of Aβ. They led to the identification of the Amyloid Precursor Protein (APP), and the Presenilin proteins (PS1 and PS2) that are the catalytic subunits of the γ-secretase that releases Aβ upon amyloidogenic processing of APP (Goate et al., 1991; Rogaev et al., 1995; Sherrington et al., 1995). However, clinical trials targeting the amyloid pathology have failed so far to improve cognitive deficits, even though they reduce the amyloid load under certain conditions (Ceyzeriat et al., 2020). Amyloid deposition appears to take place very early in pre-clinical stages and might be associated to brain dysfunctions that appear prior to clinical symptoms. Metabolic aspects of the pathology are widely studied in that context. For instance, the ^{18}F-FDG-PET scan is an established imaging standard for neuronal dysfunction in the diagnostic workup of AD-patients (Mosconi et al., 2010; Herholz, 2012; Shivamurthy et al., 2015). It was used to evidence that hypometabolism and brain atrophy appear before clinical symptoms in patients.

Mitochondria play a central role in cellular metabolism, and mitochondrial function is known to be particularly affected in the disease (Garcia-Escudero et al., 2013). Several studies have put forth a "mitochondrial cascade hypothesis" (Swerdlow and Khan, 2004; Stanga et al., 2020), according to which mitochondrial alterations are capable of initiating compensatory events that would result in the histopathological sequence of AD, including an increased production of the amyloid-β (Aβ) peptide, and thus they can be considered as an upstream event in the development of the pathology. Alternatively, mitochondrial dysfunction has also rather been suggested as a consequence of pathological processes such as amyloid deposition (Pagani and Eckert, 2011; Swerdlow et al., 2014; Swerdlow, 2018). Despite this controversy, there is little doubt that mitochondrial dysfunction contributes to AD pathogenesis, as evidenced in animal models of AD (Yao et al., 2009; Dixit et al., 2017; Long and Holtzman, 2019) and samples from AD patients (Wang et al., 2009; Martin-Maestro et al., 2017; Adav et al., 2019). Major consequences of mitochondrial dysfunction such as increased Reactive Oxygen Species (ROS) production (Dixit et al., 2017), impaired balance of fusion/fission with altered morphology (Wang et al., 2009), decreased oxidative capacity and decreased motility (Correia et al., 2016) are described in AD pathogenesis.

Mitochondrial alterations found in AD have been associated to functional changes of the major AD proteins, namely APP, Tau, and PSs (Garcia-Escudero et al., 2013). Only Presenilin 1 and 2 (PS1 and PS2) were yet clearly shown to be involved in mitochondrial function. PS1 and PS2 are encoded by two homologous genes, *PSEN1* and *PSEN2*, respectively. Mutations in *PSEN* genes are responsible for the majority of inherited AD cases (Hardy, 2006). Apart from their involvement in Aβ production (as the catalytic subunits of the γ-secretase), functions attributed to PSs can be divided into two categories: γ-secretase-dependent or γ-secretase-independent (Vetrivel et al., 2006; Zhang et al., 2013). The γ-secretase independent functions of PSs are known to be involved in synaptic transmission, endosome-lysosome trafficking, Wnt signaling, and calcium homeostasis. The involvement of PSs in calcium homeostasis has been particularly investigated. PSs could interact with Inositol trisphosphate receptor (IP3R) (Cheung et al., 2008), sarco-/endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA), or Ryanodine receptors (RyR) (Green et al., 2008; Wu et al., 2013) to regulate intracellular calcium signaling. PSs were also suggested to directly act as a low-conductance, passive ER Ca^{2+} channel (Nelson et al., 2007). Finally, PSs could also regulate the calcium crosstalk between the mitochondria and the ER by regulating their apposition through a particular domain called mitochondria-associated membranes (MAMs) (Filadi et al., 2016). Indeed, studies have shown that PSs are enriched in MAMs, which are lipid-raft-like structures. MAMs are considered as functional compartments (Area-Gomez et al., 2009) due to their implication in cellular pathways such as inflammation, mitophagy, and lipid production (Filadi et al., 2017). PS2 was found to physically interact with Mitofusin 2 to regulate MAMs organization and calcium shuttling in mouse embryonic fibroblasts (MEF) (Filadi et al., 2016). Disruption of this interaction and of the consecutive calcium crosstalk was reported in the SH-SY5Y cell line transfected with siRNA targeting PS2 (Zampese et al., 2011). Importantly, impaired calcium influx from the ER to mitochondria is implicated in the regulation of the oxidative phosphorylation (OXPHOS) and can lead to mitochondrial defects. Using MEF cell lines, we observed an altered mitochondrial phenotype related to the absence of PS2 and not PS1 (Contino et al., 2017). PS2 deficiency results in defective mitochondrial cristae correlated to an impaired OXPHOS capacity and a modified redox state (NAD^+/NADH ratio). This was compensated by an increased glycolytic capacity, sustaining a stable ADP/ATP ratio. Together with other studies (Wang et al., 2009; Pagani and Eckert, 2011; Correia et al., 2016; Dixit et al., 2017), this led to the hypothesis that PS-dependent

Abbreviations: ΔΨ, mitochondrial membrane potential; 2R2, PS2 knockout rescued PS2; CI-CV, complexes I-V; Ct, control; DTAB, dodecyltrimethylammonium bromide; ETC, electron transport chain; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; GFAP, glial-fibrillary acidic protein; MAM, mitochondria-associated membranes; MAP-2, microtubule-associated protein 2; MEF, mouse embryonic fibroblasts; MiNA, mitochondrial network analysis; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PS, presenilin; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester.

mitochondrial dysfunction could represent a major pathway in AD pathogenesis.

In this context, we investigated for the first time mitochondrial activity in primary cultures of neurons and astrocytes, which are more relevant to AD than immortalized cells. We performed primary cultures of cells expressing both PSs (wild-type, WT), one of them (PS single-knockdown/knockout, referred to as PS1KD and PS2KO), or none (combined PS1KD-PS2KO, referred to as PSdKO). Our aim was to investigate (i) if mitochondrial-related deficits appear in the absence of PSs in these cells and, if so, (ii) to identify which PS is involved in this phenotype and the possible mechanisms underpinning mitochondrial dysfunction in neurons and/or astrocytes. Surprisingly, we did not find any metabolic deficit in the different PS knockdown/knockout primary nerve cells, even in the absence of both PSs (PSdKO). Likewise, mitochondrial morphology and content were not altered in PS2KO primary neurons, contrary to what was observed in PS2-deficient MEF cell lines (Contino et al., 2017). We further studied the mitochondrial activity in PS2KO primary MEF and did not observe any mitochondrial defect. The obtained evidence leads us to conclude that PS-dependent mitochondrial alterations observed in immortalized cells may provide information about PS-dependent cancer processes, but give no straightforward indication about mitochondrial dysfunction in AD.

MATERIALS AND METHODS

Animal Models

Presenilin 2 knockout (KO) (#005617) (Herreman et al., 1999) and Presenilin 1 floxed (#004825) (Yu et al., 2000) mice, both in a C57BL/6 background, were obtained from Jackson Laboratories (Bar Harbor, USA). All animal procedures and experiments were approved and performed in agreement with the UCLouvain animal care committee's regulations (code number 2016/UCL/MD/016). Animals were housed on a 12 h light/dark cycle in a standard animal care facility with access to water and food *ad libitum*.

For primary cell cultures, generation of the different genotypes in the same litter was obtained from crossing PS2 heterozygous and PS1 floxed ($PS2^{+/-}$; $PS1^{flox/flox}$) animals followed or not by viral transduction. Indeed, *PSEN1* gene deletion was achieved by viral transduction of Cre recombinase in floxed PS1 primary cultures. Lentivirus expressing GFP (mock control) or CRE-GFP were used for transduction at DIV1 (one day of *in vitro* culture) for neurons and DIV17 for astrocytes. Following genotyping and infection we could obtain in the same litter: control non-infected **Ct** ($PS2^{+/+}$; $PS1^{flox/flox}$, non-infected), **PS2KO** ($PS2^{-/-}$; $PS1^{flox/flox}$, non-infected); control infected named **Mock** ($PS2^{+/+}$; $PS1^{flox/flox}$, infected with GFP); **PS1KD** ($PS2^{+/+}$; $PS1^{flox/flox}$, infected with CRE-GFP); and **PSdKO** ($PS2^{-/-}$; $PS1^{flox/flox}$, infected with CRE-GFP).

Primary Neuronal Cultures

Primary cultures of neurons were performed as previously described (Opsomer et al., 2020) on E17 mouse embryos. Cortices and hippocampi were isolated by dissection on ice cold HBSS (Thermo Fisher Scientific, Waltham, USA) and meninges were removed. Tissues were then dissociated by pipetting up and down 15 times with a glass pipette in HBSS glucose 5 mM medium. Dissociation was repeated 10 times with a flame-narrowed glass pipette. After sedimentation for 5 min, the supernatant containing the neurons was settled on a bed of 4 ml of Fetal Bovine Serum (FBS) and centrifuged at 1,000 × *g* for 10 min. The pellet was resuspended in Neurobasal® medium enriched with 1 mM L-glutamine, 5 mM glucose, and 2% v/v B-27® supplement medium. Cells were plated at 200,000 cells/cm^2 on pre-coated poly-L-lysine dishes and cultured (37°C, 5% CO_2 and humidified atmosphere). Half media changes were performed every 2 days and neurons were cultured for 11 days (DIV11) before being utilized for experiments. To get PS1KD and PSdKO, neurons were infected at DIV1 and all media were changed at DIV2.

Primary Astrocyte Cultures

For primary astrocyte cultures, the brains of mouse pups aged 2 days were dissected to isolate cortices on ice cold HBSS. Tissues were triturated 15 times with a glass pipette and 10 times with a flame-narrowed glass pipette. Tubes were centrifuged at 1,000 × *g* for 5 min. Pellets were resuspended in HBSS and centrifuged at 1,700 × *g* for 20 min on a 30% Percoll gradient. Astrocytes were collected from the interphase, washed in 10 ml of HBSS, and centrifuged for 5 min at 1,500 × *g*. Pellets were resuspended and plated in DMEM-glutaMAX medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% FBS (Biowest, Nuaillé, France), 50 mg/ml penicillin–streptomycin, and 50 mg/ml fungizone. Cells were left to proliferate in flasks for 15 days at 37°C and 5% CO_2, and media were changed every 4–5 days. After 15 days, astrocytes were plated and further cultured in DMEM-glutaMAX with 10% FBS. Two days later, transduction with lentivirus was achieved when necessary and differentiation was induced by reducing the concentration of FBS to 3% for 7 days before performing experiments.

Primary and Immortalized Mouse Embryonic Fibroblasts (MEF)

Immortalized MEF were cultured as previously described (Stanga et al., 2018). Primary cultures of MEF were performed on E16 embryos. Chest skin was isolated on ice and then grinded into pieces with a blade. These pieces were dissociated and incubated twice at 37°C for 10 min in trypsin (Life Technologies, Carlsbad, USA). DMEM low glucose (5.5 mM) (Sigma-Aldrich, St Louis, USA) enriched with 10% FBS and 1% pen/strep was added for an incubation of 5 min at room temperature (RT). Supernatants were collected and centrifuged at 1,000 × *g* for 5 min at RT. Pellets were resuspended in 10 ml of DMEM medium and plated in petri dishes. Once at confluence, cells were plated for experiments.

Lentiviral Particles

Lentiviral particles expressing CRE recombinase were used to delete floxed *PSEN1*. Plasmids pCMV-GFP (Mock) and pCMV-CRE-GFP for lentiviral production were purchased from Cellomics Technology (Halethorpe, USA). Amplification and purification of the different plasmids were performed using

the Plasmid Midi kit (Qiagen, Hilden, Germany). Lentiviral production was carried out by transfecting HEK293-T cells in 10 cm dishes (2×10^6 cells/dish) with CRE-GFP or GFP vectors, pMD2.G (Addgene#12259), and pCMV-dR8.2 (Addgene#12263). At 48 h after transfection, cells were harvested by flushing with the medium and centrifuged at 1,500 × *g* for 10 min at 4°C. The supernatant was filtered with Acrodisc® 0.45 μm filters (Pall, NYC, USA). Then, 1/3 (v/v) of LentiX™ Concentrator reagent (Clontech, Mountain View, USA) was added and incubated overnight (o/n). After centrifugation at 1,500 × *g* for 45 min at 4°C, the pellet was resuspended in 20 μl per dish of DMEM without serum and stored at −80°C; 10 μl of concentrated virus was used to infect 1,600,000 neurons or 300,000 astrocytes.

Western Blotting (WB)

WB was performed on cell lysates obtained by harvesting cells with lysis buffer (Tris 125 mM pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) with Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and sonicating, as previously described (Stanga et al., 2016). Protein concentration was determined using the BCA Protein assay kit (Pierce, Rockford, USA). Then, 15 μg of total proteins diluted in lysis buffer with NuPAGE® LDS Sample Buffer and 50 mM DTT was heated at 70°C (except for the detection of OXPHOS subunits, heated at 37°C). Samples were loaded and separated onto NuPAGE® 4–12% Bis-Tris gel (Life Technologies, Carlsbad, USA) with a NuPAGE® MES SDS running buffer (Life Technologies, Carlsbad, USA). Proteins were then transferred onto an Amersham™ nitrocellulose membrane (Little Chalfont, UK) with NuPAGE® transfer buffer (Life Technologies, Carlsbad, USA) for 2 h at 30 V. After blocking with non-fat dry milk (Darmstadt, Germany) (5% in PBS and 0.05% Tween-20) for a minimum of 30 min, the membrane was incubated o/n with the primary antibody diluted in PBS 0.05% Tween. Secondary antibody coupled to horse radish peroxidase was incubated 1 h at RT. Membranes were detected using ECL PerkinElmer® (Waltham, USA). ImageJ software was used for densitometric analysis of the different immunoreactive bands.

Antibodies used and their dilutions are the following: Anti-Actin (1:3,000; Abcam, Cambridge, United Kingdom); Anti-APP C-ter (1:2,500; generous gift of N. Sergeant, INSERM U422, Lille, France); Anti-DRP1 (1:1,000; Cell Signaling, Danvers, USA); Anti-mouse (1:10,000; GE Healthcare, Little Chalfont, United Kingdom); Anti-OPA1 (1:1,000; Cell Signaling, Danvers, USA); Anti-TIM23 (1:500; Santa Cruz, California, USA); Anti-OXPHOS Cocktail (1:1,000; Abcam, Cambridge, United Kingdom); Anti-Presenilin 1 (1:1,000; Cell Signaling, Danvers, USA); Anti-Presenilin 2 (1:1,000; Cell Signaling, Danvers, USA); Anti-rabbit (1:10,000; GE Healthcare, Little Chalfont, United Kingdom); Anti-TOM20 (1:1,000; Proteintech, Rosemont, USA); Anti-Tubulin (1:3,000, Abcam, Cambridge, United Kingdom).

Immunocytochemistry (ICC)

ICC were performed as previously described (Hage et al., 2014). Cells were seeded on pre-coated poly-L-lysine coverslips in 24 well plates at the density of 200,000/well for neurons or 50,000/well for astrocytes. Cells were fixed with PBS/paraformaldehyde 4% for 10 min and rinsed three times for 5 min with PBS. Cells were then permeabilized with a solution of PBS/Triton 0.3% for 30 min and non-specific sites were blocked with PBS/Triton 0.3%/FBS 5% for 30 min. Primary antibodies diluted in the blocking solution were incubated o/n at 4°C. After 3 washes of 10 min with PBS, cells were incubated with DAPI (1:2,000; Sigma-Aldrich, St Louis, USA) and secondary antibodies diluted in the blocking solution. Dilutions of the antibodies were as follows: chicken anti-glial-fibrillary acidic protein (GFAP2; 1:1,1000; Abcam, Cambridge, United Kingdom); mouse anti-microtubule associated protein (MAP2; 1:1,000; Sigma-Aldrich, St Louis, USA); rabbit anti-TOM20 (1:500, Proteintech, Rosemont, USA); Alexa 488 anti-rabbit (1:500; Life Technologies, Carlsbad, USA); Alexa 568 anti-mouse (1:500; Life Technologies, Carlsbad, USA); Alexa 647 anti-chicken (1:500; Life Technologies, Carlsbad, USA).

To characterize neuronal and astrocyte general morphology, images from MAP2 and GFAP stained-cells were acquired on EVOS FL Auto microscope (Invitrogen) with RFP (Alexa Fluor 554), and CY5 (Alexa Fluor 647) EVOS LED light cubes and analyzed with ImageJ software.

To evaluate mitochondrial morphology after TOM20 staining, samples were examined by confocal microscopy using a confocal server spinning disc Zeiss platform equipped with a ×100 objective. For each image, z-stacks were taken from the entire three-dimensional structure and the maximum intensity projection was obtained in ImageJ.

In silico Analysis of Mitochondrial Morphology

Morphological analysis of mitochondria was performed *in silico* using a plug-in of ImageJ, the toolset MiNA (Mitochondrial Network Analysis) (Valente et al., 2017). MiNA allows semi-automated analysis and consists in images' preprocessing, to ensure quality, conversion to binary image, and in the production of the final skeleton for the quantitative analysis, as previously described (Calabrese et al., 2020). Briefly, images were opened on ImageJ and processed as follows: 1-Process/Filters/UnsharpMask; 2-Process/EnhanceLocal Contrast (CLAHE); 3-Process/Filters/Median; 4-Process/Binary/MakeBinary; 5-Process/Binary/Skeletonize; 6-Analyze/Skeleton/AnalyzeSkeleton(2D/3D); 7-Plugins/StuartLab/MiNAScripts/MiNAAnalyzeMorphology.

Mitochondrial Membrane Potential (ΔΨ)

Fluorescent cationic probe tetramethylrhodamine methyl ester (TMRM) (Sigma-Aldrich, St. Louis, USA) was used to evaluate the ΔΨ. As a control, we used the uncoupling agent Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (Sigma-Aldrich, St. Louis, USA). Cells were plated in 96-well plates at a density of 60,000/well for neurons or 15,000/well for astrocytes. Cells were incubated for 30 min at 37°C with TMRM (30 nM) with or without FCCP (10 μM) diluted in KREBS medium. Cells were then washed with KREBS solution and fluorescence was read with the plate reader VICTOR® Multilabel Plate Reader (PerkinElmer). Data were normalized to the total

amount of protein measured by the Bradford assay kit (Bio-Rad Laboratories, California, USA).

Mitochondrial Oxygen Consumption

Oxygen consumption rate (OCR) was measured with the Seahorse XF96 bioenergetic analyzer (Seahorse Bioscience; Massachusetts, USA). Cells were seeded in a Seahorse 96-well plate at different densities (60,000/well for neurons, 15,000/well for astrocytes, or 20,000/well for MEF). To analyze the effect of the inhibition of γ-secretase activity on OCR, cells were treated with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-sphenylglycine t-butyl ester (DAPT) (Calbiochem, Camarillo, CA, USA) 24 h before performing the experiment. Once differentiation was completed, the medium was exchanged with the conditional medium (culture medium without sodium bicarbonate and FBS) and incubated without CO_2 at 37°C for 1 h. Inhibitors targeting the different mitochondrial complexes (Cell Mito Stress Test kit, Seahorse Bioscience) were added automatically and sequentially to the cells during the experiment to measure the basal respiration, the coupling, and the spare respiratory capacity. The sequence of the inhibitors used was: Oligomycin (1 μM); FCCP (0.5 μM for neurons and 1 μM for astrocytes and MEF); Rotenone and antimycin A (0.5 μM). Results were normalized to the total amount of protein measured by the Bradford assay kit (Bio-Rad Laboratories, California, USA).

NAD^+/NADH Ratio

NAD^+/NADH ratio was measured with the bioluminescent NAD^+/NADH-Glo assay kit (Promega, Wisconsin, USA) according the manufacturer's instructions. Cells were seeded in a 96-well plate at different densities (60,000/well for neurons, 10,000/well for astrocytes or 20,000/well for MEF). Once the differentiation was completed, cells were rinsed with PBS and then lysed with the basis solution 1 % dodecyltrimethylammonium bromide (DTAB). Samples were split for a basic or acid treatment and were heated at 60°C for 15 min. The reduced form was decomposed in the acidic solution and the oxidized form was selectively decomposed in the basic solution. After neutralization, samples were mixed with NAD^+/NADH-Glo™ detection reagent and incubated for 45 min to induce reaction. Luminescence was read on the GloMax® 96-well plate luminometer (Promega, Wisconsin, USA).

Glycolytic Flux

Glycolytic rate was analyzed by the measurement of the detritiation rate of [3-^{3}H] glucose as previously described (Contino et al., 2017). Briefly, cells were seeded in a 12-well plate at density of 800,000/well for neurons, 100,000/well for astrocytes, or 200,000/well for MEF. Tritiated glucose (0.2 μCi/ml; Perkin-Elmer; Massachusetts, USA) was added to the medium (including 5.5 mM glucose) for 30 min. After medium collection, the tritiated water resulting from detritiated glucose was separated from the non-transported tritiated glucose by column chromatography and measured with the Tri Carb 2,810 liquid scintillation analyzer (Perkin Elmer; Massachusetts, USA) as described previously (Marsin et al., 2002). Data were normalized to the total amount of protein measured by BCA assay (Thermo Scientific, Rockford, USA).

ATP Level

ATP level was measured with the bioluminescent ATPlite® assay kit (Promega, Wisconsin, USA) according the manufacturer's instructions. Cells were seeded in a 96-well plate at a density of 60,000/well for neurons or 10,000/well for astrocytes. On the day of the experiment, medium was replaced by 75 μl of fresh medium for 30 min at room temperature. Then, 75 μl of kit's solution was added and after 10 min of incubation, luminescence was read on the GloMax® 96-well plate luminometer (Promega, Wisconsin, USA).

RT-qPCR Quantification of Mitochondrial DNA Content

Relative mitochondrial DNA (mtDNA) content was quantified from isolated total DNA as previously described (Missios et al., 2014). Briefly, neurons were digested with proteinase K (100 μg/ml) in lysis buffer [100 mM NaCl, 100 mM Tris–HCl (pH 8.0), 1 mM EDTA, (pH 8.0) and 1% (w/v) SDS] at 50 °C o/n and genomic DNA was purified with phenol/chloroform method. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using different primer pairs to detect the mitochondrial genes *Cyt-b* and *Nd1*, and the nuclear gene *H19*. The relative mitochondrial DNA content was calculated by the ΔΔCt method. Previously described primers (Ferrara-Romeo et al., 2020) were used as follows: CYTB-F 5′-ATTCCTTCATGTCGGACGAG-3′, CYTB-R 5′-ACTGAGAAGCCCCCTCAAAT-3′, ND1-F 5′-AATCGCCATAGCCTTCCTAACAT-3′, ND1-R 5′-GGCGTCTGCAAATGGTTGTAA-3′, H19-F 5′-GTACCCACCTGTCGTCC-3′, H19-R 5′-GTCCACGAGACCAATGACTG-3′.

Statistical Analysis

Number of samples are indicated in figure legends with "n =" and number of independent experiments with "N =." GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used to analyze the data and perform the statistical analyses. Normality was assessed with Shapiro Wilk test (GraphPad Prism). Parametric test (Student's *t*-test, ANOVA and Tukey's multiple comparison test) was applied if the data followed normal distribution. Otherwise, non-parametric test (Mann-Whitney test, Kruskal-Wallis and Dunn's multiple comparison test) was used. Significance is indicated as: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

RESULTS

Generation of PS1KD, PS2KO, PSdKO Primary Nerve Cells and Characterization of the Primary Cultures

Metabolic analyses were performed on primary neurons and astrocytes produced at embryonic day 17 (E17) and at postnatal day 2 (P2), respectively. Neurons were cultured for 1 day (DIV1) and astrocytes for 17 days (DIV17) prior to lentiviral

transduction and were further maintained in culture until DIV11 for neurons and DIV24 for astrocytes. The experimental workflow is shown in **Figure 1A**. The different culture times (DIV) were chosen in order to obtain differentiated and active primary neurons and astrocytes (Schildge et al., 2013; Charlesworth et al., 2015). Our previous studies indicated that under these culture conditions, neurons acquire properties of differentiated neurons from DIV7 on (Opsomer et al., 2020) and that the spontaneous calcium oscillation of a functional neuronal network are readily observed at DIV10 (Santos et al., 2009, 2010). Thus, we can reasonably assume that the primary neurons in culture studied here were mature and interconnected in a functional network. Generation of the different genotypes was obtained in the same litter by crossing PS2 heterozygous and PS1 floxed ($PS2^{+/-}$; $PS1^{flox/flox}$) animals, followed when necessary by viral transduction of the Cre recombinase. This approach has been used because although PS2 knockout ($PS2^{-/-}$; PS2KO) cells can be obtained from the viable PS2 full KO mice (Herreman et al., 1999), PS1KO and PSdKO mice present a lethal phenotype at E17 and E12, respectively (Shen et al., 1997; Donoviel et al., 1999). Therefore, to obtain PS1KO and PSdKO primary cultures, *PSEN1* deletion was achieved by viral transduction of Cre recombinase in $PS1^{flox/flox}$ primary cultures (**Figure 1A**). Lentivirus expressing GFP (mock control) or CRE-GFP were used at DIV1 (primary neurons) or at DIV17 (primary astrocytes). Efficiency of infection was monitored by evaluating GFP fluorescence intensity. Three days after infection, most cells were GFP positive and GFP expression was maintained until the day of the analysis. An example of GFP and CRE-GFP infected neurons is shown in **Supplementary Figure 1**. We evaluated the expression level of PS1 and PS2 by WB in neuronal and astrocyte cultures (**Figure 1B**). Cre-mediated excision of *PSEN1* was very efficient under these conditions, with 90% decrease in PS1 expression when compared to mock-infected control (**Figure 1B**). However, since a residual PS1 expression was still observed after the infection, we consider the obtained cultures as a model of PS1 knockdown (PS1KD). Given that APP is a major substrate of the γ-secretase and critically involved in AD, we evaluated by WB the accumulation of APP C-terminal fragments (α-CTFs and β-CTFs) following PSs deletion. CTFs are the direct substrates of the γ-secretase that accumulate when γ-secretase activity is blocked. An important accumulation of the CTFs (likely α-CTFs) was observed in PSdKO neurons and astrocytes (**Figures 1B,C**). CTF accumulation is also observed in PS1KD neuronal cultures, in line with the fact that PS1 is more expressed in neurons than astrocytes (Lee et al., 1996; Lah et al., 1997). The accumulation of CTFs is very low in the absence of PS2 in primary neurons. This is in agreement with the fact that PS2 only marginally contributes to APP processing when PS1 is present. To further characterize our model, we checked the overall morphology of the various PS-deficient cells at DIV11 (neurons) and DIV24 (astrocytes) by immunostaining cells with astrocytic (GFAP) and neuronal (MAP2) markers (**Figure 2**). Protoplasmic astrocytes cultures were pure while some activated astrocytes were present in neuronal cultures (±10%). There were no apparent differences in terms of morphology or growth between Ct, mock, PS1KD, PS2KO, and PSdKO cells.

Membrane Potential (ΔΨ) and OXPHOS Complexes Expression Are Not Affected in PS-Deficient Primary Nerve Cells

As a primary indicator of mitochondrial mass (Whitaker-Menezes et al., 2011), the expression level of the mitochondrial import receptor subunit TOM20 (**Figure 3A**) was first evaluated in cell lysates from primary neurons and astrocytes. No changes were observed, suggesting that deletion of PSs did not affect the mitochondrial mass in the cells tested. Mitochondrial membrane potential (ΔΨ) is crucial for energy production and it is the driving force generated by the electron transport chain (ETC) for ATP synthesis. ΔΨ is known to stay stable since its decrease is a strong indication signal of cell death (Uechi et al., 2006). We evaluated the ΔΨ with the TMRM probe (**Figure 3B**), using FCCP (an uncoupling agent abolishing ΔΨ) as a positive control. No significant differences in ΔΨ were observed between controls (Ct and Mock) and PS2KO, PS1KD, and PSdKO cells, neither in neurons nor in astrocytes. Since ATP synthase might work in reverse to keep ΔΨ stable (Uechi et al., 2006), a defect in ETC could still occur without being readily detectable by mitochondrial membrane potential measurements. We checked the expression of the ETC subunits by WB using a cocktail of antibodies targeting representative subunits of the five mitochondrial complexes (**Figure 3C**). We found no differences in the expression of any ETC subunit in PS1KD, PS2KO, or PSdKO neurons or astrocytes. This was rather unexpected since we previously reported a defect in oxidative phosphorylation (OXPHOS) capacity along with expression changes of the ETC subunits in PS2KO MEF cell lines (Contino et al., 2017).

Mitochondrial Oxidative Phosphorylation and Bioenergetics Are Not Affected by the Absence of PS1 and/or PS2 in Primary Neurons and Astrocytes

The absence of changes in ETC complexes levels is a biochemical indication that does not rule out the hypothesis that OXPHOS could be impaired in PS-deficient primary neurons or astrocytes. Indeed, the cocktail of OXPHOS antibodies used in our study targets only one subunit of each of the massive ETC complex. We evaluated the activity of the ETC by measuring the overall profile of oxygen consumption rate (OCR) and several related parameters (**Figures 4A–C**). The parameters measured were basal respiration, coupling (oxygen consumption devoted to ATP synthesis under resting conditions), and spare respiratory capacity (maximal uncoupled rate of respiration minus the basal rate). The absence of PS2 affected all these parameters in MEF PS2KO cell lines (**Supplementary Figure 2**). Strikingly, the general OCR measured was similar in the presence (Ct, Mock) or absence of PSs (PS1KD, PS2KO, PSdKO) in primary neurons and astrocytes (**Figures 4A–C**). To note, the OCR at basal state is running near the maximal respiratory capacity in astrocyte cultures. This could suggest that the cells are stressed. However, this kind of OCR profile is commonly observed in primary astrocyte cultures (Damiano et al., 2014; Logan et al., 2018; Neal et al., 2018). The GFAP staining (**Figure 2**) indicates that astrocytes in culture are indeed activated astrocytes (Liddelow

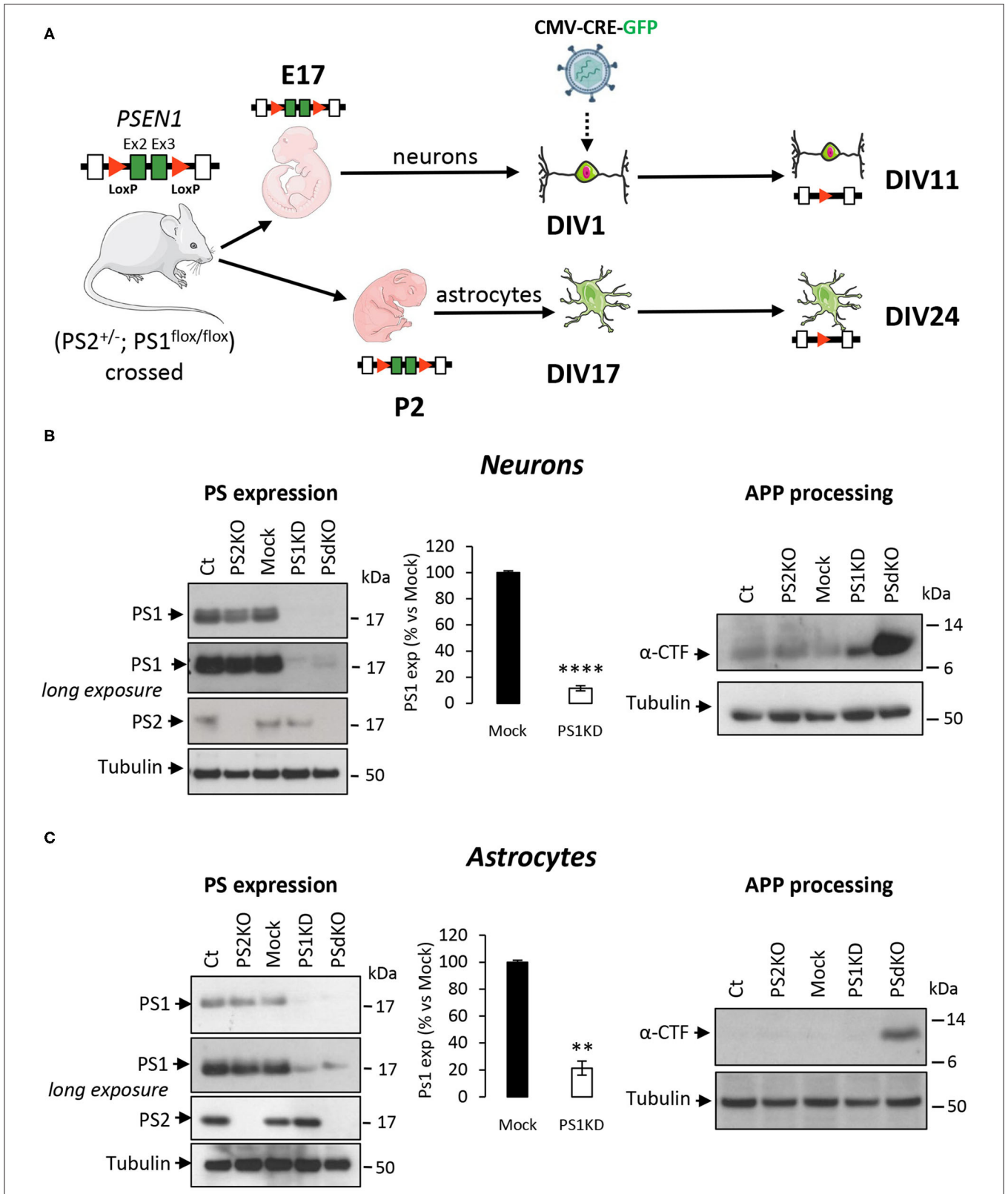

FIGURE 1 | Experimental workflow and model characterization in PS-defective primary nerve cells. **(A)** Workflow description of the experiments with specificity for *PSEN1* deletion. Primary neuronal and astrocyte cultures were performed on embryos at day 17 (E17) and postnatal pups at day 2 after birth (P2), respectively.

(Continued)

FIGURE 1 | Generation of the different genotypes in the same litter were obtained from crossing PS2 heterozygous and PS1 floxed ($PS2^{+/-}$; $PS1^{flox/flox}$) animals followed where necessary by viral transduction. At DIV1 for neurons and DIV17 for astrocytes, cells were either non-infected (Ct and PS2KO) or infected with GFP (Mock) or CRE-GFP to induce *PSEN1* gene deletion (PS1KD and PSdKO). Experiments were performed after 11 days for neurons and 24 days (including 7 days of differentiation) for astrocytes. Ex2 and Ex3 = *PSEN1* exons 2 and 3, respectively. **(B,C)** Representative WBs showing PS1 and PS2 expression profiles (left panel) and APP C-terminal fragment (α-CTFs; right panel) in total cell lysates from primary neuronal **(B)** and astrocyte **(C)** cultures. Tubulin was used as loading control. Quantification of PS1 expression (means ± sem) is given as percentage of signal measured in control cells (Mock); $^{****}p < 0.0001$; $^{**}p < 0.01$; Student's *t*-test for neurons results and Mann-Whitney test for astrocytes results ($N = 6$).

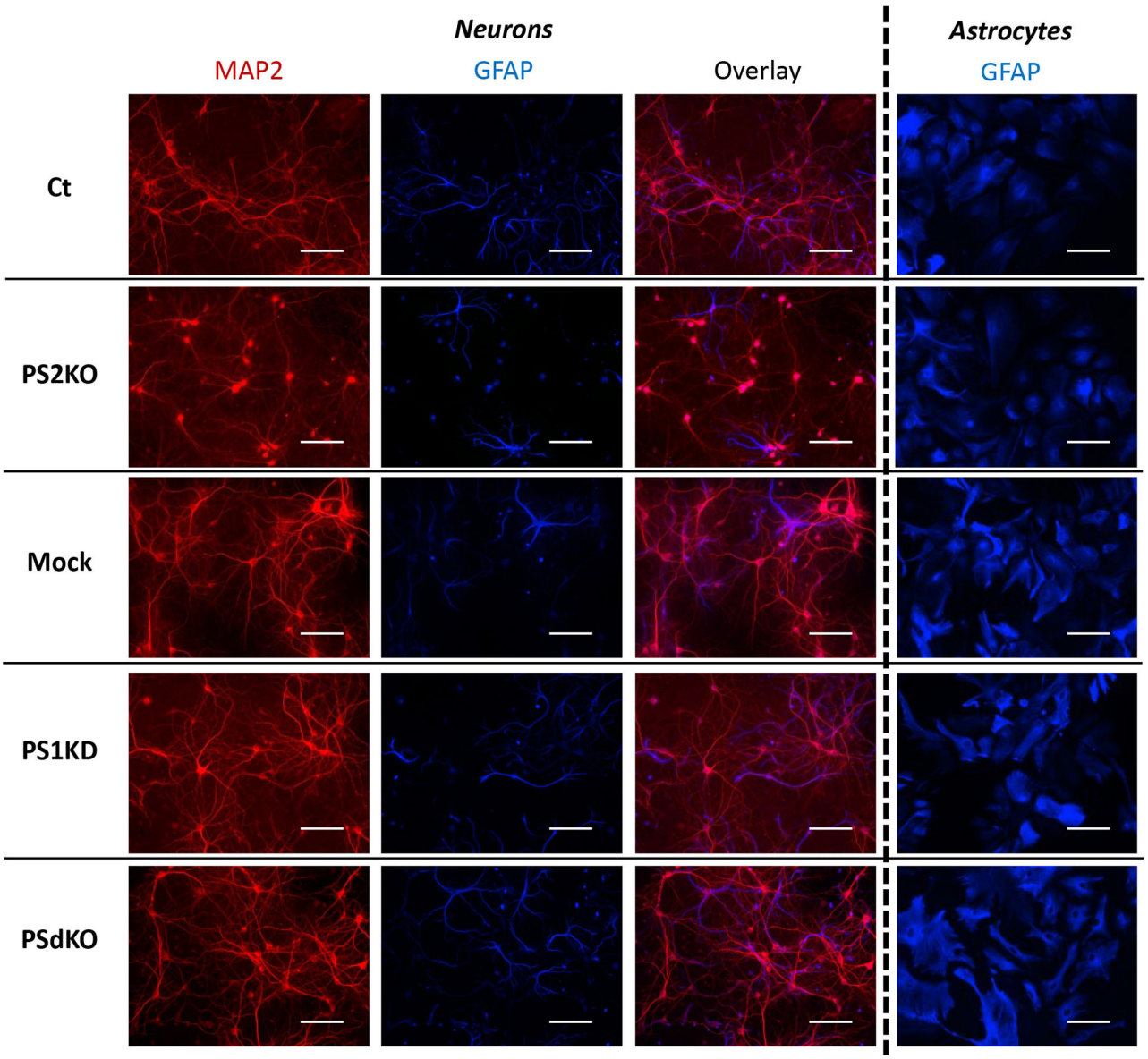

FIGURE 2 | Morphology of PS-defective primary neuronal and astrocyte cultures. On the left side, a primary culture of neurons at DIV11 immuno-stained with glial-specific protein GFAP (blue) and the neuron-specific protein MAP2 (red) antibodies. On the right side, a primary culture of astrocytes at DIV24 immuno-stained with GFAP (blue) antibody. Scale bar = 100 μm.

and Barres, 2017; Dubovy et al., 2018). This activated astrocyte profile could explain the similar OCR values measured in astrocytes at basal state and upon stimulation with FCCP.

The major outcome of genetic deletion of PSs is to down-regulate or abolish γ-secretase activity in cells. Another way to address the role of γ-secretase activity in OXPHOS is

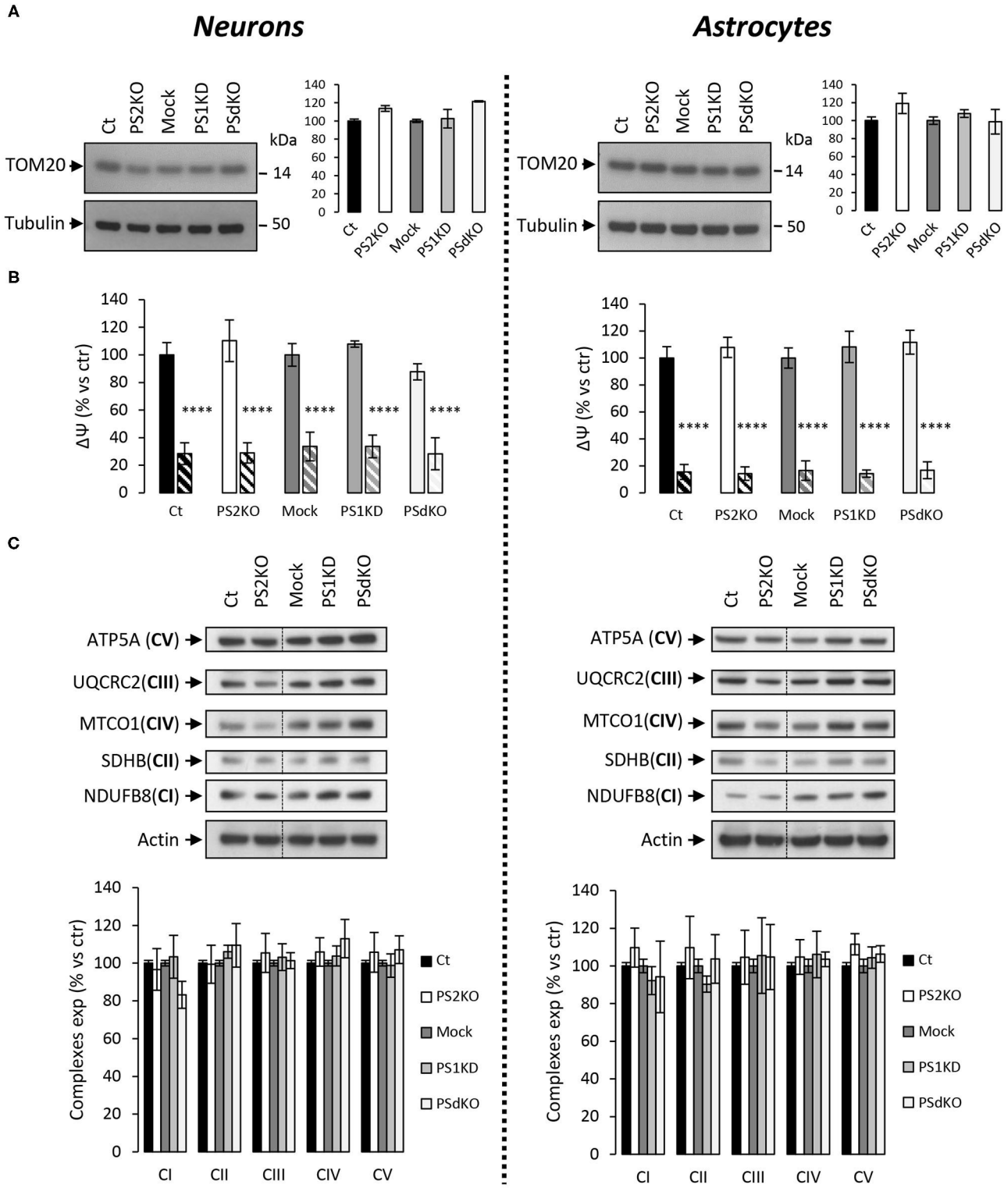

FIGURE 3 | $\Delta\Psi$ and OXPHOS complexes expression in PS-deficient primary nerve cells. Experiments were performed on primary DIV11 neuronal cultures and primary DIV24 astrocytes cultures. Cell conditions were Ct vs. PS2KO, Mock (infection control) vs. PS1KD and PSdKO. **(A)** Representative WBs and quantifications of TOM20 profile expression; in primary neuronal cell lysates (left panel) and in primary astrocyte cell lysates (right panel). Tubulin was used as a loading control. Results (mean ± sem) are expressed as percentage of the respective control (ctr = Ct or Mock) (min $N = 2$). Kruskal–Wallis test and Dunn's multiple comparison test. **(B)** $\Delta\Psi$ was evaluated with the TMRM probe in the presence or absence of FCCP, an uncoupling agent used as control (striped columns). Fluorescence signal was measured

(Continued)

FIGURE 3 | with a plate reader and results are expressed as the percentage of the relative mean fluorescence of the respective control cells (ctr = Ct or Mock) (min $N = 3$). ANOVA and Tukey's multiple comparison test. ****$p < 0.0001$. **(C)** The expression level of representative protein subunits from each of the five mitochondrial complexes (NDUFB8 for CI; SDHB for CII; UQCRC2 for CIII; MTCO1 for CIV; ATP5A for CV) was evaluated by WB on cell lysates. Actin was used as a loading control. Dashed lines indicate that proteins were run on the same gel, but lanes are not contiguous. Quantifications of the different WBs (means ± sem) are given as percentage of signal measured in the respective control cells (Ct or Mock) (min $N = 3$). ANOVA and Tukey's multiple comparison test.

to block γ-secretase activity with pharmacological inhibitors. To that end, we treated primary neurons and astrocytes for 24 h with 10 μM of DAPT, a concentration that efficiently blocks γ-secretase activity (**Figure 5A**). We measured the OCR (**Figures 5B,C**) and observed no changes in DAPT-treated cells when compared to non-treated cells. This confirms that γ-secretase activity is not involved in OXPHOS, in agreement with the results obtained in PS-deficient neurons and astrocytes. We next measured the NAD^+/NADH ratio (**Figure 6A**) and glycolytic flux (**Figure 6B**), parameters related to bioenergetics. NADH is an electron donor used by the first complex of the ETC. Glycolysis can either produce intermediates for OXPHOS or produce ATP and lactate depending on the oxidative status. These parameters were found to be altered in PS2KO MEF cell lines (Contino et al., 2017). We did not observe any changes in these indicators in primary neurons or astrocytes in the absence of PSs. In agreement, ATP levels were stable in all cell types (**Figure 6C**). All these data strongly support that the mitochondrial activity and related bioenergetics are not dependent on PSs in neurons and astrocytes, on the contrary to what was observed in MEF (Contino et al., 2017). Finally, we checked for possible mitochondrial fusion/fission defects in PS-deficient cells. Mitochondria are very dynamic entities and their shape and length can change erratically due to fusion and fission processes under certain circumstances such as cell division or especially in response to stress. We evaluated by WB two key proteins of mitochondrial fusion (OPA1) and fission (DRP1) as indicators of altered mitochondrial dynamics (**Figures 7A,B**). We found only a very small but significant increase in OPA1 in PS2KO and PSdKO neurons that is not mirrored by the DRP1 profile. Thus, subtle changes in mitochondrial dynamics could occur in PS2-deficient cells without affecting mitochondrial OXPHOS and related bioenergetics (**Figures 4**, **5**).

To further investigate the cellular effects of such protein changes in PS2KO neurons, and since previous results from our group demonstrated specific alterations in mitochondrial function and content in immortalized PS2KO MEF cells (Contino et al., 2017), we decided to evaluate mitochondrial morphology in WT (Ct) and PS2KO neurons by immunofluorescent staining against TOM20 (**Figure 8A**). A semi-automated and detailed characterization was accomplished by using the Mitochondrial Network Analysis (MiNA) toolset in ImageJ, which allows the obtention of parameters to quantitatively capture the morphology of the mitochondrial network (Valente et al., 2017). Briefly, confocal images of mature neurons (positive for MAP2) were processed to enhance their resolution and converted to binary images, finally producing the mitochondrial skeleton morphology. In all analyzed cells mitochondria were interconnected, thus forming an intracellular network, and no differences were observed when evaluating the mean number of mitochondria (individuals: unbranched, punctate organelles) or networks (branched, reticular structure of fused mitochondria) (**Figure 8B**). Additionally, no differences were observed in the mean number of junctions, end-points, and slab voxels (data not shown), suggesting an unaltered shape and distribution of the mitochondrial network together with the same degree of network complexity between genotypes. Finally, in order to confirm the lack of mitochondrial alterations in PS2KO neurons, a RT-qPCR-based mtDNA content analysis was performed in those cells, indicating normal mitochondrial mass in response to PS2 depletion (**Figure 8C**).

Metabolic Characterization of PS2 Deficient Primary MEF

Since no metabolic defect was observed in primary neuronal and astrocyte cells, we hypothesized that the phenotype previously observed in MEF cells could be exclusively peripheral. Indeed, the general OCR profile and related parameters were defective in PS2KO MEF cell lines and restored after stable re-expression of human PS2 (**Supplementary Figure 2**). To further investigate this idea, we decided to generate primary MEF derived from E16 WT (Ct) or PS2KO mice. We measured the expression of TOM20 and subunits of OXPHOS complexes (**Figure 9A**) and did not observe any differences between Ct and PS2KO fibroblasts. The activity of the ETC was also stable in the absence of PS2 as shown with the general OCR profile and the related parameters (**Figure 9B**). Finally, NAD^+/NADH ratio and glycolytic flux (**Figures 9C,D**) were not modified either, indicating a metabolic stability in primary fibroblasts lacking PS2. The metabolic phenotype observed in primary fibroblasts is not consistent with the one reported in immortalized MEF, and could reflect deep differences between primary and immortalized cells.

DISCUSSION

PSs play a major role in cell physiology and AD pathology as catalytic subunits of the γ-secretase complex. The γ-secretase is a multiprotein membrane complex, involved in regulated intramembrane proteolysis (RIP). Up to 90 membrane proteins have been identified as substrates of the γ-secretase (Haapasalo and Kovacs, 2011). The major substrates are Notch and the Amyloid Precursor Protein (APP), which respectively play a critical role during development and in the amyloid pathology found in AD (Hardy, 2006). PSs have also been implicated in other cellular functions, including calcium homeostasis, cell-cell adhesion, membrane trafficking, and Wnt signaling (Otto et al., 2016). The most reported non-catalytic functions of PSs are related to calcium homeostasis (Nelson et al., 2007; Cheung et al., 2008; Wu et al., 2013). PSs contribute to the building

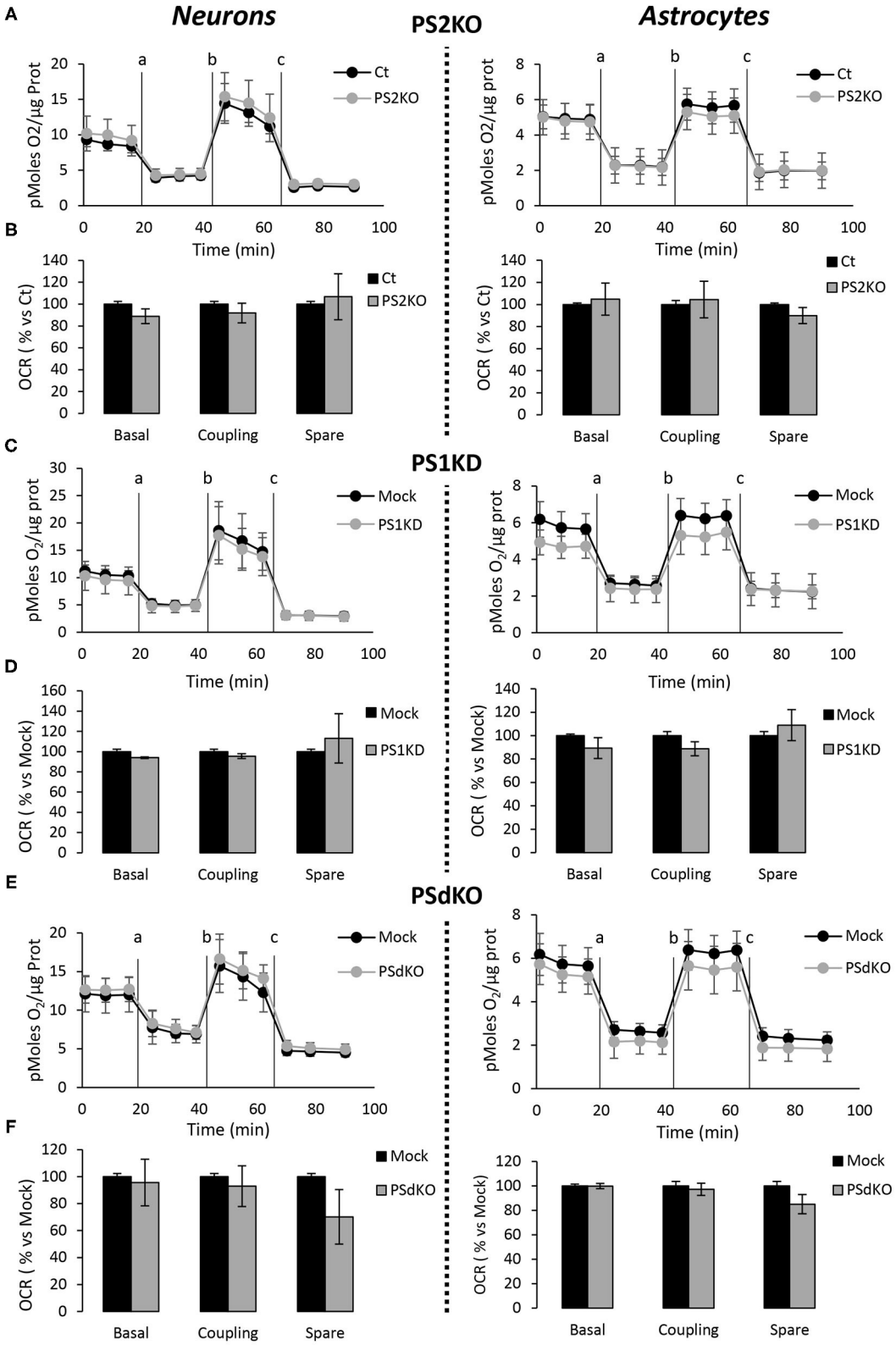

FIGURE 4 | Assessment of the OXPHOS capacity in primary nerve cultures genetically deleted for PSs. Oxygen consumption rate (OCR) was evaluated by using the Seahorse XF96 bioenergetic analyzer. Experiments were carried out in primary DIV11 neuronal cultures (left panel) and in primary DIV24 astrocyte cultures (right panel). *(Continued)*

FIGURE 4 | Cell conditions were wild-type non-infected (Ct) vs. PS2KO; control infection (Mock) vs. PS1KD and PSdKO. **(A,C,E)** General profile of the OCR with vertical lines indicating the time point at which the different compounds have been added: a. Oligomycin (CV inhibitor) b. FCCP ($\Delta\Psi$ uncoupler) c. Rotenone (CI inhibitor) and antimycin A (CIII inhibitor). Values (means ± sem) are given in pmol O_2/min/μg protein (min $N = 3$). **(B,D,F)** The basal respiration, the coupling ratio and the spare respiratory capacity were calculated according to the Cell Mito Stress Test kit's recommended protocol. Values (means ± sem) are given as percentage of signal measured in the respective control cells (Ct or Mock) (min $N = 3$). ANOVA and Tukey's multiple comparison test.

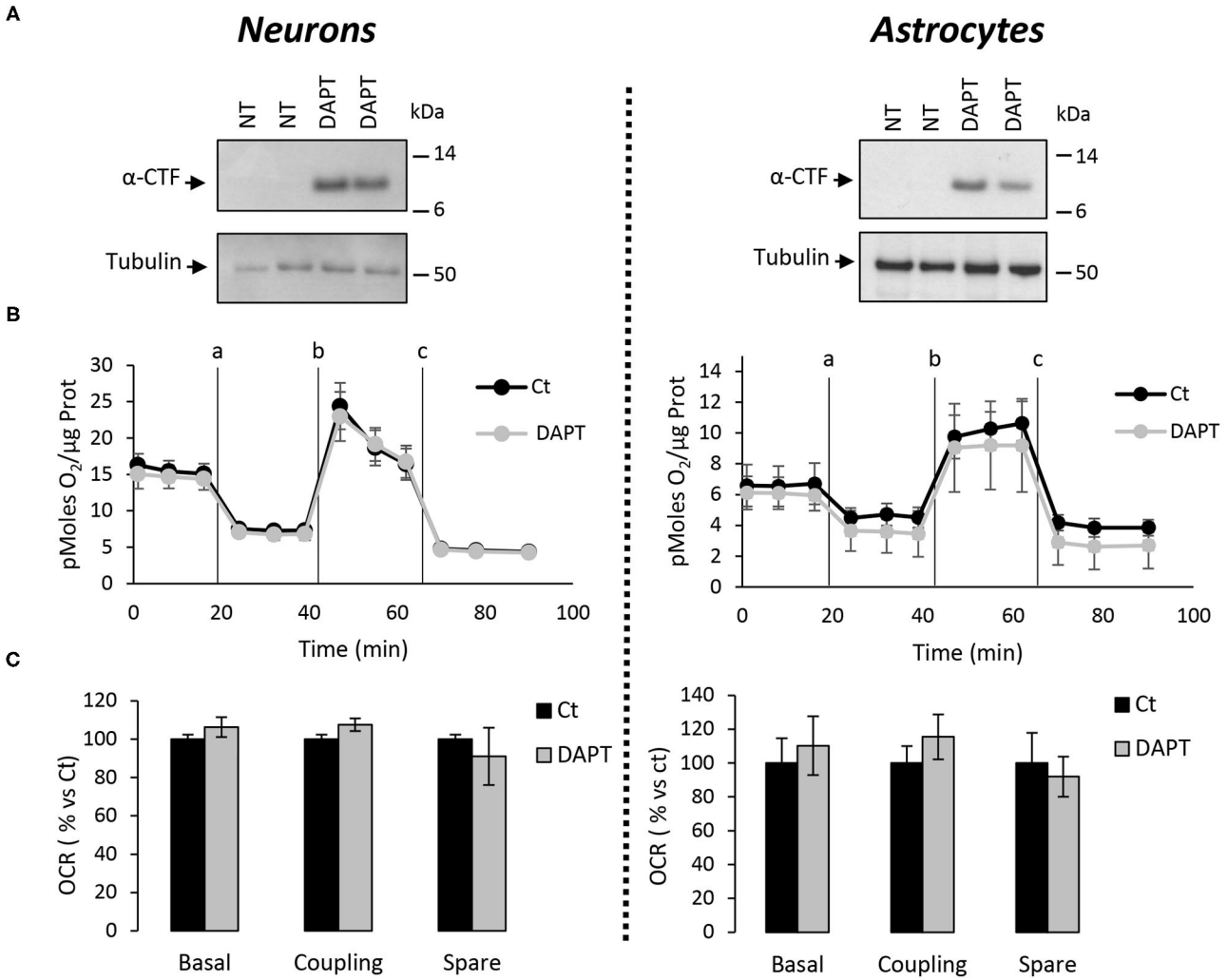

FIGURE 5 | Assessment of the OXPHOS capacity in primary nerve cultures treated with DAPT. OCR was evaluated by using the Seahorse XF96 bioenergetic analyzer. Experiments were performed in primary neurons (DIV11) or primary astrocytes (DIV7) wild-type non-treated (NT or Ct) or treated with DAPT. **(A)** Accumulation of α-CTF upon treatment with 10 μM of DAPT for 24 h of control primary nerve cultures was evaluated by WB with an antibody targeting the C-terminal region of APP. Tubulin was used as loading control. **(B)** General profile of the OCR with vertical lines indicate the time point at which the different compounds have been added: a. Oligomycin (CV inhibitor) b. FCCP ($\Delta\Psi$ uncoupler) c. Rotenone (CI inhibitor) and antimycin A (CIII inhibitor). Values (means ± sem) are given in pmol O_2/min/μg protein ($N = 3$). **(C)** The basal respiration, the coupling ratio, and the spare respiratory capacity were calculated according to the Cell Mito Stress Test kit's recommended protocol. Values (means ± sem) are given as percentage of signal measured in control cells (Ct). Student's *t*-test for neurons results ($N = 3$) and Mann-Whitney test for astrocytes results ($N = 2$).

of functional ER/mitochondria interfaces called mitochondria-associated membranes (MAMs) (Area-Gomez et al., 2009; Brunello et al., 2009; Filadi et al., 2016). MAMs control calcium shuttling between ER and mitochondria, interconnecting calcium homeostasis and mitochondrial function. We previously observed in MEF cell lines that the absence of PS2 led to a decrease in OXPHOS activity and in the expression of ETC proteins, along with an increased anaerobic glycolysis that sustains the ATP production. The effects observed were cell-autonomous since defects in bioenergetics were rescued by the

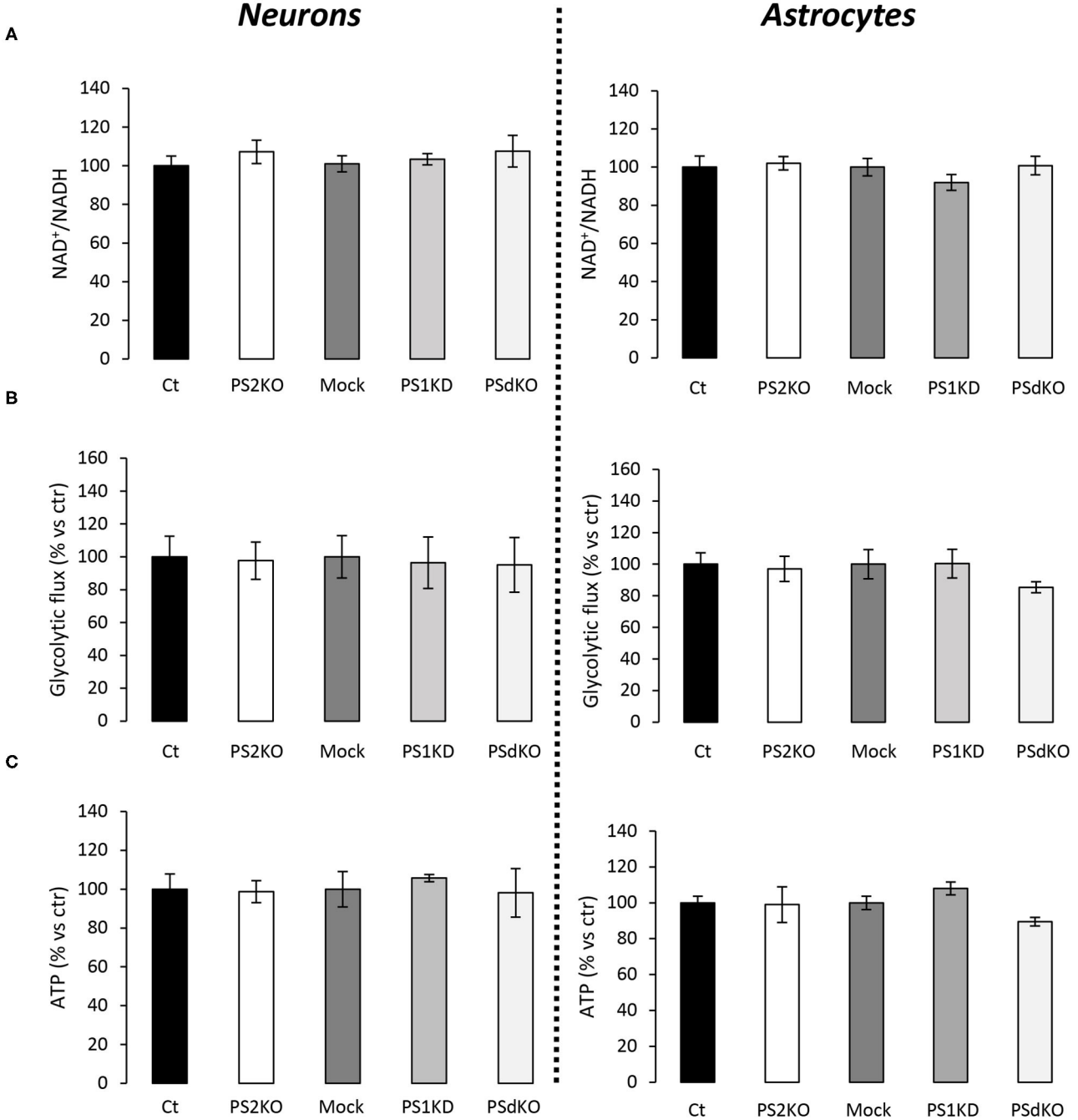

FIGURE 6 | Evaluation of ATP levels, NAD^+/NADH ratio, and glycolytic flux in primary nerve cultures. Experiments were performed respectively at DIV11 and DIV24 in neuronal (left panel) and astrocytes (right panel) cultures. Cell conditions were wild-type non-infected (Ct) vs. PS2KO; Mock (infection control) vs. PS1KD and PSdKO. (ctr = Ct or Mock). **(A)** NAD^+/NADH ratio was quantified by a bioluminescent kit (min $N = 3$). **(B)** Glycolysis rate was determined by the detritiation rate of [3-^{3}H] glucose after a 30 min incubation. Data were normalized to protein content (min $N = 3$). **(C)** ATP level was quantified by a bioluminescent kit (min $N = 3$). ANOVA and Tukey's multiple comparison test.

stable expression of human PS2 in PS2KO cells (Contino et al., 2017). These indications supported the role of presenilins, and more precisely PS2 in mitochondrial function, in agreement with other studies carried out in the same model (MEF) or in neuronal cell lines. PS2 but not PS1 deficiency was reported to alter mitochondrial respiration (Behbahani et al., 2006). PS2 was found to modulate calcium shuttling between the ER and mitochondria, a critical process for OXPHOS stimulation

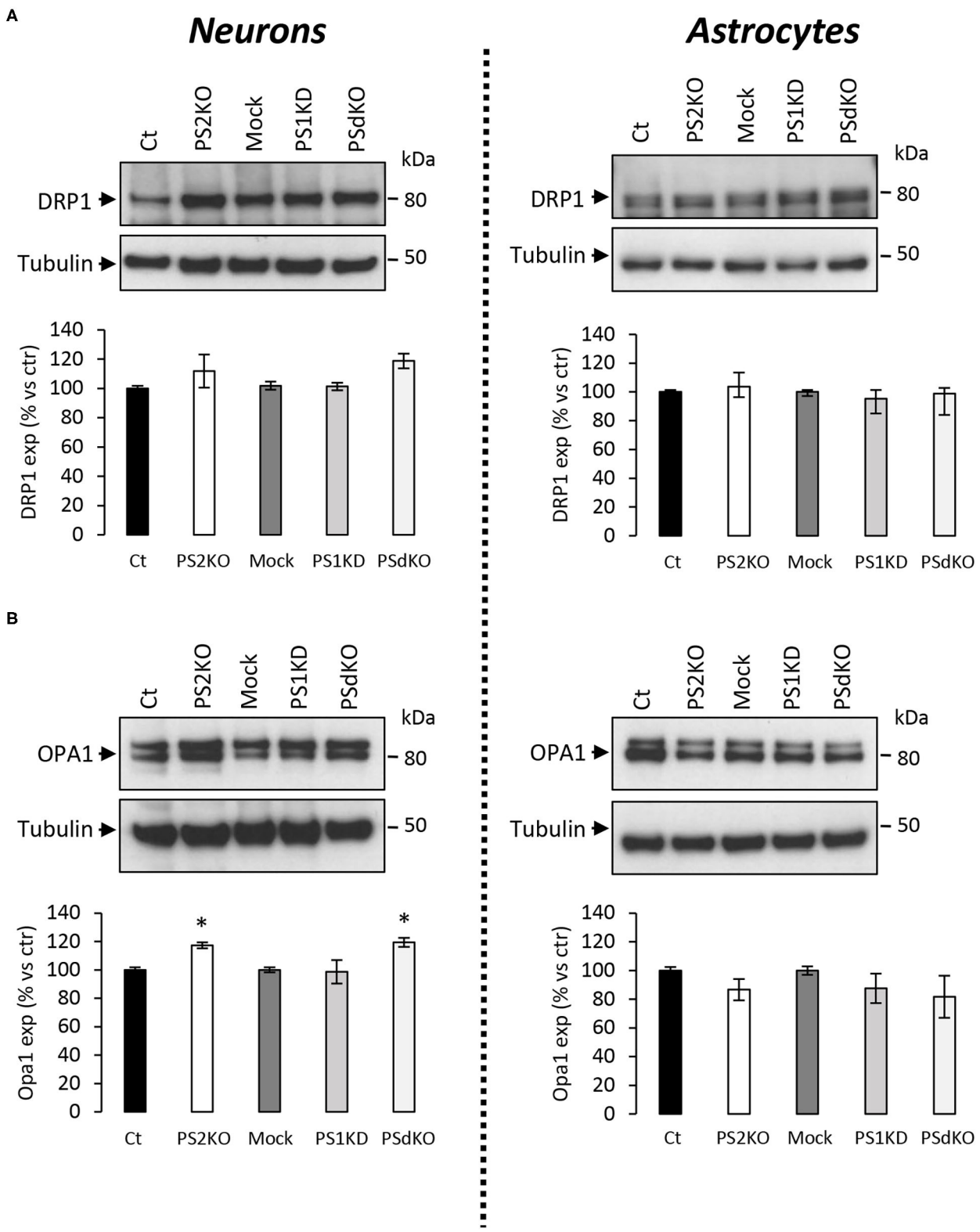

FIGURE 7 | Expression of mitochondrial fusion and fission proteins in PS-deficient primary nerve cells. Experiments were performed on primary DIV11 neuronal cultures and primary DIV24 astrocytes cultures. Cell conditions were Ct vs. PS2KO, Mock (infection control) vs. PS1KD and PSdKO. **(A)** Representative WBs and

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FIGURE 7 | quantifications of DRP1 profile expression; in primary neuronal cell lysates (left panel) and in primary astrocyte cell lysates (right panel). Tubulin was used as a loading control. Results (mean ± sem) are expressed as percentage of the respective control (ctr = Ct or Mock) (min $N = 2$). Kruskal–Wallis test and Dunn's multiple comparison test. **(B)** Representative WBs and quantifications of OPA1 profile expression; in primary neuronal cell lysates (left panel) and in primary astrocyte cell lysates (right panel). Tubulin was used as a loading control. Results (mean ± sem) are expressed as percentage of the respective control (ctr = Ct or Mock) (min $N = 4$). ANOVA and Tukey's multiple comparison test, $^*p < 0.05$.

and thereby mitochondrial activity (Zampese et al., 2011). In agreement, a study reported that PSs are enriched in MAMs (Area-Gomez et al., 2009).

We further investigated in this study the role of PSs in mitochondrial function by using mouse primary neurons and astrocytes. Contrary to what was previously observed, we found that the absence of PS1 or PS2 and even of both (PSdKO) did not affect mitochondrial ETC and related bioenergetic parameters in primary nerve cells. Moreover, the morphology of the mitochondrial network and the quantity of mtDNA were not changed either in PS2KO neurons. Importantly, we also did not observe any change in primary PS2-deficient fibroblasts, in opposition to the results obtained in immortalized fibroblasts (MEF cell lines). This raises important points about the interpretation of the role of PSs in mitochondrial functions across cellular models. First, the PS2KO MEF cell lines, which have been widely used so far, can genetically derive and acquire clonal properties, with the inherent risk of artifacts or misinterpretation of the data (Kaur and Dufour, 2012). Immortalized cell lines can acquire mutations with subcultures and these mutations can interfere with the cellular phenotype. However, the rescue experiments that we performed in a previous study indicated that the mitochondrial defects observed in PS2KO MEF cell lines are truly PS2-dependent (Contino et al., 2017). Second, the immortalization process might be responsible for the PS2-dependent phenotype observed in MEF. The fact that a PS2-dependent phenotype is observed only in immortalized cells can be related to data from the literature. PSs are involved in different cellular pathways related to cancer, like Notch and Wnt pathways (Xia et al., 2001; Andersson and Lendahl, 2014; Li et al., 2016). An increase in lung tumor formation through peroxiredoxin 6 (PRDX6) activation was also reported in PS2KO mice. The proposed underlying mechanism involves the PS2-dependent γ-secretase cleavage of PRDX6 that inhibits its critical activity in cell growth (Yun et al., 2014; Park et al., 2017). Another study showed that PSs are involved in epidermal growth and transformation by regulating the epidermal growth factor receptor (EGFR) signaling (Rocher-Ros et al., 2010). It is also important to take into account that the immortalization process used for the generation of MEF cell lines relied on SV40 Antigen T expression (De Strooper et al., 1999), which shows similarities with tumor development due to the large T antigen forming complexes with pRB-1 and p53 (Hubbard and Ozer, 1999; Pipas, 2009). In agreement, the inhibition of γ-secretase activity is suggested as a potential approach for cancer treatment. Interestingly, PSs have been associated to regulation of proteins such as Akt, HIFα, or β-catenin (Xia et al., 2001; Kang et al., 2005; De Gasperi et al., 2010). Those proteins contribute to the Warburg effect, which states that most cancer cells produce lactic acid from glucose even under non-hypoxic conditions and despite functional mitochondria (Koppenol et al., 2011). Many pieces of evidence relate thus PSs function to cancer processes, not only to neurodegenerative diseases. Our data clearly indicate that the absence of PS2 has a different outcome when measured in primary fibroblast or in immortalized fibroblast. The large amount of data reported so far in MEF cell lines (Behbahani et al., 2006; Tu et al., 2006; Brunello et al., 2009; Filadi et al., 2016; Pera et al., 2017) might be relevant to describe the role of PSs and γ-secretase activity in cancer models, which are difficult to transpose to a neurodegenerative context.

To that end, we addressed the role of PSs in mitochondrial function in primary neurons and astrocytes. Neurons are a prime target of neurodegeneration, and a major function of astrocytes is to support neuronal activity. Impairment of the neuronal network and activity underlies the gradual memory and other cognitive deficits in AD (Long and Holtzman, 2019). Although neurons are the most studied nerve cell type, astrocytes also play a key role in AD pathogenesis (Verkhratsky et al., 2010). Astrocytes and neurons are known to be metabolically different. Neurons rely primarily on mitochondrial oxidative phosphorylation for energetic supply and astrocytes on glycolysis. We did not observe any metabolic defects in our PS-deficient primary neuronal cultures. Indeed, the OXPHOS system (activity and expression) as well as the $\Delta\Psi$, glycolysis, and NAD^+/NADH ratio were comparable between control, PS2KO, PS1KD, and PSdKO cells. Since astrocytes are more glycolytic than neurons (Kasischke et al., 2004; Castelli et al., 2019), we hypothesized that astrocytes would be close, in terms of metabolic phenotype, to the MEF cell line we previously analyzed (Contino et al., 2017). However, all the metabolic parameters evaluated in primary astrocytes were not altered by the absence of PSs. This leads us to conclude that the lack of PSs does not affect neither the OXPHOS, nor related metabolic aspects, such as glycolysis and NAD^+/NADH ratio in primary nerve cells. In support of these observations, the analysis of the morphology and complexity of the mitochondrial network, as well as its mass, does not show differences between control and PS-deficient neurons. This result is coherent with all the data obtained regarding mitochondrial function; indeed, the preservation of a correct and interconnected network of mitochondria is crucial to cell function (Chen and Chan, 2004; Lackner, 2014). To note, differences between nerve cells and peripheral cells have been reported in a metabolic study comparing primary neurons, astrocytes, and fibroblasts cultures deficient for the mitochondrial complex I subunit NDUFS4, a model for the mitochondrial Leigh syndrome, a severe neurological disease (Bird et al., 2014). The $\Delta\Psi$ and ATP synthesis were impaired in the NDUFS4 KO primary MEF, with an increase in ROS generation and an altered sensitivity to cell death. In contrast, NDUFS4 KO primary neurons and astrocytes

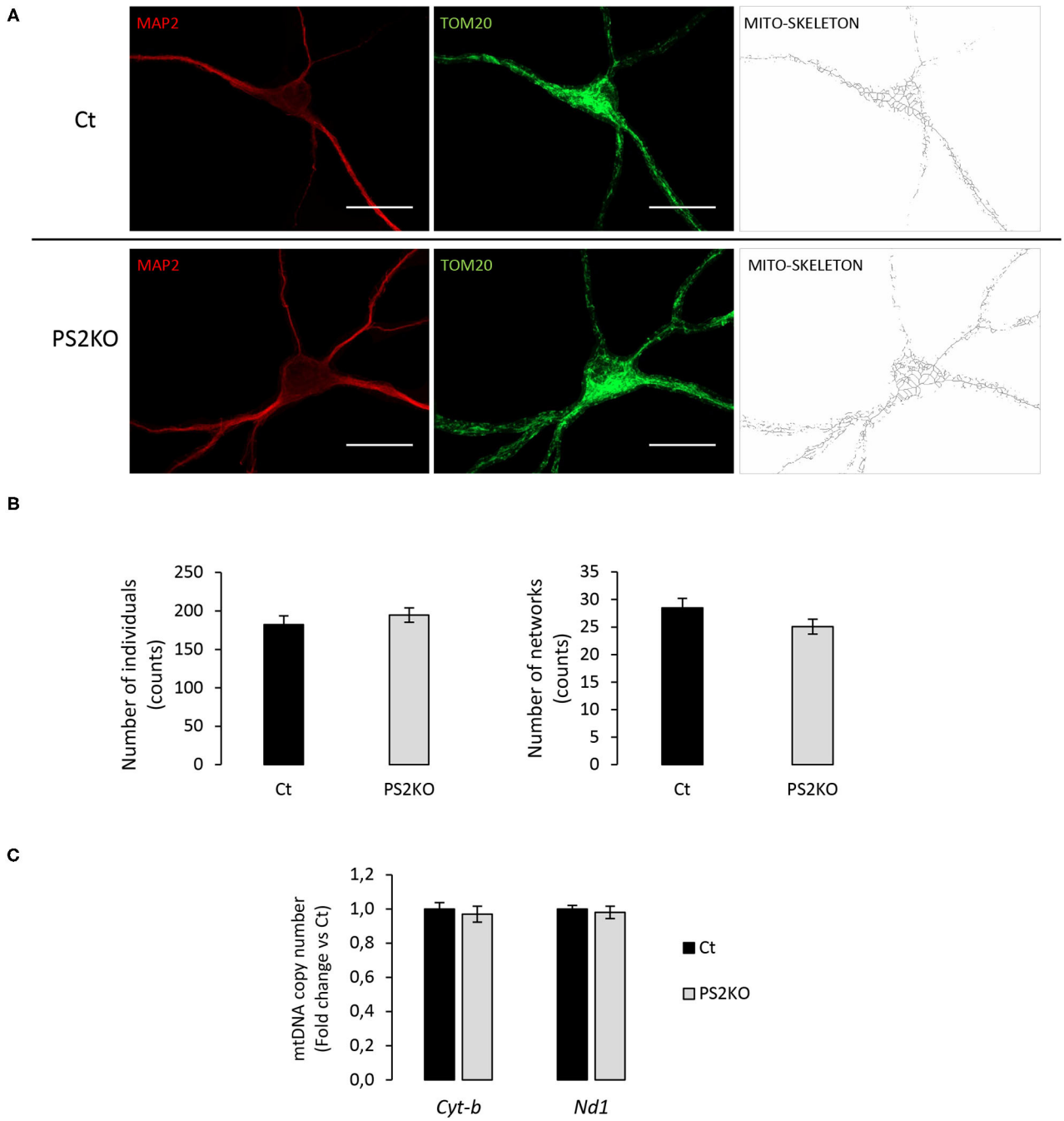

FIGURE 8 | Evaluation of mitochondrial morphology and content in PS2KO neurons. **(A)** Mitochondria from wild-type non-infected (Ct) and PS2KO neurons at DIV11 were stained with an anti-TOM20 antibody (green) and mature neurons were identified with MAP2 (red). Confocal microscope images were acquired and TOM20 images were processed using the MiNA toolset to generate accurate mitochondrial skeletons for further analysis. Scale bar = 20 μm. **(B)** Quantification of individuals (counts) and networks (counts) are shown in the graphs. Results are presented as mean ± sem. Student's *t*-test ($n = 55$ from $N = 3$ independent experiments). **(C)** RT-qPCR analysis of mitochondrial DNA content (*Cyt-b* and *Nd1* mitochondrial genes) in wild-type non-infected (Ct) and PS2KO neurons at DIV11. Results (mean ± sem) are expressed as percentage of the respective control (Ct) (min $N = 4$).

displayed only impaired ATP generation. This underlines the importance of the cellular model and experimental set-up when investigating alteration of mitochondrial function related to a neuronal pathology.

Still, the fact that the absence of PSs does not affect the basal mitochondrial-related bioenergetics in astrocytes and neurons is rather intriguing. Considering the expression profiles of the *PSEN1* and *PSEN2* genes throughout the body, one could

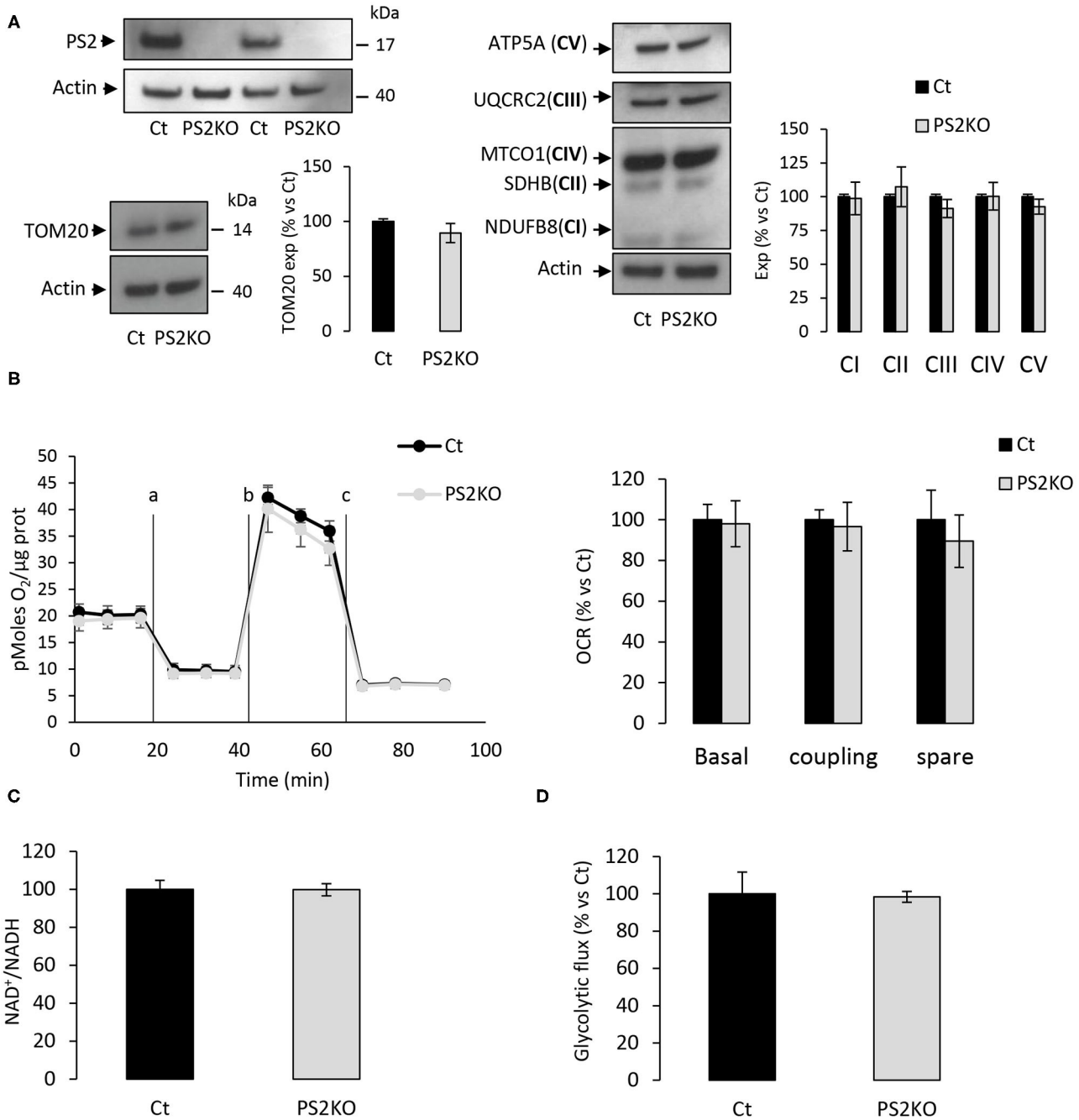

FIGURE 9 | Metabolic characterization of PS2 deficient primary MEF. Experiments were carried out in primary MEF derived from E16 WT mice (Ct) or PS2KO mice (PS2KO). **(A)** Expression profile of PS2 (left panel, up), TOM20 (left panel, down), and representative protein subunits from each of the five mitochondrial complexes (right panel; NDUFB8 for CI; SDHB for CII; UQCRC2 for CIII; MTCO1 for CIV; ATP5A for CV), evaluated by WB on cell lysates. Actin was used as a loading control. Quantifications of the different WBs (means ± sem) are given as percentage of signal measured in control cells (Ct) (min $N = 3$). Student's *t*-test. **(B)** Left panel: General OCR profile of primary MEF Ct vs. PS2KO. Values (means ± sem) are given in pmol O_2/min/µg protein. Right panel: The basal respiration, the coupling ratio and the spare respiratory capacity. Values (means ± sem) are given as percentage of signal measured in control cells (Ct) ($N = 3$). **(C)** NAD^+/NADH ratio was quantified by a bioluminescent kit ($N = 4$). **(D)** Glycolytic rate was determined by the detritiation rate of [3-^{3}H] glucose after a 30 min incubation. Data were normalized to protein content ($N = 3$). Student's *t*-test.

expect to have distinct phenotypes related either to PS1 or PS2 deficiency. PS1 was suggested to be more important in CNS and PS2 in peripheral organs (Lee et al., 1996). The absence of mitochondrial defects in PSdKO is even more unexpected, considering the broad array of functions attributed to PSs (Zhang et al., 2013; Wolfe, 2019), which process more than 90 membrane

proteins (Haapasalo and Kovacs, 2011; Wolfe, 2019). Cortical brain sections from conditional PSdKO mice show several pathological features such as neurodegeneration, astrogliosis, and even swollen mitochondria (Saura et al., 2004; Wines-Samuelson et al., 2010). The experimental set-up and environment should be taken into consideration to give a precise interpretation to data obtained across different models. All our experiments were performed on primary cells that were cultured in low glucose (5 mM) medium, to reflect a more physiological condition. Neurons and astrocytes were mature and differentiated (DIV11 for neurons and DIV24 for astrocytes), and tested at basal and resting state. Challenging the cells might be a key point for further investigations to unravel a deficit that could be masked in basal conditions. The time of the culture, especially for neurons, is also an important parameter. Performing the experiments on aged cultures would be of interest to check if mitochondrial functions evolves distinctly in PS-deficient cells upon aging. Cultures could be supplied with different types of cell fuels (pyruvate, fatty acid…), cells could be challenged with depolarization or hypoxia to perhaps unravel a PS-dependent phenotype in specific contexts. Indeed, the brain tissue is much more complex than *in vitro* cultures, in which disrupted PS-dependent cell interactions could account for changes in energy homeostasis (Saura et al., 2004; Wines-Samuelson et al., 2010).

In conclusion, our study provides evidence for the lack of mitochondrial alterations in PS1KD, PS2KO, and even PSdKO neurons and astrocytes, as well as in PS2KO primary MEF. This is in contradiction with previous observations in neuronal cell lines and immortalized fibroblasts. Thus, immortalized cells might provide relevant results regarding the role of PSs in mitochondrial activity and bioenergetics related to cancer processes, in which PSs involvement have already been reported. However, the contribution of PSs to alterations in mitochondrial activity related to neurodegenerative processes, such as AD, needs to be critically readdressed and further explored in brain models.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by UCLouvain animal care committee's regulations (code number 2016/UCL/MD/016).

AUTHOR CONTRIBUTIONS

SC performed research, analyzed data, and wrote the paper. NS and CV performed research and analyzed data. DV performed research. VP, SS, and LB analyzed data. PK-C designed research and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.586108/full#supplementary-material

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REVIEW
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APOE and Alzheimer's Disease: From Lipid Transport to Physiopathology and Therapeutics

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Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterized by extracellular amyloid β (Aβ) and intraneuronal tau protein aggregations. One risk factor for developing AD is the *APOE* gene coding for the apolipoprotein E protein (apoE). Humans have three versions of *APOE* gene: ε2, ε3, and ε4 allele. Carrying the ε4 allele is an AD risk factor while carrying the ε2 allele is protective. ApoE is a component of lipoprotein particles in the plasma at the periphery, as well as in the cerebrospinal fluid (CSF) and in the interstitial fluid (ISF) of brain parenchyma in the central nervous system (CNS). ApoE is a major lipid transporter that plays a pivotal role in the development, maintenance, and repair of the CNS, and that regulates multiple important signaling pathways. This review will focus on the critical role of apoE in AD pathogenesis and some of the currently apoE-based therapeutics developed in the treatment of AD.

Keywords: Alzheimer's disease, APOE, APOE receptors, lipidation, amyloid β, therapeutics

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OVERVIEW

Polymorphism in the apolipoprotein E (APOE) gene is a major risk for developing late onset Alzheimer disease (LOAD), whose symptoms are more frequently appearing after the age of 65 years (Yamazaki et al., 2019). The ε4 allele of *APOE* gene is the strongest risk factor for LOAD (Yamazaki et al., 2019). The differences in the structure of apoE isoforms influence their ability to bind lipids, receptors, and amyloid-β (Aβ), which aggregates in plaques within the brain. Human and animal studies clearly indicate that apoE isoforms differentially regulate neuroinflammation, tau hyperphosphorylation, Aβ aggregation and clearance (Tachibana et al., 2019; Kloske and Wilcock, 2020; Vasilevskaya et al., 2020). ApoE regulates lipid homeostasis by mediating lipid transport from one tissue or cell type to another (Holtzman et al., 2012; Chernick et al., 2018; Zhao et al., 2018b). Since lipids such as cholesterol and triglycerides are insoluble in water, they must be carried in the circulation by hydrophile-lipophile particles named lipoproteins. These lipoproteins play a major role in the absorption and transport of dietary lipids between the small intestine, liver and peripheral tissues to the brain where they are essential. In the periphery, it is established how lipids travel in the blood using the different types of lipoproteins (Holtzman et al., 2012; Chernick et al., 2018; Zhao et al., 2018b), whereas within the CNS, lipoproteins are often designated as high-density lipoproteins (HDL)-like, yet their size, shape, and distribution remain unclear. ApoE, present in the CNS and the periphery, represents a critical link between these two compartments and could influence Alzheimer's disease (AD) pathogenesis by disrupting the blood–brain barrier

(BBB) integrity from both sides (Chernick et al., 2019). In this review, the possible mechanisms by which apoE exerts its modulatory effect on AD physiopathology are discussed and new therapeutic perspectives targeting apoE for AD treatment are also described.

HUMAN APOE GENE

Gene Polymorphism

All species have one version of *APOE* gene while humans have three versions: APOE-ε2 (APOE2), APOE-ε3 (APOE3), and APOE-ε4 (APOE4) allele (McIntosh et al., 2012). The human *APOE* gene comprises of several single-nucleotide polymorphisms (SNPs) distributed across the gene (Nickerson et al., 2000). The most common SNPs are categorized as rs429358 (C > T) and rs7412 (C > T) that lead respectively in amino acid change at position 112 and 158 within the apoE protein (Belloy et al., 2019). The haplotype combination at the two SNPs results in three apoE protein isoforms: apoE2 (Cys112, Cys158), apoE3 (Cys112, Arg158), and apoE4 (Arg112, Arg158). In non-human mammals, APOE genotype is (Thr61/Arg112/Arg158) while all human APOE alleles have an Arginine in position 61 (Arg61). Combinations of these specific amino acids modify the protein structure and functions. The world-wide frequency of human APOE alleles varies considerably (**Figure 1**). APOE3 is the most common among all human populations and its frequency ranges from 85% (Asia) to 69% (Africa) (Singh et al., 2006). APOE4 allele frequency varies considerably in native populations of Central Africa (40%), Oceania (37%), and Australia (26%) (Corbo and Scacchi, 1999). The distribution across Europe and Asia follows an apparent gradient from north to south, with low APOE4 allele frequencies in the Mediterranean or South China and higher frequencies in northern regions (up to 25%) (Egert et al., 2012). APOE2 is the least common allele with a global prevalence of 7.3% and is absent in many indigenous people without any clear regional pattern (Corbo and Scacchi, 1999; Singh et al., 2006).

APOE Allele and Risk of Diseases

The different alleles of *APOE* confer differential risks of developing pathologies (Wu et al., 2018). A meta-analysis of clinical and autopsy-based studies on five ethnic groups (Caucasian, African American, Hispanic, and Japanese) revealed that among Caucasian subjects, the risk of developing AD was increased in individuals with one APOE4 copy compared to individuals homozygote for APOE3 (Farrer et al., 1997). Compared to non-carriers of APOE4, the increased risk of AD is 3–4 fold in heterozygotes and about 9–15 fold in APOE4 homozygotes (Farrer et al., 1997; Neu et al., 2017). The APOE4-AD correlation was weaker among African–Americans and Hispanics, and greater in Japanese people compared to Caucasian cases (**Table 1**). The risk of AD was however decreased in people carrying APOE2 compared to those carrying APOE3 (Farrer et al., 1997). According to a population-based cohort study, lifetime risk of mild cognitive impairment (MCI) or dementia is 30–35% for APOE4 homozygote individuals, 20–25% for APOE4 heterozygote individuals (ε3/ε4 and ε2/ε4), and 10–15% for non APOE4 individuals (ε3/ε3, ε3/ε2, and ε2/ε2) (Qian et al., 2017). With respect to other APOE genotypes, familial type III hyperlipoproteinemia is associated to those homozygous for APOE2 (Yang et al., 2007; McIntosh et al., 2012). Altogether, these APOE-related risks for diseases point toward differences in the structure and function of the proteins involved in lipid metabolism.

APOE2, Guardian Angel Against AD?

A study on 38,537 people from six population-based cohorts showed a survival benefit for APOE2 carriers (Wolters et al., 2019). They identified 239 APOE2 homozygotes, who have lived the longest lives. Another group reported that compared with the ε3/ε3 genotype, individuals with the ε2/ε2 genotype have larger gray-matter volume in brain areas subjected to AD (i.e., hippocampi, medial temporal inferior temporal, cortex, precuneus, superior parietal regions, and temporal pole) and in areas related to cognitive resilience during aging (i.e., anterior cingulate and medial prefrontal areas) (Arenaza-Urquijo et al., 2019). APOE2 homozygotes have a 66% reduction in AD risk compared to ε2/ε3 carriers, an 87% reduction in AD risk compared to APOE3 homozygotes, and a 99.6% reduction in AD risk when compared to APOE4 homozygotes (Reiman et al., 2020). These recent studies on APOE2-related genotypes might stimulate research interest for characterizing the molecular advantages of apoE2 protein over the other isoforms.

APOE PROTEIN

Structure

Human *APOE* gene is located on the chromosome 19 at position q13.32 (**Figure 2A**) and codes for a 299 amino acid protein (∼36 kDa) whose primary function in the brain is to transport cholesterol. ApoE contains three main regions: a N terminal region containing the receptor-binding site and four helices, a C-terminal region containing the lipid-binding site and three helices, and an intervening flexible hinge region that links the N- and C-terminal regions (**Figure 2B**) (Liu et al., 2013; Flowers and Rebeck, 2020). ApoE isoforms differ by a unique amino acid combination at position 112 and 158: apoE2 (Cys112, Cys158), apoE3 (Cys112, Arg158), and apoE4 (Arg112, Arg158) (Yu et al., 2014). The genotype-related change of one or two amino acids within the apoE protein modifies its structure. For instance, Cys-158 in apoE2 removes a salt-bridge between Arg158 and Asp154, reduces the positive potential, and consequently changes the receptor binding region (Wilson et al., 1991). Arg-112 changes the lipid binding region of apoE4 and shifts the lipid binding preference from HDL to very-low-density lipoproteins (VLDL) (Mahley and Rall, 2000). The existing interaction between the amino acid 61 and 112 influences the lipoprotein binding; it is the main reason explaining the high affinity of apoE4 to VLDL while apoE2 and apoE3 bind to HDL (McIntosh et al., 2012). Even though apoE of the non-human mammals such as chimpanzee is similar to the human ε4 allele, the presence of a threonine at position 61 (Thr61) makes it work more like the human ε3 allele (McIntosh et al., 2012).

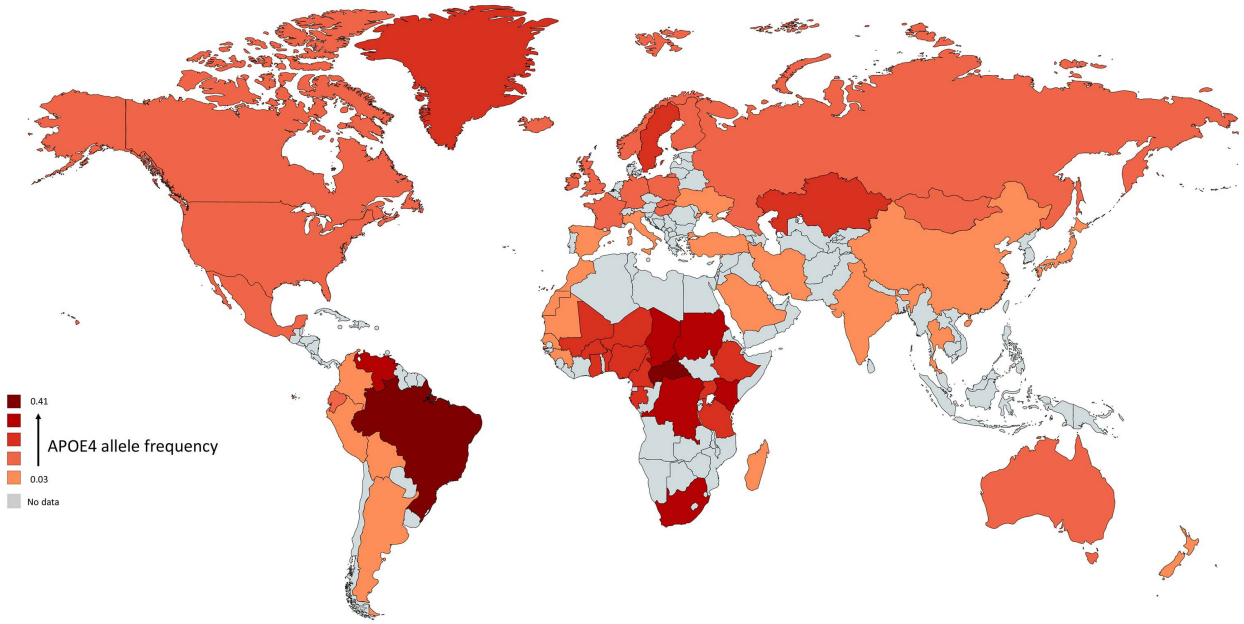

FIGURE 1 | Global distribution of APOE4 allele in *Homo sapiens*. Frequency of ε4 is low (light red regions) and high (dark red regions). The gray color indicates that there are no data available for this country. The geographical map of APOE4 frequency was generated using the package, rworldmapR. State estimates were generated by averaging the frequency of APOE4 allele across the various studies conducted in a specific country.

TABLE 1 | APOE genotypes, allele frequency distribution, and odds ratio for developing AD, stratified by AD patient cases and controls on five ethnic groups (Farrer et al., 1997).

		APOE genotype {(frequency (%) / AD Odds ratio (95 % confidence interval)}						APOE allele frequency (%)		
Ethnic groups	**No**	**E2/E2**	**E2/E3**	**E2/E4**	**E3/E3**	**E3/E4**	**E4/E4**	**E2**	**E3**	**E4**
CAUCASIAN CASE PATIENTS	5107	0.2/0.9	4.8/0.6	2.6/1.2	36.4/1.0	41.1/2.7	14.8/12.5	3.9	59.4	36.7
Controls	6262	0.8	12.7	2.6	60.9	21.3	1.8	8.4	77.9	13.7
AFRICAN AMERICAN CASE PATIENTS	235	1.7/2.4	9.8/0.6	2.1/1.8	36.2/1.0	37.9/1.1	12.3/5.7	7.7	59.1	32.2
Controls	240	0.8	12.9	2.1	50.4	31.8	2.1	8.3	72.7	19.0
HISPANIC CASE PATIENTS	261	0.4/2.6	9.6/0.6	2.3/3.2	54.4/1.0	30.7/2.2	2.7/2.2	6.3	74.5	19.2
Controls	267	0.4	12.0	0.8	67.4	17.6	1.9	6.7	82.3	11.0
JAPANESE CASE PATIENTS	336	0.3/1.1	3.9/0.9	0.9/2.4	49.1/1.0	36.9/5.6	8.9/33.1	2.7	69.5	27.8
Controls	1977	0.4	6.9	0.8	75.7	15.5	0.8	4.2	86.9	8.9

Tissue Expression

The human *APOE* gene is expressed in several organs and in various cell types. Ninety percent of the circulating apoE is produced by the liver (Yu et al., 2014) and to a lesser extent by the adrenal gland and macrophages (Kockx et al., 2008). Other cells capable of synthesizing apoE include astrocytes, macrophages, and endocrine cells such as ovarian and adrenal cells (Huang et al., 2015). In the CNS, apoE secretion is sustained by astrocytes, oligodendrocytes, pericytes, choroid plexus and neurons (Kang et al., 2018; Flowers and Rebeck, 2020). ApoE lipoproteins produced by the choroid plexus are directly secreted into the cerebrospinal fluid (CSF) (Achariyar et al., 2016). ApoE is also synthesized by glial cells and associates with lipids to form lipid-transport particles in the CSF (Koch et al., 2001).

The expression level of apoE varies by genotype. For instance, individuals carrying APOE2 have higher concentration of apoE proteins in the CSF whereas APOE4 carriers have lower levels (Castellano et al., 2011; Cruchaga et al., 2012). The same observation was made in a mouse model whose *APOE* gene was replaced by the human APOE2 (hAPOE2) or hAPOE4 (Ulrich et al., 2013). ApoE levels in the CNS of AD patients

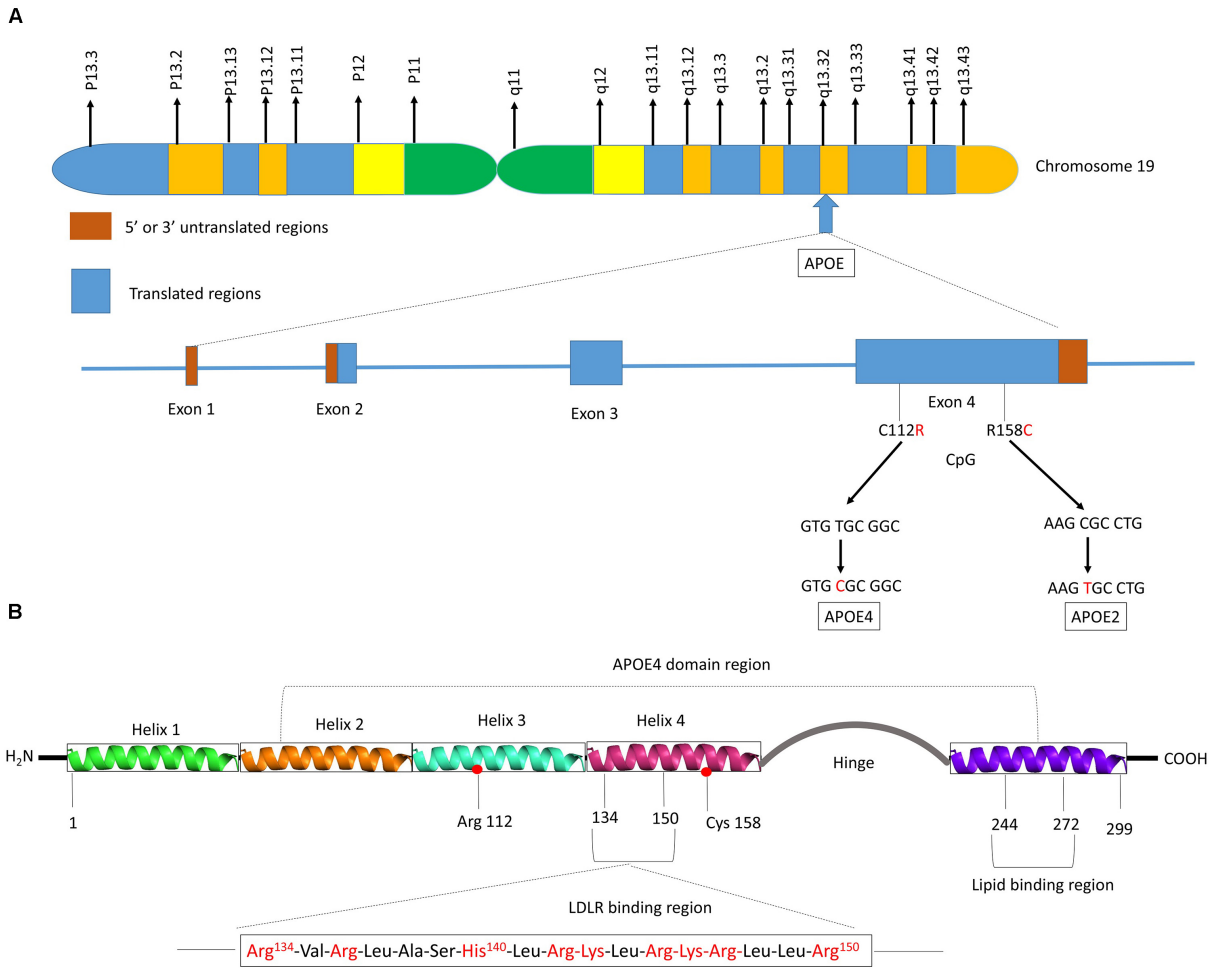

FIGURE 2 | Schematic illustration of structural and functional regions of apoE protein. **(A)** Location and structure of the *APOE* gene on chromosome 19 at position q13.32. The *APOE* gene has 4 exons, respectively consisting of 44, 66, 193, and 860 nucleotides, with exon 4 coding for over 80% of the protein. Exon 1 contains the 5′ untranslated region (5′UTR). The wild-type sequence is apoE3 (Cys112, Arg158). Point mutations in the exon 4 generate two single nucleotide polymorphisms (SNPs): a T → C point mutation produces apoE4 (C112R), while C → T mutation gives apoE2 (R158C). **(B)** Diagram of human apoE structural domains. The N-terminal region contains the receptor-binding site (residues 134–150) and four helices (1–4), the C-terminal region contains the lipid-binding region (residues 244–272) and the two domains are joined by a hinge region. The N-terminal region contains the two polymorphic positions (112 and 158) that discriminate the three apoE isoforms. The lower part of the figure shows the α-helical segment (residues 134–150) that recognizes the LDL receptor. This segment is rich in positively charged arginine and lysine residues.

are inconsistent with studies reporting either higher (Baker-Nigh et al., 2016), lower (Cruchaga et al., 2012; Talwar et al., 2016) or unchanged level of apoE (Martínez-Morillo et al., 2014) compared to healthy individuals. However, the expression levels of APOE mRNA in post-mortem AD brain tissues are elevated compared to controls (Gottschalk et al., 2016), emphasizing the difficulty to correlate APOE mRNA and protein levels.

APOE RECEPTORS

Affinity for apoE Isoforms and Their Tissue Expression

ApoE is a ligand for cell surface lipoprotein receptors belonging to the low-density lipoprotein receptor (LDLR) family (Holtzman et al., 2012). The LDLR family consists of eight receptors i.e., LDLR, very-low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (apoER2 or LRP8), LRP4, LDLR-related receptor 1 (LRP1), LRP1b, megalin (LRP2), and LR11/SorLA (Lane-Donovan and Herz, 2017). All LDLR family members share structural properties that allow them to interact with apoE isoforms but with distinct affinities. ApoE3 and apoE4 isoforms bind with high affinity to the LDLR and LRP1 (Ruiz et al., 2005; Zhao et al., 2018b) whereas apoE2 binding to LDLR is 50 times weaker than that of apoE3 or apoE4 (Hatters et al., 2006a). VLDL receptor recognizes all apoE isoforms with equal affinity (Ruiz et al., 2005). Co-immunoprecipitation assays revealed differences in the formation of the apoE-LR11 complex for each isoform (apoE4 > apoE3 > apoE2, apoE4 binding the most), however the lipidation and oxidation status of apoE was

not addressed in this study (Yajima et al., 2015). It remains unclear whether apoE differentially activates apoE receptors.

These receptors are expressed by many tissues. LDLR, is expressed by neurons and hepatocytes (Goldstein and Brown, 2015). LRP1 is ubiquitously expressed, but more abundantly in vascular smooth muscle cells (SMCs), hepatocytes, and neurons (Etique et al., 2013). LRP1 is present on plasma membrane of various cells including microglia, astrocytes, and neurons. LRP1b is primarily expressed in the brain, and a differentially spliced form is present in the adrenal gland and in the testis (Marschang et al., 2004). VLDLR is expressed by neurons and in tissues throughout the body while apoER2 is restricted to the brain, testis, and placenta (Dlugosz and Nimpf, 2018). Megalin is highly expressed in the proximal tubule of the kidney (Christensen et al., 1992) and in many other absorptive epithelia, e.g., lung, retina, yolk sac, inner ear, and brain (Kounnas et al., 1994). LR11 is expressed in neurons of the central and peripheral nervous system (Kim et al., 2009).

ApoE Receptors and AD

The role in AD pathogenesis for each member of the LDLR family remains unclear. Overexpression of LDLR in the brain could lower apoE levels, increase the clearance of Aβ which is one of the neurological hallmarks of AD, and decrease the deposition of Aβ (Kim et al., 2009). LRP1 could affect AD pathogenesis by controlling amyloid precursor protein (APP) processing and Aβ catabolism (Cam and Bu, 2006). Alterations in APP cellular trafficking and localization directly impact its processing to Aβ, and disrupting the interaction between LRP and APP could decrease Aβ production which in turn affects development of AD (Cam and Bu, 2006). LRP1b binds to APP and decreases the processing of APP to Aβ (Cam et al., 2004). Hence, the expression of LRP1b might also be involved in protecting against the pathogenesis of AD by decreasing the generation of Aβ proteins within the CNS (Cam et al., 2004). ApoE isoforms also affect the endocytosis of receptors. For instance, carrying the APOE4 allele significantly impairs the recycling of apoER2 and VLDLR compared to APOE3 and APOE2 (Chen et al., 2010). Since apoER2 interacts with APP and affects APP processing, lower recycling might increase output of Aβ (He et al., 2007). Unlike other members of the LDLR family, SorLA expression does not affect APP endocytosis, but rather mediates APP intracellular transport processes (Kim et al., 2009). Megalin was thought to clear Aβ from the brain at the choroid plexus across the blood-CSF barrier (Spuch et al., 2015). It has been shown that binding of megalin to Aβ is decreased in the CSF of AD patients, suggesting that decreased Aβ sequestration in the CSF could be associated with defective Aβ clearance and increased brain Aβ levels (Spuch et al., 2015).

METABOLISM AND APOE ISOFORMS

Lipidation of apoE Isoforms

To perform its important functions (i.e., cholesterol transport, immune modulation, synapse regeneration, and clearance/degradation of Aβ), apoE must be secreted and properly lipidated (Kanekiyo et al., 2014; Hu et al., 2015). ApoE lipidation is facilitated by the cholesterol-efflux protein ATP-binding cassette A1 (ABCA1). ABCA1 is present in a wide variety of body cells, including the brain's astrocytes, neurons, BBB and in the choroid plexus (Flowers and Rebeck, 2020). ABCA1 is essential for the proper lipidation of apoE and absence of ABCA1 in knockout mice leads to a decrease in the overall apoE level in the CNS (Flowers and Rebeck, 2020). ApoE isoforms differ in their lipid binding and lipoprotein preferences. The C-terminal domain of apoE (273–299) is critical for the lipoprotein binding and therefore determines apoE isoform lipidation specificity and efficiency (Hu et al., 2015). Contrary to apoE2 and apoE3, apoE4 is poorly lipidated (Kanekiyo et al., 2014). ApoE3 and apoE2 preferentially bind to small phospholipid-rich HDL while apoE4 strongly binds to large triglyceride-rich VLDL (Nguyen et al., 2010). Reduced binding affinity of apoE4 for HDL results in a greater proportion of unlipidated apoE, hence forming aggregates (Hatters et al., 2006a). ApoE4 large aggregates are more toxic for neurons than apoE2 and apoE3 aggregates (Hatters et al., 2006b). Biophysical studies have shown that lipid-free apoE appears to aggregate *in vitro* in an isoform-dependent manner (apoE4 > apoE3 > apoE2), and lipidation of apoE impedes aggregates formation (Hubin et al., 2019). Unlipidated apoE monomers form multimers such as dimers and tetramers, and apoE can additionally aggregate to form fibrils (Flowers and Rebeck, 2020). Free cholesterol, phospholipids, and triglycerides are the main lipids present in apoE-containing CNS particles (Peng et al., 2003). ApoE4-containing particles have less cholesterol than those containing ApoE3 (Zhao N. et al., 2017). APOE genotypes also modify lipidation states in the periphery. In the homozygous and heterozygous (ε3/ε4) genotypes, APOE4 is frequently associated with increased LDL-cholesterol levels in plasma while in homozygous and heterozygous (ε2/ε3) genotypes, APOE2 is correlated with mild or low levels (Villeneuve et al., 2015). APOE2 genotype correlates with higher triglyceride levels compared to APOE3 and APOE4 (Zhao N. et al., 2017). The amino acid substitution in apoE2 impairs its binding with LDLR and impairs clearance of triglyceride-rich lipoprotein remnant particles, leading to the onset of type III hyperlipoproteinaemia (Phillips, 2014).

Lipid Transport Within the CNS

ApoE mediates delivery of cholesterol and other lipids to neurons and glial cells. Cholesterol cannot not cross the BBB and the choroid plexus Blood-CSF Barrier (BCSFB), but it is converted into 24S-hydroxycholesterol by the enzyme 24-hydroxylase cholesterol, specifically located in neurons and 24S-hydroxycholesterol can bind to apoE and easily cross the BBB (Russell et al., 2009). It was suggested that apoE4 is involved in AD pathogenesis by mechanisms linked to the metabolism of brain lipids (Hauser et al., 2011). In cultured neurons, apoE4 was less effective than apoE2 and apoE3 to transport brain cholesterol (Rapp et al., 2006). Because cholesterol and phospholipids transport relies on apoE isoforms (apoE2 > apoE3 > apoE4) (Hara et al., 2003), the lower efflux of cholesterol and phospholipids by apoE4 might be involved in the increased risk of LOAD in APOE4 carriers. Two recent studies on

human iPSCs-derived astrocytes have shown higher cholesterol accumulation inside apoE4-expressing astrocytes than inside apoE3 astrocytes (Lin et al., 2018; Tcw et al., 2019), supporting that the transport of cholesterol out of the astrocytes might be deficient in APOE4 carriers. A more recent study using iPSC-derived astrocytes showed that apoE4 is less lipidated than apoE3, potentially impacting apoE4 neurotrophic role (Zhao J. et al., 2017). In the CSF of middle-aged adults (average age 54.5 years) with no dementia, apoE particles were smaller in both ε3/ε4 and ε4/ε4 individuals than in ε3/ε3 individuals, but larger in ε2/ε3 individuals (Heinsinger et al., 2016; Nelson and Sen, 2019). The cholesterol efflux ability of individuals homozygous for APOE4 is reduced in CSF (Yassine et al., 2016). The larger particle size in APOE4 homozygote AD patients may inhibit particle binding or endocytosis, thus depriving neurons of enough cholesterol for repair (Yassine et al., 2016). In the CNS, apoE lipid transport capability could also be influenced by the quantity of apoE (Rebeck, 2017). In mice, the amount of apoE in brain parenchyma (Riddell et al., 2008) and CSF (Ulrich et al., 2013) has an isoform-dependent gradient (apoE2 > apoE3 > apoE4). In humans, there was however no isoform-dependent variations in levels of apoE in the CSF in young control subjects (average 34.5 years) (Baker-Nigh et al., 2016), cognitively healthy subjects (average 61 years) and AD patients (average 78 years) (Martínez-Morillo et al., 2014). In a cohort of Aβ-positive cognitively healthy individuals as well as with MCI, levels of apoE in CSF were significantly lower in APOE4 carriers relative to non-carriers (Baker-Nigh et al., 2016). This indicates that extensive lipid homeostasis studies are required to unravel a more comprehensive mechanism.

APOE ISOFORMS AND AD PHYSIOPATHOLOGY

APOE genotypes can affect many cellular functions such as synaptic integrity, lipid transport, glucose metabolism, Aβ clearance, BBB integrity or mitochondria regulation (**Figure 3**). For instance, recent reviews came to the conclusion that apoE4 increases the pro-inflammatory response, which in turn causes the dysfunction of BBB, and leads to cognitive deficits (Marottoli et al., 2017; Teng et al., 2017; Kloske and Wilcock, 2020). ApoE isoforms also affect the primary neuropathological markers of AD: neuroinflammation, Aβ plaques and tau protein aggregations. Studies in humans and transgenic mice showed that brain Aβ levels and amyloid plaque loads are higher in APOE4 carriers than the other genotypes, with the lowest levels in APOE2 carriers (Huang et al., 2017; Safieh et al., 2019; Tachibana et al., 2019). The increase in Aβ plaques in APOE4 carriers may be due to the enhanced ability of apoE4 to bind Aβ but also its inability to completely remove Aβ from the brain (Kloske and Wilcock, 2020). Other mechanisms involve apoE4 in different pathways such as interstitial fluid (ISF) drainage, uptake by microglial phagocytosis (**Figure 4**), that could contribute to the decrease of Aβ removal (Castellano et al., 2011; Tarasoff-Conway et al., 2015; Ma et al., 2018). Tau protein hyperphosphorylation as well as the formation of tangles differ by APOE genotype. Overexpression of apoE4 in neurons abnormally increases tau phosphorylation while apoE3 overexpression has no effects (Cao et al., 2017; Shi et al., 2017; Wang et al., 2018; Vasilevskaya et al., 2020). ApoE directly inhibits GSK-3β-mediated phosphorylation of tau (Hoe et al., 2006). In the next sections we will discuss synergy between apoE lipidation and sex specificity with LOAD.

Crosstalk Between apoE Lipidation and AD

Lipidation of apoE and lipid transport within the CNS are currently under investigation to clarify their roles in the development of LOAD. ApoE is unique among apolipoproteins with its minimal intracellular degradation (Yassine and Finch, 2020). Internalized lipids are dissociated from apoE into late endosomal compartments after intracellular absorption of apoE-containing lipoprotein particles, followed by recycling of apoE into early endosomes and its re-secretion within or into HDL particles (Yassine and Finch, 2020). ApoE4 has lower recycling capacity due to its greater affinity for lipid binding. This property reduces the efflux of cholesterol and enriches the cell membrane with cholesterol (Yassine and Finch, 2020). ApoE recycling controls the expression of several cell surface proteins, such as ABCA1 (Rawat et al., 2019), the insulin receptor (IR), or LRP1 (Yassine and Finch, 2020). The reduced apoE4 recycling traps ABCA1 in endosomes, away from the cell surface. Reduced activity of ABCA1 hence contributes to lower efflux of cholesterol to HDL and redistributes cholesterol to cell membranes (Rawat et al., 2019). Greater cell membrane cholesterol enhances TLR4 signaling in macrophages which in turn, activates NFkB and induces inflammatory genes response (Singh et al., 2020; Yassine and Finch, 2020). Lower apoE4 recycling in the brain also traps insulin receptor (IR) away from cell surface in the endosome (Zhao N. et al., 2017), hence modifying its preferences for cellular energy sources. Consequently, it reduces glucose utilization to generate ATP and encourages oxidation of fatty acids (Svennerholm et al., 1997). It is reported that the level of phospholipids (PL) decreases in the brain by 42% between the age of 20 and 100 years old, and that there is an additional loss of 20% in the AD brain (Mesa-Herrera et al., 2019). In humans, our group also showed that beta-oxidation of docosahexaenoic acid (DHA), a polyunsaturated fatty acid that is highly concentrated in brain membranes, is higher in APOE4 carriers than the non-carriers (Chouinard-Watkins et al., 2013). Since the brain largely relies on glucose to fulfill its high energy-demand, the decrease of glucose uptake through the BBB during aging had been identified as a risk factor for developing AD (Fernandez et al., 2019). This is especially true in APOE4 carriers since it was reported that human astrocytes expressing apoE4 have half of the glucose uptake capacity compared to apoE3 ones whereas astrocytes expressing apoE2 have the highest glucose uptake (Williams et al., 2020). Moreover, apoE4 proteins secreted from primary astrocytes are poorly lipidated compared to apoE3. Increasing the activity of ABCA1 could therefore provide a therapeutic approach to promote the recycling of apoE4 from endosomes and restore its function at the membrane level (Yassine and Finch, 2020). A greater distribution of cholesterol at the neuronal plasma membrane increases the expression of

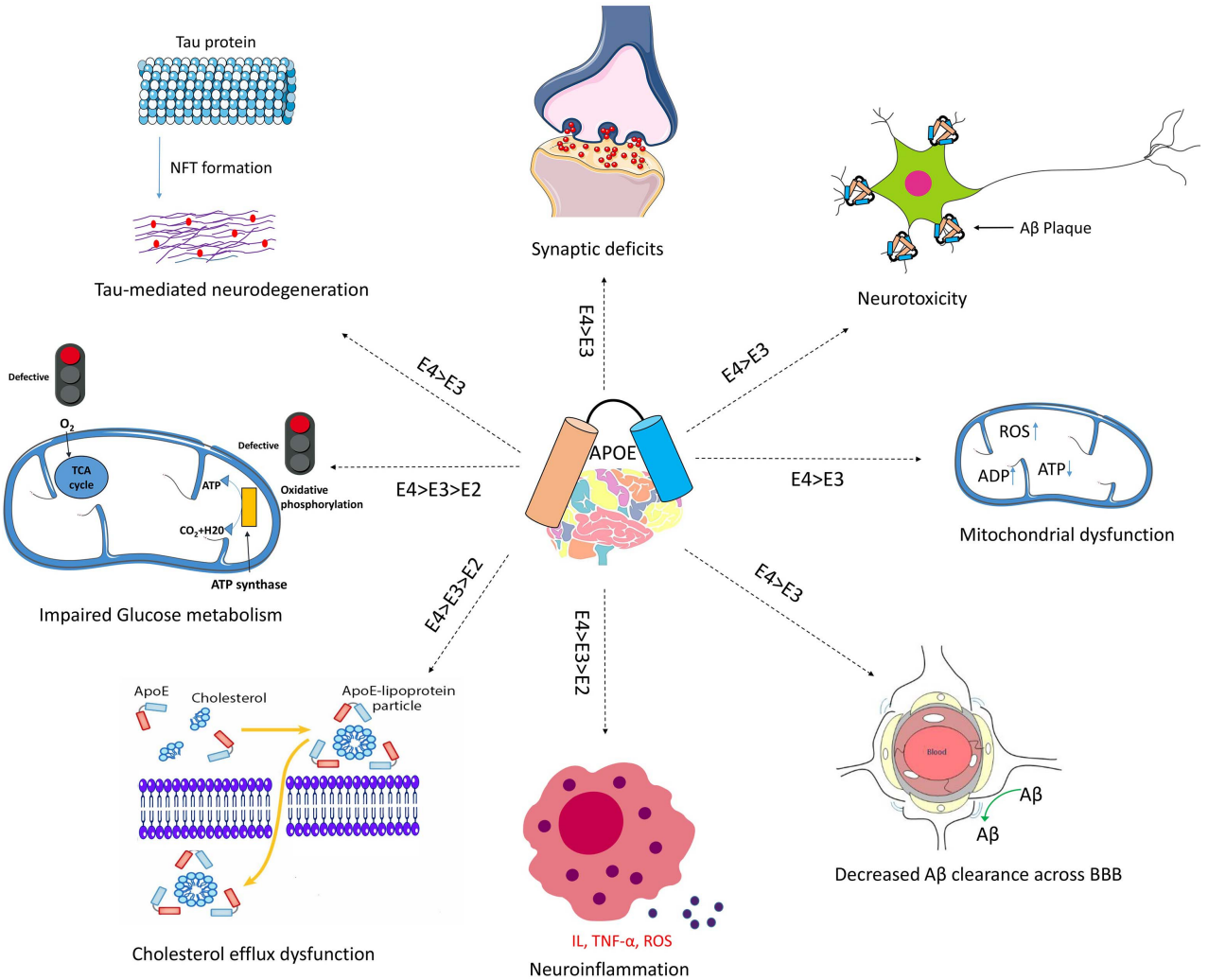

FIGURE 3 | Schematic overview of Aβ-independent roles for apoE in Alzheimer's disease pathology. The isoform-dependent effects of *APOE* are indicated. Abbreviations: apoE, apolipoprotein E; NFT, neurofibrillary tangle; BBB, blood brain barrier; IL, interleukin; TNF-α, Tumor necrosis factor-α; ROS, reactive oxygen species.

BACE1 and APP processing to generate more Aβ (Cui et al., 2011). In astrocytes and microglia, less efflux of cholesterol decreases Aβ degradation which in turn might accentuate its aggregation to form plaques. In astrocytes, LRP1 complexes with apoE and the reduced plasma membrane recycling of LRP1 decreases the ability of astrocytes to degrade Aβ peptides (Prasad and Rao, 2018), providing one mechanism for the increased development of apoE4 associated amyloid plaques. Altogether, there are emerging evidence that in APOE4 carriers, there are metabolic shifts in the energy metabolism that could contribute to LOAD pathogenesis during aging.

One interesting observation in individuals carrying one or two APOE4 copies have usually "normal" brain functions until older ages despite having low lipidation of their apoE4 protein. Therefore, apoE4 lipid transport capabilities to supply neurons and astrocytes are probably decreased in late stages of life in APOE4 carriers. Hence, a young brain might have mechanisms in place to cope with inadequate lipid transport related to apoE4 protein structure (Yassine and Finch, 2020). During aging, there is a potential loss of these alternative mechanisms while there is also a decreased production of cholesterol (Boisvert et al., 2018), and both could lead to neuronal lipid deficits (Fernandez et al., 2019). Identification of the coping mechanisms that are lost during aging would highly benefit the field in moving forward this research area.

Sex-Specificity in LOAD and for apoE4 Lipidation

The risk of developing LOAD is much higher for women than men (Barnes et al., 2005). Women with a single APOE4 copy have a significant increase risk of AD compared to men carrying two APOE4 copies (Farrer et al., 1997). The risk factor for women with one APOE4 copy is around 4-fold whereas in

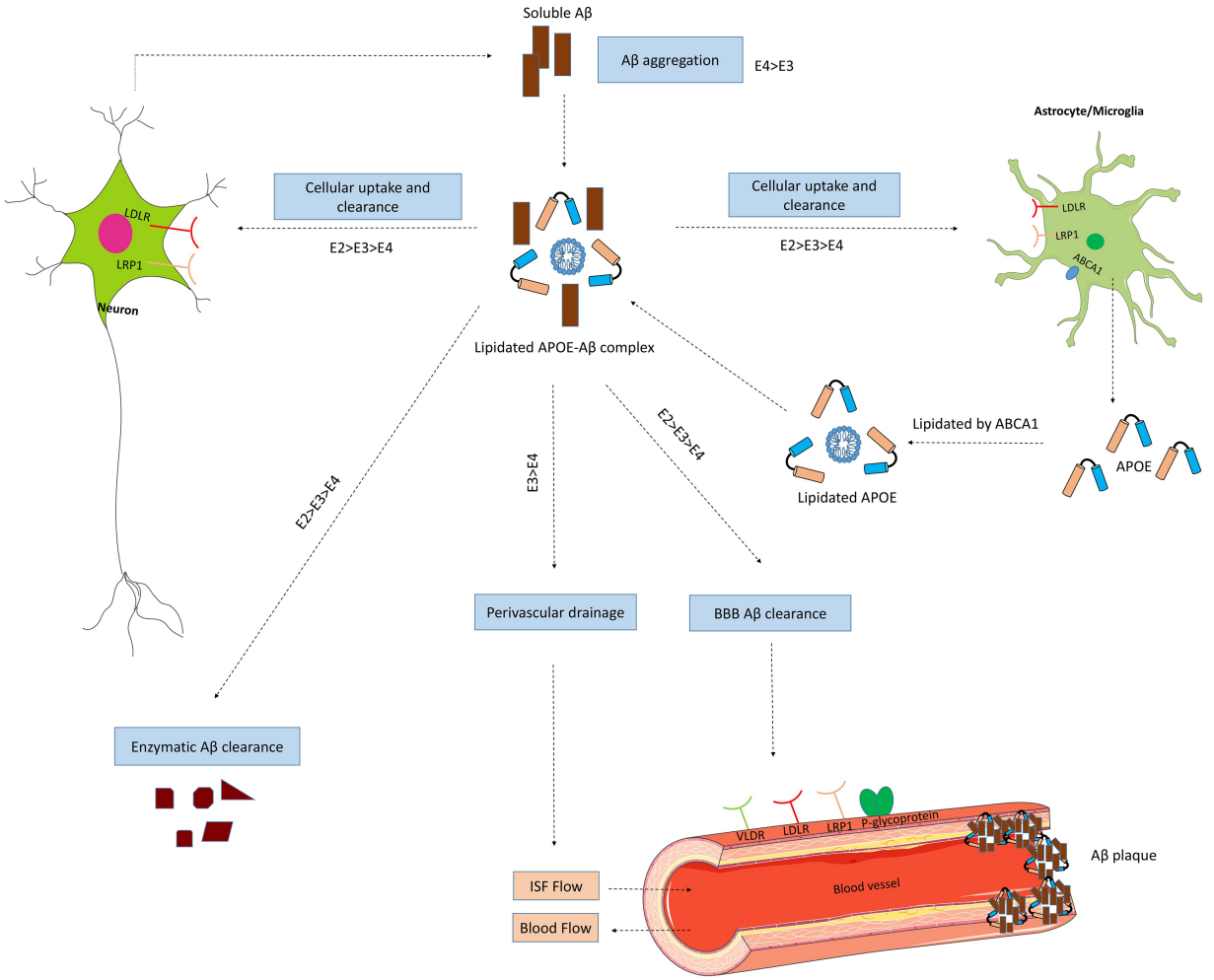

FIGURE 4 | ApoE isoform-dependent effects on Aβ metabolism and clearance. Aβ is mainly produced by neurons via proteolytic cleavage of APP. In the brain, apoE is primarily produced by astrocytes and microglia, and is then lipidated by ABCA1 to form lipoprotein particles. ApoE accelerates isoform-dependent aggregation and deposition of Aβ, but also promotes the cellular uptake and clearance of Aβ by astrocytes or microglia by endocytosis of the lipidated APOE-Aβ complex. This endocytosis is mediated by various receptors, including LDLR and LRP1. ApoE facilitates isoform-dependent extracellular proteolytic degradation of Aβ. At the BBB, soluble Aβ is mostly transported via LRP1 and P-glycoprotein from the interstitial fluid (ISF) into the bloodstream. ApoE also mediates perivascular drainage of Aβ. Insufficient Aβ clearance can cause Aβ aggregation in brain parenchyma and can contribute to the formation of Aβ oligomers and amyloid plaques.

men with one APOE4 copy, the risk is 1-fold higher only (Payami et al., 1994; Altmann et al., 2014). There are also marked regional variations between men and women in the regulation of fatty acid metabolism. Triglycerides (TG) are distributed differently in the adipose tissue of male and females. Moreover, concentrations of polyunsaturated fatty acids in the adipose tissue are higher in pre-menopause women compared to men (Lohner et al., 2013; Lim et al., 2020). Estrogen levels, higher in women before menopause, play a key role in the transport of lipids, increasing metabolic enzyme expression, and reducing α-linolenic acid (ALA) oxidation, an essential polyunsaturated fatty acid (Palmisano et al., 2018). In post-menopausal women, the decrease in estrogen levels is associated with increased plasma TG levels and lower HDL, both of which increase the risk of cognitive decline (Ancelin et al., 2014; Chew et al., 2020). Higher rate of cognitive decline was observed with increased TG content and lower HDL levels (Ancelin et al., 2014). APOE4 allele increases the risk of abnormal Aβ aggregation in men and women equally, but impacts tau hyperphosphorylation more prominently in women (Altmann et al., 2014). Women APOE4 carriers with MCI had higher CSF tau and tau/Aβ ratios compared to APOE4 males with MCI (Payami et al., 1994). Likewise, women APOE4 carriers with mild LOAD had a greater risk of developing both neurofibrillary tangles and Aβ plaques than APOE4 men with mild LOAD (Corder et al., 2004). However, women and men carrying two APOE4 copies have both a 15-fold higher risk of developing LOAD. The sex-specific differences of APOE4 genotype can also occur at the level of gene regulation as apoE4 functions as a transcription factor in the brain, binding to the promoter regions of genes linked to microtubule disassembly, programmed cell death, synaptic function, and aging (Theendakara et al., 2016). ApoE4-mediated

transcriptional activity is sex-specific for genes involved in the response of the immune system, inflammation, oxidative stress, aging and estrogen signaling as different patterns of activation have been observed between female and male ε4-positive brains (Hsu et al., 2019). Collectively, these findings show that there are sex differences in the risk of developing LOAD based on the APOE genotype. Therefore, sex should be considered when investigating the impact of different therapeutic strategies on the modulation of lipid metabolic pathways.

APOE-TARGETED THERAPIES FOR LOAD

Carrying the APOE4 allele is associated with higher deposition of Aβ in the brain however it remains to be proved that Aβ is AD-causative (Bu, 2009) since Aβ deposition in human brain without significant cognitive impairment are frequently observed (Aizenstein et al., 2008). Moreover, clinical trials reporting reduction of brain Aβ accumulation do not necessarily show improvement of cognition supporting that there is no direct link between Aβ plaque reduction and improved cognition (van Veluw et al., 2017). As the disease is complex and multifactorial, it is likely that the current developed drugs target the wrong pathological substrates, or that a multi-target drug approach could be required. We describe here apoE-targeted therapies tested in animals and those in the early phases of clinical trials. The current therapeutic strategies targeting apoE to treat LOAD include: (1) targeting apoE structural properties and interaction with Aβ, (2) modulating apoE level and lipidation, (3) targeting APOE receptors, and (4) apoE gene therapies.

Targeting apoE Structural Properties and Interaction With Aβ

Blocking apoE-Aβ interaction with peptide mimics might be advantageous since the peptide can be very selective due to its precise target (Cesa et al., 2015). Aβ12-28P, a peptide corresponding to residues 12–28, reduces Aβ deposition and insoluble tau accumulation in the brain of mice (Liu et al., 2014), Treatment with Aβ12-28P reduces accumulation of Aβ in brain, co-deposition of apoE within Aβ plaques and neuritic degeneration in APOE2-TR and APOE4-TR Aβ mouse models (Pankiewicz et al., 2014). 6KApoEp is a peptide that inhibits apoE binding to the N-terminus of APP (Sawmiller et al., 2019). Notably, in 5XFAD mouse model, 6KApoEp injection reduces both Aβ and tau pathologies concomitantly with improved memory and hippocampal-dependent learning (Sawmiller et al., 2019). These findings indicate that blockers of apoE-Aβ interaction may potentially be used to reduce the therapeutic burden of Aβ and tau in the CNS.

It is possible to control the lipidation and secretion of apoE using apoE mimic peptides (Osei-Hwedieh et al., 2011). Mimic peptides correspond to the LDL receptor binding domain (130–150 residues) of the apoE protein. These peptides are designed to promote cholesterol trafficking but also alter APP trafficking and processing, and anti-inflammatory signaling within macrophages (Yao et al., 2012). ApoE mimetic peptides, such as 4F, COG112, COG133, and COG1410, increase apoE lipidation and apoE secretion, decrease Aβ levels and tau hyperphosphorylation, inhibit neurodegeneration and neuroinflammation, and improve cognitive functions (Chernick et al., 2018). A recent study in E4FAD mice showed that transient treatment with CN-105 decreases Aβ pathology and rescued memory deficits (Krishnamurthy et al., 2020). Mimic peptide CN-105 has completed Phase I clinical trial (NCT03168581 and NCT03802396) in patients with intracerebral hemorrhage (Guptill et al., 2017). This peptide is derived from the receptor binding region of apoE-α helix and decreases neuronal injury and neuroinflammation in acute brain injury mouse models (Laskowitz et al., 2017; Liu et al., 2018). However, in the context of human apoE isoforms, the effects of these peptides on Aβ deposition and other LOAD-related pathologies have not been thoroughly identified. Therefore, this strategy seem to have gather some success although it still requires to be improved, tested in specific population and to prove its efficacy on cognition to become a therapy.

Another therapeutic strategy is to disrupt apoE4 interaction domain with small molecules, modify apoE4 overall structure and therefore modulate its adverse effects in LOAD pathogenesis (Brodbeck et al., 2011). At least three regions (15–30, 116–123, and 271–279 peptides) vary between apoE isoforms, and targeting these regions with small molecules to switch apoE4 toward an apoE2 and apoE3-like structure appears to be a more direct approach to modulate apoE4 pathobiology (Frieden and Garai, 2012). Small molecules such as PH-002, GIND-25, and CB9032258 (a phthalazinone derivative) target the interaction domain and thus modify the detrimental effects of apoE4 in human neurons (Lin et al., 2018; Wang et al., 2018). This approach is currently being further developed to verify whether it has therapeutic benefits *in vivo*.

Modulating apoE Level and Its Lipidation

Instead of converting apoE4 structure, another strategy is to use anti-apoE4 antibodies to neutralize the toxic effects of apoE4 (such as Aβ plaques), like the anti-Aβ-based immunotherapies. Such antibodies can cross the BBB even though only a small amount will penetrate the brain effectively (Kim et al., 2012). Anti-apoE antibodies in mouse models were shown to effectively prevent deposition of Aβ when added with pre-existing Aβ deposits (Kim et al., 2012). In a subsequent study, administration of anti-apoE antibodies directly into the brain prevented deposition of new Aβ plaques as well as pre-existing plaques that were cleared. It is very exciting that these anti-apoE antibodies can interfere with the direct binding of apoE to Aβ deposits, as this may act synergistically with anti-Aβ immunotherapy in APOE4 patients to attain a higher degree of Aβ reduction (Liao et al., 2014). A recent study has shown that the antibody HAE-4, which preferentially binds the non-lipidated forms of apoE4/apoE3, is highly effective in reducing the deposition of Aβ in an APP/APOE4 mouse model when delivered directly into the brain by intracerebroventricular injection (Liao et al., 2018). Further testing of this approach are underway to verify the off-target effect of these antibodies that could also detrimentally interfere with the physiological functions of apoE.

Instead of using an antibody to neutralize apoE4, another strategy is to use antisense oligonucleotides (ASO) which can target APOE4 mRNA and decrease its expression (Schoch and Miller, 2017). Reduction of apoE expression by ASO significantly decreased Aβ pathology during the early stages of plaque formation in APP/PS1-21 mice homozygous for APOE4 or APOE3 (Huynh et al., 2017). ASO therapies targeting APOE receptors have also been tested in AD mouse models and treatment of AD mouse with an anti-APOER2 oligonucleotide resulted in increased synaptic function and improved learning and memory functions (Hinrich et al., 2016). There are only few ASO-mediated therapies in clinical trials for AD, the most prominent one being the anti-tau ASO in phase1/2 trial (BIIB080 from Ionis/Biogen/Washington University) (DeVos et al., 2017) but results are, to our knowledge, not yet published.

ApoE4 is hypolipidated suggesting that the pathological effects depend on how much apoE4 is lipidated. Since ABCA1 plays an important role in apoE lipidation, some groups worked on increasing ABCA1 activity to improve apoE4 lipidation. Bexarotene and 9-cis retinoic acid are drugs able to regulate ABCA1 expression (Boehm-Cagan et al., 2016; Tachibana et al., 2016). In an Aβ mouse model expressing human apoE4 and apoE3, treatment with bexarotene and 9-cis retinoic acid increased ABCA1 levels in both mice groups and reversed Aβ and hyperphosphorylated tau accumulation in hippocampal neurons, as well as cognitive deficits (Tachibana et al., 2016). Intraperitoneal injection of CS-6253 injection, an ABCA1 agonist peptide, increased apoE4 lipidation, decreased Aβ accumulation and tau hyperphosphorylation as well as reduced cognitive deficits in APOE4-TR mice (Boehm-Cagan et al., 2016). Taken together, these studies show that modulating apoE4 lipidation by increasing ABCA1 expression reduced Aβ accumulation and thereby cognitive deficits.

Targeting apoE Receptors

Considering that Aβ clearance in the brain is partially mediated by apoE receptors, especially LRP1, LDLR, and APOER2, increasing the expression of these receptors is a possible therapeutic strategy for reducing Aβ pathology. Fluvastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, decreases Aβ deposition and enhances Aβ clearance in cultured brain microvessel endothelial cells, possibly by increasing LRP1 expression (Qosa et al., 2012). Another study found that increased Aβ clearance in brain endothelial cells and isolated mice brain microvessels treated with rifampicin or caffeine (Shinohara et al., 2010). In Aβ mouse models, conditional LRP1 knockout in neurons (Kanekiyo et al., 2013), astrocytes (Liu et al., 2017), and vascular SMCs (Kanekiyo et al., 2012) resulted in increased deposition of Aβ. In APOE4-TR mice but not in the corresponding APOE3 or APOE-deficient (KO) mice, APOER2 levels in hippocampus are also reduced (Gilat-Frenkel et al., 2014). Regulating expression levels of APOER2 could therefore be considered as a good anti-AD strategy.

ApoE Gene Therapy

Adeno-associated viruses (AAVs) can mediate gene transfer directly to the CNS (Ittner et al., 2019). AAVs have become the most widely used gene therapy vectors for the CNS due of their safety, nonpathogenic nature, and capability to infect dividing and quiescent cells *in vivo*, particularly neurons (Ittner et al., 2019). An ongoing trial is scheduled to start soon to test the safety of AAV-APOE2 expression in APOE4 carriers[1]. Estimated completion date of this trial is December 2021. Patients will be injected with AAV-APOE2 in the cisterna magna and then followed for at least 2 years about their general health. This trial was made possible because previous studies in animals including APP/PS1 and Tg2576 mice showed that AAV-APOE2 reduces Aβ load after intracerebral administration of AAV-APOE4 (Hudry et al., 2013). Another group performed an intracerebral injection of AAV-APOE2 to APP/PS1/APOE4 TR mouse and reported that it reduced Aβ deposition (Zhao L. et al., 2016). More recently, a group used a non-viral delivery of plasmid encoding apoE2 (pApoE2) in the brain of mice using liposomes and showed a significant increase of apoE levels in the brain of mice with one single injection (dos Santos Rodrigues et al., 2019). Together these studies show that increasing the expression of apoE2, but not apoE4, could be efficient in reducing Aβ pathology.

While apoE-targeted therapies remain in an early phase of development, they hold great promises in the fight against LOAD. Up-regulation of apoE3 is likely to support synapses and other apoE-related functions, while down-regulation of apoE4 decreases its toxic effects and minimize Aβ deposition. The isoform-specific targeting approach would be an encouraging strategy for treating AD due to the differential functions of apoE isoforms in AD pathogenesis (Zhao et al., 2018a).

Current Challenges and Considerations in Light of apoE-Targeted Therapies

All the recent phase three clinical trials for AD failed, highlighting that this challenge always remains a priority. It is likely that AD has a spectrum of diseases without a common trigger, with slightly different initiators, accelerators and exacerbators (Suidan and Ramaswamy, 2019). The mystery and ambiguity around the cause of AD is primarily what to blame for the lack of successful current therapeutics. It is highly probably that a cocktail of drugs for different targets might be required and adapted along with the disease progression.

One limitation in the recent studies is the small sample size with regards to the APOE genotype stratification. Two important questions in the field remain to be addressed: Is there a specific patient population for which an apoE-directed therapy would benefit the most? At what stage of the disease this therapy could be most successful? People with one copy of APOE2 have half the risk of developing LOAD as opposed to those with the most common ε3/ε3 genotype. We do not know whether the risk of ε2/ε2 is substantially lower than that of ε2/ε3 but new studies specifically focused on APOE2 are likely to reinvigorate interest among drug manufacturers. While it may be advantageous to increase apoE2 levels in the brain, long-term expression of APOE2 could increase the risk of cerebral amyloid angiopathy (CAA), CAA-associated intracerebral hemorrhagic and possibly primary tauopathy (Yamazaki et al., 2019).

[1]https://clinicaltrials.gov/ct2/show/NCT03634007

Another limitation is the lack of understanding concerning the relationship between apoE lipidation status and markers of LOAD progression, such as Aβ ratio, phosphorylated tau, total tau protein, and cognitive end points. ApoE biology is highly complex and several factors must be addressed when targeting this protein in LOAD, in particular the lipidation status of apoE. It has proven difficult to develop molecules that modify the conformation of APOE4 to APOE3 or APOE2 because the variable degree of lipidation of APOE may affect its tertiary conformation. On the other hand, for APOE4 homozygote patients, approaches aiming at apoE4 reduction may be sufficient (Suidan and Ramaswamy, 2019). It is possible that such therapies slow the rate of cognitive decline in APOE4 carriers, but it is likely that the neurodegenerative process will not be completely halted (Safieh et al., 2019). Modulating the quantity of apoE or peripheral expression of apoE receptors may also increase the risk of atherosclerosis, hyperlipidaemia, and cardiovascular problems due to defective lipoprotein metabolism.

While we strive to better understand LOAD and find successful therapeutics, maintaining a healthy lifestyle (nutritional guidance, physical exercise, cognitive training, and management of metabolic and vascular risk factors) improve memory and cognitive function of older people carrying APOE4 (Solomon et al., 2018) and can assist with the onset of disease and symptoms. Indeed, not all APOE4 carriers will develop AD hence supporting that there are potential lifestyle conditions lowering the expression of the disease in this population.

CONCLUSION

Carrying APOE4 is the major genetic risk factor for developing LOAD, although not everyone carrying APOE4 develops the disease. APOE not only impacts lipid metabolism but various CNS functions in an isoform-dependent manner. In addition of controlling blood cholesterol levels, apoE proteins also regulate Aβ deposition, aggregation and clearance. However, the exact molecular mechanisms behind Aβ regulation observed in human and animal models remain to be elucidated. It is still unclear whether APOE4 allele affects LOAD pathogenesis by a gain of toxic functions or a loss of defensive functions (or a combination of both). To date, no drugs have been developed to cure/delay AD or to target apoE4 pathways, and a long list of failures already pave the road to the discovery of successful LOAD therapies. This multifactorial disease might require a multi-target treatment likely to be adapted toward the disease progression. The current apoE-targeted strategies need to consider apoE lipidation and global lipid homeostasis in the periphery and into the brain. Combined therapy of increased lipidation with simultaneously decreasing lipid-free apoE4 would be an appealing approach to prevent the progression of AD. Exploring the biology of apoE isoforms may also provide more promising approaches. Finally, improving the lifestyle and diet also need to be considered to minimize the risks associated with the APOE4 isoform. Therefore, there is a need to generate fundamental knowledge not specifically oriented on one biomarker such as Aβ but to adopt an integrative systematic approach to tackle the understanding of this complex disease.

AUTHOR CONTRIBUTIONS

MAH, MP, and BL designed the review. MAH prepared the first draft. BL and MP revised and improved the draft. All authors read and approved the final submitted version of the manuscript.

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Sialometabolism in Brain Health and Alzheimer's Disease

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Sialic acids refer to a unique family of acidic sugars with a 9-carbon backbone that are mostly found as terminal residues in glycan structures of glycoconjugates including both glycoproteins and glycolipids. The highest levels of sialic acids are expressed in the brain where they regulate neuronal sprouting and plasticity, axon myelination and myelin stability, as well as remodeling of mature neuronal connections. Moreover, sialic acids are the sole ligands for microglial Siglecs (sialic acid-binding immunoglobulin-type lectins), and sialic acid-Siglec interactions have been indicated to play a critical role in the regulation of microglial homeostasis in a healthy brain. The recent discovery of CD33, a microglial Siglec, as a novel genetic risk factor for late-onset Alzheimer's disease (AD), highlights the potential role of sialic acids in the development of microglial dysfunction and neuroinflammation in AD. Apart from microglia, sialic acids have been found to be involved in several other major changes associated with AD. Elevated levels of serum sialic acids have been reported in AD patients. Alterations in ganglioside (major sialic acid carrier) metabolism have been demonstrated as an aggravating factor in the formation of amyloid pathology in AD. Polysialic acids are linear homopolymers of sialic acids and have been implicated to be an important regulator of neurogenesis that contributes to neuronal repair and recovery from neurodegeneration such as in AD. In summary, this article reviews current understanding of neural functions of sialic acids and alterations of sialometabolism in aging and AD brains. Furthermore, we discuss the possibility of looking at sialic acids as a promising novel therapeutic target for AD intervention.

Keywords: sialic acid, sialylation, ganglioside, neural cell adhesion molecule, PSA-NCAM, Siglec, CD33, late-onset Alzheimer's disease

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SIALIC ACID STRUCTURE AND METABOLISM: AN OVERVIEW

Sugars have diverse physiological functions beyond serving as a source of energy via cellular respiration or as a component of cell wall polysaccharides and the nucleic acid backbone (Gabius and Roth, 2017). The extent of structural and compositional variability offered by sugars is unsurpassed in nature. Mono-, oligo-, or polysaccharides attached proteins or lipids are termed as glycoconjugates. Sugars of the glycoproteins and glycolipids are commonly referred to as glycans (Taylor and Drickamer, 2011), and they are mostly terminated with sialic acids, an acidic sugar unit with a 9-carbon backbone (Varki, 2008; Schnaar et al., 2014). The process of covalent addition of sialic acid to glycoconjugates is termed sialylation (Li and Ding, 2019).

Sialic acid (also known as neuraminic acid) nomenclature originated from its discovery (Siddiqui et al., 2019). The Swedish chemist Gunnar Blix first isolated it from salivary mucins and

called it "sialic acid" after the Greek word for saliva. The same substance was then independently discovered by German scientist Ernst Klenk in 1941 in brain glycolipids and named "neuraminic acid" to relate to neural tissue, the source tissue in which it was found. Structurally, sialic acid or sia refers to any of the identified 50 members of the family of neuraminic acid (Neu, 5-amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid) (Karim and Wang, 2006). Neu represents C-5 free amine form and is rarely encountered in nature. More prevalent sialic acids are N-acetyl and N-glycolyl derivatives of Neu commonly referred to as Neu5Ac and Neu5Gc, respectively (Schnaar et al., 2014). The term sialic acid is generally used to refer to Neu5Ac, the most abundant sialic acid in humans (Schauer, 2004).

Cytosolic conversion of a nucleotide sugar UDP-N-acetylglucosamine (UDP-GlcNAc) to N-acetyl-D-mannosamine (ManNAc) and subsequently to N-acetyl-D-mannosamine 6-phosphate (ManNAc-6-P) by a bifunctional enzyme GNE (UDP-GlcNAc 2-epimerase/ManNAc kinase) are the first steps in the biosynthesis of sialic acid in mammals (Schwarzkopf et al., 2002; Li and Chen, 2012). Condensation of ManNAc-6-P with phosphoenolpyruvate (PEP) by N-acetylneuraminic acid 9-phosphate synthase forms N-acetylneuraminic acid 9-phosphate (Neu5Ac-9P). Neu5Ac-9P dephosphorylation by N-acetylneuraminic acid-9-phosphate phosphatase gives rise to free sialic acids in the cytoplasm, mainly Neu5Ac. In the nucleus, sialic acids are then converted to their activated nucleotide form (CMP-Sia) by CMP-Sia synthases using cytidine triphosphate (CTP) as a donor. CMP-Sia then returns to the cytoplasm and is further translocated into the lumen of Golgi apparatus via an antiporter in exchange for CMP. Sialylation occurs when a newly synthesized glycoconjugate is terminated by sialic acid during its passage through the golgi compartment by sialyltransferase (ST) (Li and Chen, 2012). The enzyme mediates the attachment at C-2 carbon of sialic acid via one of the following linkages: α 2–3 or α 2–6 to galactose (Gal), α 2–6 to N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc), or α 2–8 when bound to another sialic acid (Harduin-Lepers et al., 2005). Twenty mammalian STs have been identified and comprise four groups: ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia based on their primary substrates, Gal, GalNAc, and Sia, as well as the linkage generated (α2–3, α 2–6, or α 2–8) (Schnaar et al., 2014).

Removal of sialic acid from a sialoglycan is mediated by sialidase also known as neuraminidase, which is found in lysosomes, on the cell surface or in cytoplasm (Varki and Schauer, 2009). The regenerated sialic acids can be further utilized in the sialylation of glycoconjugates. There are 4 different types of neuraminidases in humans, NEU1-NEU4 (Giacopuzzi et al., 2012; Monti and Miyagi, 2012). The most abundant sialidase is NEU1 expressed in the lysosome, which is responsible for removing sialic acid from oligosaccharides and glycoproteins with no action on gangliosides. NEU2 is a cytosolic sialidase involved in the removal of sialic acid from a wide variety of glycan structures. NEU3 is localized on the cell plasma membrane and specifically desialylates gangliosides. NEU4 is primarily found on intracellular membranes with a broad range of glycan specificity. Studies have identified and implied various physiological roles for these neuraminidases. NEU1 gene mutation has been linked to a congenital lysosomal storage disorder called sialidosis that affects the nervous system in humans (Seyrantepe et al., 2003). NEU1 has also been shown to be upregulated and localized to plasma membrane in activated T-cells (Nan et al., 2007) and differentiating monocytes (Liang et al., 2006). Furthermore, NEU1-NEU3 are found to be important for skeletal muscle differentiation (Fanzani et al., 2012).

SIALIC ACID PHYSIOCHEMICAL AND BIOLOGICAL PROPERTIES

The location and ubiquitous distribution of sialic acid allows it to mediate a diverse range of physiological and pathological processes (Varki, 2008). Sialic acid functions can be categorized into two types. The first is more of a general role because of its charge and hydrophilicity. This function primarily affects physiochemical properties of the underlying glycoconjugates (Schauer et al., 1995). Negatively charged sialic acid on human erythrocytes provides charge repulsion and prevents unwanted cellular interactions in the blood (Born and Palinski, 1985). An unusually high concentration of sialic acid has also been reported on the luminal surfaces of vascular endothelia (Varki, 2008). This results in a mutual repulsion between blood cells and endothelial surfaces and prevents impediment of circulation. In addition to its role in the circulatory system, the importance of sialic acid in the renal system has also been recognized. Sialic acid is expressed in foot processes of podocytes, a specialized group of cells in kidney glomerulus. Podocytes prevent entry of plasma proteins into urinary ultrafiltrate by forming a barrier consisting of filtration slits. The polyanionic nature of podocyte epithelial cells that sialic acid offers maintains the slit integrity (Dekan et al., 1991). Intraperitoneal injection of sialidase to remove sialic acid was found to cause proteinuria and renal failure in a dose dependent manner in mice (Gelberg et al., 1996). Therefore, sialic acid is essential for normal and efficient kidney filtration function. Sialic acid also lines the epithelial border of airways and is a primary component of mucins, the building blocks for mucus (Schauer et al., 1995). Sialic acid contributes to the anionic and hydrophilic properties of mucins and thus maintains the required rheological activities of mucus to lubricate airways and trap pathogens from inhaled air.

The second category of sialic acid functions is more specific and deals with cellular and molecular recognition (Schauer et al., 1995). This can either mask or facilitate biological identification, allowing sialic acid to exert dual and opposite effects. Binding and uptake of desialylated glycoproteins by hepatocytes is a well-characterized example of the masking function (Harford et al., 1984). In the absence of sialic acid, underlying glycoproteins are recognized by receptors in organs such as the liver and are rapidly cleared (Weigel and Yik, 2002). Therefore, sialic acid plays an important role in determining half-lives of circulating glycoproteins (Raju et al., 2001). For this reason, therapeutic glycoproteins are increasingly being synthesized with sialic acid capping to prolong their serum half-lives (Raju et al., 2001). In addition to increasing molecular life span, sialic acid can also promote cell survival in a pathological

condition like cancer. Sialic acid expression is altered during the period of tumor transformation and malignant progression (Zhou et al., 2020). Hypersialylation is commonly reported in different types of cancers such as leukemia, ovarian cancer, colorectal cancer, and breast cancer. Mounting evidence show that sialic acid can downregulate host immune activation to cancer cells (Rodrigues and Macauley, 2018). Sialic acid-binding immunoglobulin-type lectin (Siglec) is the major mediator through which inhibitory signals are transmitted from sialic acid to the immune system. Siglecs are type-1 membrane proteins containing an extracellular region with Ig-like domains and a homologous V-set domain responsible for sialic acid recognition. The V-set domain has an arginine residue that is critical for forming a salt bridge with negatively charged sialic acids (Pillai et al., 2012). Cytoplasmic domains of most of the Siglecs possess several immunoreceptor tyrosine-based inhibitory motifs (ITIMS) and ITIM-like signaling motifs. ITIMs are phosphorylated by Src family kinases upon ligand binding on tyrosine residues. Following phosphorylation, ITIMs recruit Src homology domain-2 phosphatase-1 (SHP-1) and SHP-2 (Crocker and Varki, 2001). SHP-1 has been shown to dephosphorylate a variety of signaling molecules in immune cells and reduce the release of inflammatory mediators such as nitric acid (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) (Zhao et al., 2006). The reduction of inflammatory response may be mediated by inhibiting MAP kinase activity (Paulson et al., 2012; Pillai et al., 2012). Thus, sialic acid-Siglec interaction causes the immune system to switch to an "off" state, resulting in immune evasion and cancer progression. Strategies for interrupting this interaction are emerging in the field of cancer therapeutics (Rodrigues and Macauley, 2018).

Sialic acid recognition can also facilitate many biological processes (Varki, 2008). It has been well established that influenza virus hemagglutinin can recognize and bind to sialic acids on the airway epithelium. Hemagglutinin is a glycoprotein expressed on the surface of influenza virus. Attachment of hemagglutinin to sialic acid causes host cell internalization of the virus. Sialic acid thus plays a critical role in the first step of influenza virus infection. In addition, sialic acid-mediated recognition is also well documented in the brain, an organ with the highest levels of sialic acid in the body (Schnaar et al., 2014). Sialic acid in the brain is most abundantly expressed in gangliosides (65%), followed by glycoproteins (32%) and less than 3% in free form (Proia, 2004). Gangliosides are amphipathic molecules made up of a ceramide lipid anchor attached to an oligosaccharide chain of variable length. They are localized on the surface of mammalian cells and are most abundant on neuronal cell surfaces. Although a wide variety of ganglioside structures have been detected, four closely related ganglioside structures, GM1, GD1a, GD1b, and GT1b, together represent 97% of gangliosides present in an adult human brain and are referred to as "complex gangliosides" (Tettamanti et al., 1973). Interactions between sialic acids on complex gangliosides and Siglecs are crucial for axon myelination (Weinhold et al., 2005). The mechanism for the interaction is discussed in detail in section "Sialic Acid and Sialylation Functions in the Brain" and **Figure 1**.

Human diseases associated with impaired sialic acid metabolism can lead to severe neurological defects (Boomkamp and Butters, 2008). A case study was reported of an infant suffering from severe mental deficits and ultimately death 3 months after birth (Schnaar et al., 2014). The postmortem showed a total deficiency of complex gangliosides in the brain with significantly reduced ganglioside synthase activity. Furthermore, the brain exhibited diminished myelin structures, emphasizing the crucial role of gangliosides in axon-myelin interactions. Salla disease is characterized by impaired lysosomal sialic acid storage that results in ataxia, hypotonia, delayed motor development, and seizures (Prolo et al., 2009). In addition, hypomyelination with thin corpus callosum is also usually reported. Infantile free sialic acid storage diseases (ISSD) is another lysosomal sialic acid storage disease with a more severe and debilitating pathology leading to failure of multiple organs and premature death (Schnaar et al., 2014). Both Salla disease and ISSD are caused by mutations in sialin, a lysosomal membrane exporter of sialic acid. Sialin is encoded by the *SLC17A5* gene, mutation in which causes lysosome to sequester sialic acid. As a result, free sialic acid fails to be transported out of the lysosomes into cytoplasm. A decrease in sialin activity is associated with an increased disease severity. Sialin-KO mouse models exhibited human disease phenotypes with poor coordination, seizures, tremors and early death (Prolo et al., 2009). A marked brain hypomyelination was also observed in these mouse models. A study aimed to understand the mechanism for this pathology in sialin-KO mice reported a significantly reduced number of postmitotic oligodendrocytes, glial cells responsible for axon myelination (Van Den Bosch, 2017). This decrease was associated with an increase in cell apoptosis during later stages of myelin formation. Thus, sialic acid deficiency causes an impaired maturation and increased apoptosis of oligodendrocytes leading to myelination defects. Sialin deficiency can also lead to alterations in ganglioside metabolism, thereby compromising axon myelination (Pitto et al., 1996).

SIALIC ACID AND SIALYLATION FUNCTIONS IN THE BRAIN

Axon Myelination

Development of the term "gangliosides" to describe sialic acid-bound glycosphingolipids in the brain underscores its abundance (Schnaar, 2004). Complex gangliosides on axons serve as receptors for myelin-associated glycoprotein (MAG), a founding member of the Siglec family (**Figure 1A**; Crocker et al., 1998). MAG is a transmembrane protein expressed only on myelinating cells–oligodendrocytes and Schwann cells in the CNS and PNS, respectively (Kelm et al., 1994). MAG is located in the innermost wrap of myelin juxtaposed to axonal surface (Schnaar and Lopez, 2009). Because of its location, MAG was first proposed to play an important role in axon-myelin interactions. Multiple studies have since confirmed this initial hypothesis (Bartsch et al., 1997; Kossmann et al., 2007). Sialic acid-MAG interaction is required for axon myelination, which promotes axon integrity, stability, and action potential conduction velocity (Kelm et al., 1994;

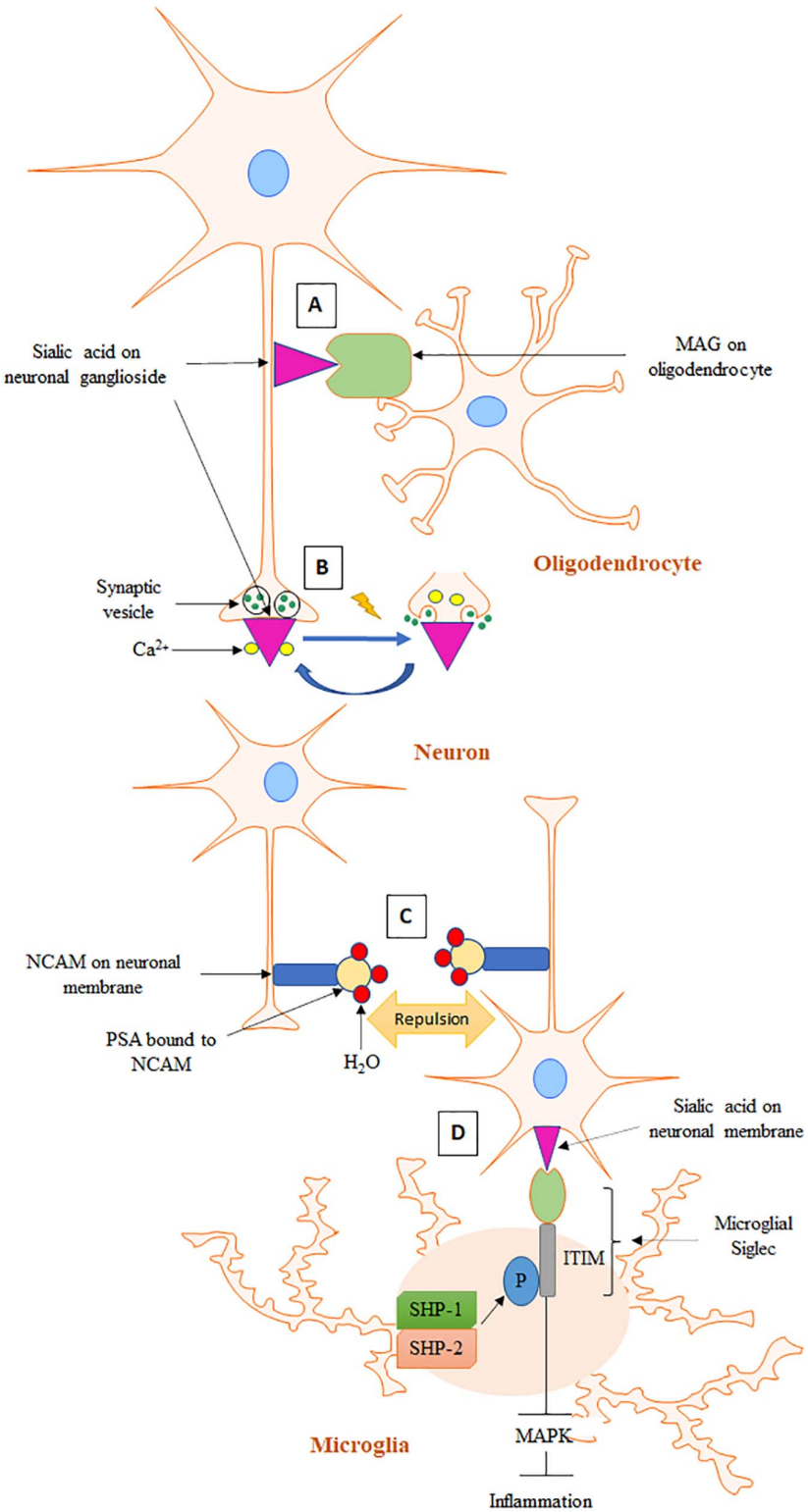

FIGURE 1 | Schematic representation of sialic acid functions in the brain. **(A)** In the process of axon myelination, MAG on oligodendrocyte interacts with sialic acid of ganglioside on axonal membrane. **(B)** At resting state, Ca^{2+} is bound to negatively charged sialic acid on synaptic membrane. In the event of an action potential, Ca^{2+} is released from sialic acid and enters presynapse for exocytosis and neurotransmitter release. **(C)** PSA on NCAM causes stereochemical repulsion that prevents homophilic contacts of NCAMs on opposing cells. **(D)** Sialic acid binds to microglial Siglec, which induces phosphorylation of ITIM present on the cytosolic domain of Siglec. Phosphorylated ITIM recruits SHP-1 and SHP-2 leading to inhibition of MAPK activity and the subsequent inflammatory cascade.

Schnaar and Lopez, 2009). *B4galnt1*-null mice are mouse models of complex gangliosides deficiency (Pan et al., 2005). These mice exhibited abnormal axon myelination, thinner axon diameters and progressive axon degeneration. MAG-KO mice exhibited the same phenotype as *B4galnt1*-null mice, underlining the importance of both gangliosides and MAG for axon myelination. All Siglecs recognize terminal sialic acid residues, however, they can show variations in sialic acid linkage specificity (Collins et al., 1999). Studies have shown that MAG preferentially binds to NeuAc 2–3 Gal 1–3 GalNAc trisaccharide, a terminal sequence shared by GD1a and GD1b gangliosides. GD1a and GD1b lacking this terminal structure did not bind to MAG, leading to a hypothesis that these two gangliosides are crucial partners of MAG in axon myelination. Despite these important leads, the detailed mechanism of MAG-sialic acid interaction and the resulting downstream signaling for axon myelination remains unknown (Lopez and Báez, 2018).

Complex gangliosides also enable MAG to shield axons from toxic insults (Schnaar et al., 2014). Vincristine is a neurotoxin that causes structural and functional damage to axons. Addition of a soluble MAG to neuronal cultures rescued them from vincristine mediated damage. However, addition of a mutant form of MAG failed to show such protective effects. The mutant MAG lacked the arginine residue shared among all Siglecs for sialic acid binding. Furthermore, wild-type MAG failed to show the same protective effect in neuronal culture of *B4galnt1*-null mice. Taken together, these observations indicate that gangliosides are a necessary component of healthy axon-MAG interactions. Moreover, it has been postulated that interaction of MAG with sialic acid regulates the expression and phosphorylation of neurofilaments, the most abundant cytoskeletal proteins in axons (Dashiell et al., 2002). Phosphorylation of neurofilaments increases negative charge and induces a side arm repositioning, allowing a larger neurofilament spacing. Increased neurofilament phosphorylation thus contributes to a reduced packing density and increased axon caliber. Axon caliber has a functional importance because diameter governs conduction velocity of myelinated nerve fibers (Hoffman et al., 1987). Higher axon caliber exhibits faster conduction velocity because of lower electrical resistance and rapid distribution of action potential (Costa et al., 2018). In summary, axon myelination increases axon caliber, an increase associated with a higher level of neurofilament phosphorylation and a lower neurofilament packing density. Thus, the axonal neurofilament packing density is regulated by a balance between kinases and phosphatases that catalyze the phosphorylation and dephosphorylation of neurofilaments, respectively (Dashiell et al., 2002). MAG is associated with an elevated level of kinases and increased neurofilament phosphorylation leading to a higher axon caliber. However, the role of MAG in phosphatase activity remains to be established (Naito-Matsui et al., 2017). The interaction between MAG and sialic acid may not always be beneficial (Schnaar et al., 2014). MAG has also been shown to inhibit axon regeneration, for which gangliosides serve as a mediator. Mutations in the sialic acid binding arginine residue on MAG decreased inhibition strength (Vinson et al., 2001). MAG binding to gangliosides activates intracellular GTPase RhoA, a small G-protein that controls actin and microtubule cytoskeletal assembly and disassembly (Hall and Lalli, 2010). GTPase RhoA activation regulates reorganization through growth cone collapse and inhibition of neurite outgrowth. The growth cone is a large, motile actin-supported structure located at growing ends of a developing or regenerating axon (Suter and Forscher, 1998). Crosslinking anti-ganglioside antibodies have been shown to mimic MAG-related axon outgrowth inhibition via RhoA pathway (Vinson et al., 2001). Furthermore, sialidase treatment has been found to increase axon outgrowth following CNS injury in animal models, validating the role of sialic acid in the inhibition (Yang et al., 2006; Mountney et al., 2010). In summary, ranging from axon myelination to protection to repressing regeneration, MAG's functions have been linked to its interaction with sialic acid in the brain.

Synaptic Development

Cell adhesion molecules are important components of synapses with well-established roles in forming and maintaining various synaptic structures during brain development (Benson et al., 2000). Neural cell adhesion molecule (NCAM) proteins can interact among themselves via homophilic contacts as well as form heterophilic contacts with other molecules such as neuron-glia cell adhesion molecule, fibroblast growth factor receptor and L1CAM to facilitate cell adhesion (Fiszbein et al., 2015). Addition of polysialic acid (PSA) is a very important posttranslational modification of NCAM that occurs during its passage though the Golgi apparatus (Schnaar et al., 2014). PSA is a large molecule made of linear homopolymers of α 2–8 linked sialic acids. PSA represents a major form of sialic acid bound to proteins in the brain with more than 95% of it bound to NCAM (Yang et al., 1994). PSA was first discovered for its role in reducing NCAM-mediated neuronal cell adhesion (Benson et al., 2000). Although the mechanism by which PSA reduces NCAM-mediated adhesion is not perfectly understood, it is postulated to involve steric hindrance. The polyanionic nature and large degree of hydration provided by PSA can significantly increase the overall size of a PSA carrier and thereby increase stereochemical repulsive forces (**Figure 1C**; Yang et al., 1994; Nakata and Troy, 2005). PSA-NCAM is the less adhesive form of NCAM and therefore an appropriate form for allowing synaptic/structural reorganization during brain development and under circumstances of synaptic plasticity during maturity (Cremer et al., 1997). PSA expression increases throughout the brain during the embryonic and perinatal stages in order to facilitate various brain development events. Axon pathfinding is a process of neural development in which neurons send out their axons to reach their appropriate targets. Axon pathfinding has cycles of fasciculation in which growing axons can travel, adhering together to form bundles (fasciculation), and separate and rearrange (defasciculation). These cycles are a prerequisite to forming new synaptic contacts and have been unequivocally demonstrated as a form of synaptic plasticity (Weledji and Assob, 2014). Endoneuraminidase-mediated selective removal of PSA from growing axons make them incapable of defasciculation generating pathfinding errors (Yin et al., 1995). Moreover, endoneuraminidase treatment impairs migrations of neurons in

the olfactory bulb (Yoshida et al., 1999). Similar effects have also been observed in NCAM-KO mice (Schnaar et al., 2014). Adult NCAM, however, has reduced PSA expression associated with mature synapse formation with the exceptions of areas characterized by a high degree of modeling of structures such as the hippocampus, hypothalamus, dentate gyrus, and olfactory bulb (Cremer et al., 1994; Seki, 2002). Therefore, PSA on NCAM is necessary for cell migration, axon outgrowth, axon defasciculation, and target recognition. Long-lasting synaptic plasticity requires a process for downregulating the expression of adhesion proteins on neuronal surfaces to promote process rearrangement (Fiszbein et al., 2015). PSA present on NCAM serves as the necessary modulator for such rearrangement. Based on these findings, PSA-NCAM has been recognized as a promoter of synaptic plasticity in the brain.

Synaptic Transmission

Sialic acids on gangliosides interact with Ca^{2+} ions via electrostatic interactions at synapse and facilitate well-regulated release of neurotransmitters (Rahmann et al., 1976; Rahmann, 1995). At resting state, Ca^{2+} ions are tightly bound by the negatively charged sialic acids at synapse and are only released when an action potential arrives at the presynaptic terminals (**Figure 1B**). Arrival of the action potential causes alterations in ionic concentrations and/or electric field strength, causing gangliosides to rearrange and thereby release Ca^{2+}. The released Ca^{2+} can then enter the nerve terminal through voltage gated Ca^{2+} channels (Mochida, 2019). Increased Ca^{2+} levels at the presynapse can then trigger release of neurotransmitters from synaptic vesicles. Following release of the neurotransmitters, Ca^{2+} ions re-attach to the gangliosides as the resting potential is restored via ganglioside-modulated Ca^{2+}-ATPase. Therefore, sialic acid on gangliosides plays an important role in mediating neurotransmission.

Microglial Homeostasis

Microglia are brain resident myeloid cells that protect the brain from pathogenic invasions and maintain brain homeostasis and plasticity (Long and Holtzman, 2019). Microglial activation can generate anti-inflammatory and immunosuppressive signals and exert protective functions. However, it can also produce pro-inflammatory mediators such as reactive oxygen species, TNF-α, and NO, leading to neuronal damage and cytotoxicity (Hansen et al., 2018). In an acute event, a balance between microglial activation and inflammation is maintained (Spangenberg and Green, 2017). However, this balance is disrupted in neurodegenerative diseases such as AD, leading to chronic neuroinflammation. Therefore, modulation of microglial activation is crucial for retaining microglial homeostasis and reducing neuroinflammation. Microglia have been found to express a number of Siglecs including CD33, Siglec-11, and Siglec-16 in humans and CD33, Siglec E, F, and G in mice (Griciuc et al., 2013). Interaction of sialic acid with Siglec allows microglia to return to an "off" state (section "Sialic Acid Physiochemical and Biological Properties" and **Figure 1D**). This is supported by a study where stimulation of Siglec-11, a CD33-related Siglec, in murine microglia by cross-linking caused a decrease in phagocytosis of apoptotic neurons (Wang and Neumann, 2010). The stimulation also prevented lipopolysaccharide (LPS)-induced transcription of proinflammatory mediators IL-1β and nitric oxide synthase-2. Moreover, Siglec-11 on microglia was found to bind to PSA-NCAM in co-cultures of microglia and neurons and protected neurons from microglia-induced toxicity. However, this protective effect was not observed when PSA was removed by endoneuraminidase treatment (Wang and Neumann, 2010). Siglec E expressed on murine microglia was also found to inhibit phagocytosis of neuronal debris and eliminated the resulting oxidative burst and proinflammatory effects (Siddiqui et al., 2019). This neuroprotective effect of Siglec E was abolished upon sialidase treatment emphasizing the role of sialic acid-Siglec interaction in microglial activity regulation. Mouse Siglec F is a paralog of human Siglec-5 that binds to sialic acids in neurons (Wielgat and Braszko, 2012). Treatment of the microglia-neuron co-culture with endoneuraminidase and α-neuraminidase prevented the binding and promoted microglial activation and phagocytosis of neurons leading to increased production of microglial proinflammatory mediators such as IL-1β and nitric oxide synthase 2 and reduced neurite and neuronal cell bodies. Furthermore, a pool of PSA-containing proteins, E-selectin ligand 1 (ESL1), and neuropilin 2 (NRP2), have been identified in the golgi compartment of stem cell-derived and primary murine microglia and THP-1 human macrophages (Werneburg et al., 2016). These proteins were found to be synthesized in response to injury-induced microglia activation in adult mice brain slices (Thiesler et al., 2020). In addition, inflammatory activation by LPS induced the release of PSA-bound ESL1 and NRP2 from the golgi to extracellular space in BV2 microglia cells. Based on these observations, it was hypothesized that the increased secretion of microglia-intrinsic pool of PSA-containing proteins could serve as a negative feedback regulator of microglia activation. Consistently, Siglec E receptor expression was also significantly increased in LPS-treated BV2 cells while CRISPR/Cas9-mediated Siglec E removal prevented the protective effect of exogenously added PSA against LPS (Thiesler et al., 2020). This is further supported by a finding where LPS-induced microglial inflammation was found to be exacerbated in ST8SIA4-deficient mice, a model for impaired production and release of PSA (Werneburg et al., 2015). Taken together, these studies indicate that sialic acid in general, whether it is localized on neuronal membrane or released from microglia itself or added exogenously, provides negative feedback inhibition of microglia activation and inflammation and protects neurons under neuroinflammatory conditions.

SIALYLATION IN BRAIN AGING

In light of the crucial role of PSA-NCAM in the formation of neuronal circuitry (section "Synaptic Development"), follow-up studies have been done to analyze whether PSA is re-expressed in mature nervous systems to accommodate structural reorganization of neuronal connections (Rønn et al., 2000). Covault and Sanes reported PSA-NCAM reappearance in denervated skeletal muscles during the process of regeneration

of lesioned motor neurons (Covault and Sanes, 1985). Consistent with this study, regenerating fibers were found to re-express PSA-NCAM in hippocampal organotypic cultures, which allowed for the formation of functional synaptic contacts between CA3-CA1 neurons across the sections (Muller et al., 1994). Furthermore, treating the sections with Endo-N, an enzyme that causes specific cleavage of PSA from NCAM, led to a less effective and functionally slower recovery from the lesion. These findings indicate that regeneration of both the central and peripheral neuronal fibers involves an increase in the levels of PSA-NCAM. However, the ability of PSA-NCAM re-expression and neuromuscular regeneration was found to be significantly reduced in aged rats as compared to young adult rats (Daniloff et al., 1986). The lower PSA-NCAM level in the aging nervous system has been linked to an age-associated decline in sialyltransferase activity, which ultimately leads to a decline in regenerative potential (Olsen et al., 1995). More recently, the effect of reduced sialylation in mice brains heterozygous for GNE, an essential enzyme for sialic acid biosynthesis (section "Sialic Acid Structure and Metabolism: An Overview") was studied (Klaus et al., 2020). It was found that the GNE ± mice had hyposialylation in various brain regions along with less synapses in the hippocampus and lower microglial arborization at 6 months of age followed by an elevated neuronal loss at 12 months. Interestingly, the neuronal loss observed in these mice was not accompanied by a pro-inflammatory signature prototypic of inflammatory neurodegenerative disorders. During development, the microglial complement receptor 3 and the complement components C3 and C1q can regulate synaptic pruning and neuronal network maturation. Cross breeding of GNE ± mice with C3-deficient mice rescued the synaptic and neuronal loss along with a significant upregulation of the microglial marker, Iba1, indicating that the complement system plays a crucial role in mediating the neuronal loss in GNE ± hyposialylated mice. It has been postulated that GNE ± mice could mimic a physiological overall decrease in sialic acid with aging. This can cause a decline in masking of glycocalyx by sialic acids and subsequent exposure of underlying aminophospholipids could increase complement binding and thereby lead to complement mediated synaptic elimination and neuronal loss by microglia with age (Klaus et al., 2020). Furthermore, microglia have been found to be hypo-motile and chronically produce pro-inflammatory cytokines in an aged brain suggesting an impaired homeostatic functioning with age (Pluvinage et al., 2019). In order to elucidate the molecular mechanism underlying this impairment and to identify an age-related genetic modifier of microglia phagocytosis, Pluvinage et al. combined CRISPR-Cas9 screening in BV2 cells with RNA sequence analysis in mice brains. The study found that the expression of CD22, a Siglec typically found on B cells, was significantly increased in aged microglia and CD22-targeted deletion promoted phagocytosis in BV2 cells. In addition, CD22 was found to interact with cytidine monophosphate N-acetylneuraminic acid synthetase (CMAS), an important enzyme for sialic acid synthesis and PTPN6, a gene that encodes for SHP-1 for sialic acid-Siglec signaling. Targeted deletion of CMAS or PTPN6 or sialidase treatment phenocopied CD22 deletion in BV2 cells indicating involvement of sialic acid in CD22-mediated inhibition of microglial phagocytosis. Although there are other Siglecs that can also modulate microglial phagocytosis such as CD33, the study found that CD22 was the sole mouse Siglec upregulated with age where aged microglia expressed three times more CD22 than young microglia. Furthermore, phagocytotic clearance of myelin debris was upregulated with an anti-CD22 antibody injection compared to IgG control antibody injected mice. To identify the transcription functions of CD22 inhibition, aged mice were implanted with osmotic pumps to deliver anti-CD22 antibody into the CSF for a month and compared with an IgG control antibody infusion. The anti-CD22 antibody infusion was found to elevate the expression of homeostatic microglia genes such as Sall1, Mef2a, Tgfbr1, Il10ra, and P2ry13 and downregulate activated and disease related microglial genes such as Ccl3, Tspo, Lgals3, H2-K1, and Tnfsf13b, thereby restoring the transcriptional hallmarks of aging associated microglia damage. The study also addressed the effects of CD22 inhibition on age-associated cognitive dysfunctions. Aged $CD22^{-/-}$ mice showed improved associative memory and spatial memory in the contextual fear conditioning and Y-maze test, respectively, as compared to aged WT mice. The same tests were also conducted in aged WT mice infused with anti-CD22 or IgG directly into the brain via an osmotic pump. The anti-CD22 infusions phenocopied the cognitive improvements exhibited by $CD22^{-/-}$ mice indicating the specificity of CD22 in negatively affecting cognitive functions in the aging brain. Hence, CD22 is a mediator of anti-phagocytotic activity of sialic acid in the microglia and inhibition of this interaction reverses the decline in microglia functions and cognitive performance in aged brain (Pluvinage et al., 2019).

Furthermore, a gradual loss of learning and memory functions is one of the earliest and most predominant consequences of brain aging (Schnaar et al., 2014); however, a definite cause of the loss is still unclear. Long-term potentiation (LTP) is a form of neural plasticity affected by aging and has been established as a cellular correlate for learning and memory (Bergado and Almaguer, 2002; Barnes, 2003). PSA-NCAM plays a role in LTP in the hippocampus and significantly declines in the dentate gyrus with increasing age (Ní Dhúill et al., 1999). Two major types of N-methyl-D-aspartate (NMDA) receptor subunit, GluN2A and GluN2B, have been demonstrated to play opposite roles in LTP (Kochlamazashvili et al., 2010, 2012). An increase in the activation of GluN2B-containing NMDA receptors and a decrease in the activation of GluN2A-containing receptors have been linked to impairment in LTP. PSA-NCAM inhibits the binding of glutamate to extra synaptic GluN2B due to steric hindrance. As a result, GluN2A activation prevails and leads to LTP induction. In the absence of PSA-NCAM, GluN2B is activated at the expense of GluN2A, which in turn activates p38MAPK (mitogen-activated protein kinase), a major player in synaptic depression. This effect, in combination with the lack of activation of GluN2A, results in impaired LTP in aged brains (Kochlamazashvili et al., 2010, 2012). Moreover, studies have looked at ganglioside composition in brains of humans ranging from 20 to 100 years of age (Chiricozzi et al., 2020). Ganglioside level progressively reduced with age, reaching about

30% in centenary compared to 20-year-old subjects. In addition to the amount, ganglioside expression pattern was found to be altered as well. An increased level of b-series gangliosides, such as GD1b, and a decreased level of a-series gangliosides, including GM1 and GD1a, were reported. Ganglioside sub-series are defined based on the number of sialic acids linked to the galactose residue of the glucosylceramide core in gangliosides: sialic acid number equals 0 is the O-series; 1, the a-series; and 2, the b-series (Kolter, 2012). GM1 and GD1a are thus the major gangliosides associated with reduced sialic acid content in aging humans. A study aimed to identify the difference in the response between young and aged rats upon brain cold injury found an increase in brain sialic acid levels in young but not aged rats in response to injury (Uslu et al., 2004). Similarly, aging was found to affect the response to thermal stimuli where aged rats exhibited a longer latency in hot plate compared to younger rats which was reversed upon treatment with GM1 (Goettl et al., 2000). Intraperitoneal injection of GM1 was found to increase the count and size of tyrosine-hydroxylase immunopositive neurons indicative of higher presynaptic dopaminergic indices in substantia nigra pars compacta of rats (Toffano et al., 1983). Furthermore, GM1 was shown to promote differentiation, protect against neuronal excitotoxicity, and facilitate response to neurotrophic factors (Chiricozzi et al., 2020). In summary, GM1 has been established as one of the major determinants of neuronal functions and its reduced biosynthesis is considered as one of the major causes for neuronal loss in aged brain.

SIALYLATION IN ALZHEIMER'S DISEASE

AD is a progressive neurodegenerative disorder that accounts for over 60% of the 46.8 million cases of dementia worldwide (Long and Holtzman, 2019). The nerve cell death causes memory loss and personality changes and disrupts one's ability to carry out daily activities (Sundaram, 2017). The deposition of extracellular amyloid beta (Aβ) plaques and intraneuronal neurofibrillary tangles in the brain are the most prominent hallmarks of AD (Nasr et al., 2018). In addition, research aimed at developing a biomarker for AD has shown elevated levels of serum sialic acid in AD (Davis et al., 2009). Studies have also demonstrated various roles of sialic acid in the development of AD pathologies.

Interaction of Gangliosides With Aβ

Elucidation of the mechanism for conversion of soluble and non-toxic α-helix-rich Aβ into aggregated and toxic β-sheet–rich structures would help to understand the early pathogenesis of AD (Ariga et al., 2011). Studies have shown that Aβ1–40 can bind to gangliosides, particularly GM1, causing changes in the secondary structure of Aβ (**Figure 2A**). The rate of fibril formation of Aβ1–40 was accelerated with an addition of ganglioside-containing vesicles as compared to vesicles without gangliosides (Choo-Smith et al., 1997). In addition to GM1, Aβ 1-40 can also bind to several other gangliosides with the following descending order of binding strength: GQ1bα > GT1aα > GQ1b > GT1b > GD3 > GD1a = GD1b > LM1 > GM1 > GM2 = GM3 > GM4 (Ariga et al., 2001). It has been hypothesized that the ganglioside-bound Aβ self-associates on the surface of cell membranes and undergoes a conformation change to form a β-sheet–ordered structure (Chi et al., 2007). This serves as an initial step in the ganglioside-mediated fibrillation of Aβ. Although the exact mechanism is not clearly understood, possible involvement of electrostatic forces between negatively charged sialic acid and Aβ has been proposed (Ariga et al., 2011). A study reported a GM1-dependent Aβ fibril binding to murine endothelioma H-END cells (Bucciantini et al., 2012). The binding was highly localized to the cell membrane and resulted in an increase in cell death in a dose-dependent manner. However, cells pretreated with neuraminidase were unable to bind to Aβ fibrils. This sialic acid-mediated Aβ fibril binding was associated with an increase in the activity of caspase-8, an apoptotic mediator. Furthermore, binding of the Aβ fibril led to an increased activation of Fas receptors. Fas receptors are death receptors expressed on cell surfaces and cause apoptosis upon ligand binding. Similar observations were seen in another study where treatment of neuroepithelial cells with either GM1 or Aβ1–40 alone did not cause any change in cell viability (Yanagisawa et al., 2010). However, a significantly reduced viability was observed in cells cultured with both GM1 and Aβ1–40. The combination was associated with an increased expression of caspase-3, another critical mediator of apoptosis. These studies suggest that sialic acid-mediated Aβ binding and fibrillation causes cytotoxicity by triggering an apoptotic cascade. Yanagisawa et al. reported that Aβ1–42, and not Aβ1–40, binds strongly to GM1 in the AD brain (Yanagisawa et al., 1995). The bound Aβ1–42, termed ganglioside-bound Aβ (GAβ), could serve as an endogenous seed for Aβ accumulation. This observation was further validated in immunoprecipitation experiments using cerebral cortices from AD patients (Yanagisawa and Ihara, 1998). The immunoprecipitates obtained with Aβ1–42 N-terminal fragment monoclonal antibody also showed reactivity for cholera toxin-B subunit, a ligand highly sensitive and specific for GM1, suggesting Aβ1–42 and GM1 binding. GAβ exhibits unique properties such as high aggregation potential and changed immunoreactivity. These properties allowed GAβ to facilitate Aβ fibril formation in the brain. The increase in Aβ aggregation has been found to correlate positively with the increase in GM1 in neuronal membranes (Kurganov et al., 2004). Furthermore, removal of GM1 from the neuronal membranes was found to reduce Aβ-mediated cytotoxicity. Remarkably, an antibody targeting GAβ has been found to suppress Aβ deposition in the brain in mouse models of AD (Yamamoto et al., 2005; Yamamoto et al., 2008). In summary, these studies indicate that gangliosides play a crucial role in Aβ fibrillation and disrupting the ganglioside–Aβ interaction could significantly decrease Aβ deposition in AD brains.

Regulation of CD33 by Sialic Acid

Late-onset Alzheimer's disease (LOAD) is the most prevalent form of AD generally affecting individuals after the age of 65 (Griciuc et al., 2013; Wu et al., 2018). CD33 has been identified as one of the highly ranked genetic risk factors for the development of LOAD, with apolipoprotein (ApoE) ε4 at the top (Harold et al., 2009; Naj et al., 2011). Two different

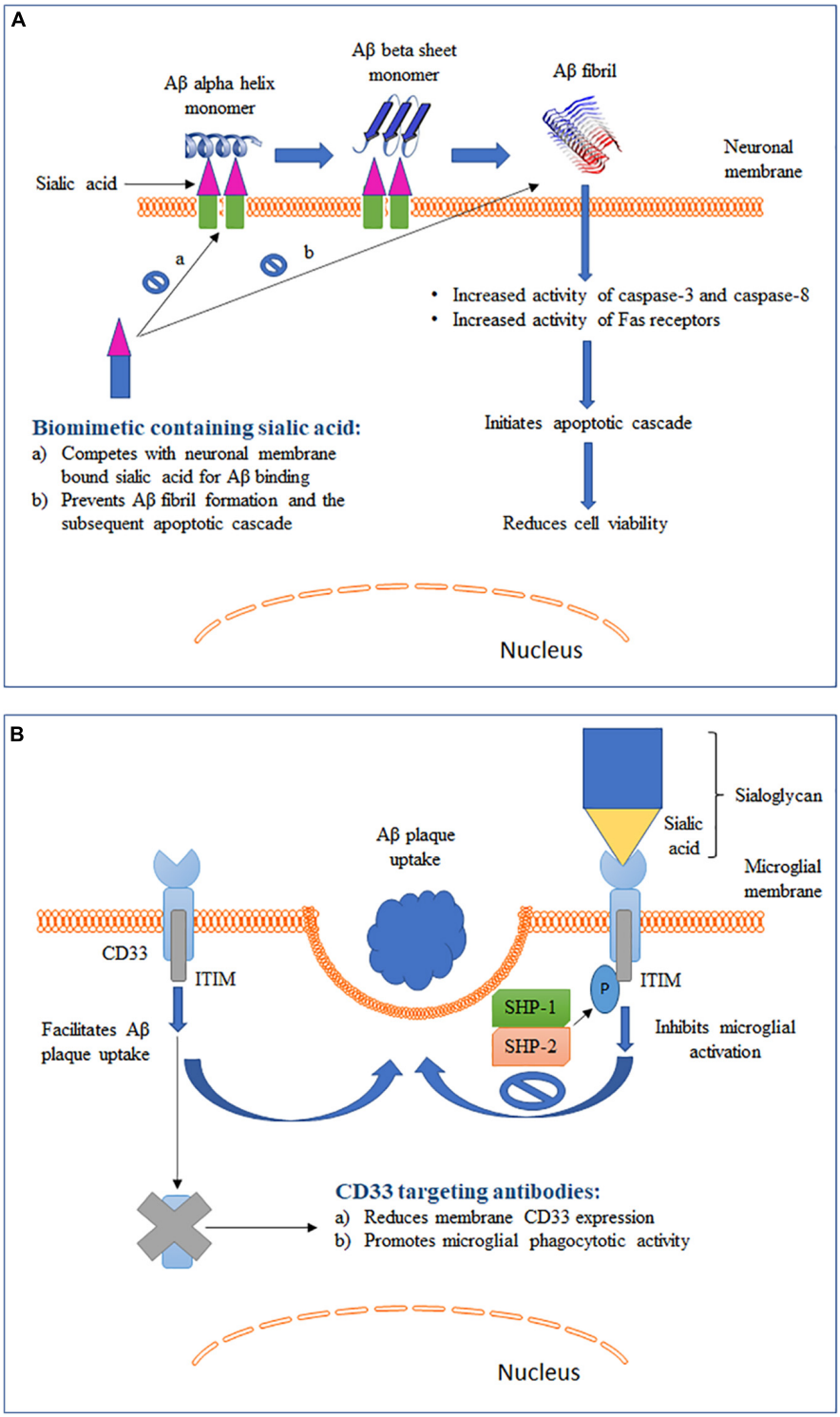

FIGURE 2 | Schematic representation of two different roles of sialic acid in AD pathology along with their therapeutic intervention strategies. **(A)** Role of sialic acid in Aβ fibrillation and the associated cytotoxicity. **(B)** Role of sialic acid in CD33-mediated microglial functions.

single nucleotide polymorphisms (SNPs) in CD33 have been reported: rs3865444C and rs3865444A. rs3865444C is a common allele (70% of the general population) and is associated with an increased expression of CD33 in the brain. Carriers of this allele had a greater likelihood of Aβ pathology and AD diagnosis (Griciuc et al., 2013; Malik et al., 2013). In contrast, rs3865444A,

which is the minor A allele of CD33, is associated with reduced CD33 expression in the brain and has been found to be protective against AD (Raj et al., 2014).

CD33, also known as Siglec-3, is an inhibitory Siglec found to be expressed by microglia, monocytes and macrophages (Zhao, 2019). Binding of sialic acid-modified glycoproteins and glycolipids activates CD33 and leads to inhibition of cellular functions (section "Sialic Acid Physiochemical and Biological Properties" and **Figure 2B**). Studies have shown that microglia-mediated phagocytosis is dependent on the level of CD33 expressed by microglia (Griciuc et al., 2013; Jiang et al., 2014). Primary microglia cells derived from CD33 KO mice showed a higher Aβ uptake as compared to wild type (WT) cells (Griciuc et al., 2013). On the other hand, BV2 microglia cells that overexpressed CD33 had a significantly impaired Aβ uptake capacity. Circulating monocytes infiltrate the brain under pathological conditions, including AD (Jiang et al., 2014). Higher CD33 expression on the surface of monocytes was found to be associated with inhibition of Aβ phagocytosis. Overall, these findings point to an important role of CD33 expression in modulating Aβ clearance in the brain. To further identity the role of sialic acid in CD33-modulated Aβ clearance, BV2 microglia cells were transfected with a mutant CD33 ($CD33^{\Delta\ V-Ig}$), which lacked the sialic acid-interacting V-type immunoglobulin-like extracellular domain (Griciuc et al., 2013). The mutant $CD33^{\Delta\ V-Ig}$ was localized to BV2 cell plasma membrane and expressed at a level comparable to that of WT CD33. Inhibition of Aβ clearance by CD33 was eliminated in cells expressing mutant $CD33^{\Delta\ V-Ig}$, indicating that sialic acid interaction is needed for CD33 to modulate microglial Aβ uptake. These findings also provide support to a recently proposed hypothesis that Aβ plaque itself can dodge microglia mediated clearance with the help of sialic acid–CD33 interaction (Jiang et al., 2014). Aβ plaque often aggregates with sialic acid-containing glycoproteins and glycolipids and this aggregated form of Aβ can directly activate CD33 signaling and downregulate microglia mediated immune activation (Jiang et al., 2014). Hence, in addition to immunosuppression of microglia by increased CD33 expression, sialic acid–CD33 interaction can also efficiently mask microglia recognition and lead to Aβ accumulation in the brain independent of altered CD33 expression levels.

PSA-NCAM in Adult Neurogenesis

Neurogenesis in an adult brain involves a complete neuronal development process, from proliferation, differentiation, and migration to synaptic integration and survival of the newly formed neurons (Gascon et al., 2010). Although the precise physiological relevance of adult neurogenesis is unclear, possible involvement in recovery from injury, learning and memory, as well as enhanced sensory discrimination of the olfactory bulb, has been demonstrated (Lledo et al., 2006). The subventricular zone (SVZ) below the ventricular walls and the subgranule layer (SGL) of the hippocampal dentate gyrus are the neurogenic niches of the adult brain (Haughey et al., 2002). These regions supply new neurons to the hippocampus and neocortex, the two major brain structures affected by AD. Aβ deposition has been observed in the SVZ and SGL and was found to disrupt proliferation and differentiation and induce apoptosis of neural progenitor cells (Kerokoski et al., 2001). Postnatal neurogenic niches are characterized by a prominent PSA-NCAM expression, which indicates that this molecule plays a role in neurogenesis (Cremer et al., 1994; Gascon et al., 2010). Hippocampal tissue analysis of AD patients showed an upregulation of PSA-NCAM with disease severity (Mikkonen et al., 1999, 2001; Jin et al., 2004). The hippocampal regions with increased PSA expression were also the regions where Aβ plaques, neurofibrillary tangles and neuronal loss occur and where neurons undergo remodeling (Jin et al., 2004). Consistent with this finding, acute injection of Aβ into rat hippocampus caused an increased PSA expression (Limón et al., 2011), which suggested an important role of PSA in neurogenesis-associated AD pathogenesis.

Studies have shown that genetic deletion of NCAM causes 30% reduction in the size of the olfactory bulb, with the overall brain size being decreased by 10% (Gascon et al., 2010). These effects were replicated by an injection of Endo-N, which indicated that the observed phenotype in NCAM-deficient animals was due to the absence of PSA-associated NCAM (Ono et al., 1994). Along with the reduced olfactory bulb size, an upregulated number of neuronal precursors were also seen in the SVZ-rostral migratory stream (RMS) in NCAM-deficient animals as compared to their WT littermates (Chazal et al., 2000). Neural stem cells present in the SVZ form neuroblasts that first migrate tangentially via RMS and then radially to the olfactory bulb (Gascon et al., 2010). It has been postulated that the accumulation of neuronal precursors in the SVZ-RMS is due to impaired migration to the olfactory bulb. Several studies have shown that PSA on NCAM are primarily responsible for the migration: (a) Migrating NCAM-positive cells have high PSA content (Kiss and Rougon, 1997; García-Verdugo et al., 1998); (b) functional inhibition of PSA on NCAM, by either enzyme-mediated removal or neutralizing antibody, with no changes to the core protein, was adequate to impair migration (Ono et al., 1994); and (c) deficiency of polysialyltransferases ST8SiaII and ST8SiaIV, the two enzymes responsible for PSA synthesis, caused abnormal tangential as well as radial migration during development (Angata et al., 2007). As discussed in section "Synaptic Development," PSA reduces NCAM-mediated adhesive interaction, which is a property that has been postulated to allow for cell motility during neurogenesis (Ono et al., 1994). In addition to migration, PSA-NCAM has also been found to be important for the survival of newly formed immature neurons in primary cortical neuronal cultures (Ono et al., 1994). It has been shown that the Endo-N-mediated removal of PSA from NCAM drastically reduced the number of newly generated neurons. Similar effects were also seen when PSA was inhibited by specific antibodies and in cultures from NCAM-deficient mice. Although the exact mechanism by which PSA regulates survival of newly generated neurons is not clearly understood, but possible involvement of brain-derived neurotrophic factor (BDNF) signaling has been considered (Vutskits et al., 2001). BDNF is a member of the neurotrophin family, a group of secreted proteins that have a profound role in neuronal differentiation, growth and survival (Gascon et al., 2007). PSA-NCAM has been shown to increase the neuronal ability to respond to BDNF by facilitating the binding of

BDNF to its receptor, tyrosine kinase receptor B (TrkB) (Muller et al., 2000). Removal of PSA from NCAM has been found to significantly reduce the level of TrkB phosphorylation and activation; however, how PSA facilitates BDNF-TrkB–induced signaling is unclear (Vutskits et al., 2001). Overall, PSA-NCAM is involved in the regulation of migration and survival of newly generated neurons and therefore is an important regulator of neurogenesis. In neurodegenerative conditions, such as AD, the ability of the brain to retain new neurons provides prospective cell replacement (Jin et al., 2004). This could result in beneficial consequences, especially in the brain regions disproportionately affected by AD, such as the hippocampus. Although there is no direct evidence in AD pathology, findings from spinal cord injury studies provide strong support (Mehanna et al., 2010). Subdural infusions of PSA glycomimetic was found to increase the number of monoaminergic axons and glutamatergic and cholinergic nerve terminals in the lumbar region of the spinal cord. Axon myelination and functional motor recovery were also found to be improved at areas in proximity to the injury site due to PSA-NCAM–mediated axonal outgrowth, branching, and defasciculation (section "Synaptic Development") (Doherty et al., 1990; Becker et al., 1996). Although an increase in PSA in AD patients could be an attempt to compensate for the neuronal damage, cell loss continues to persist (Jin et al., 2004). Several hypotheses pointing to a limited restoration capacity of neurogenesis have been postulated to explain this gap. First, the extent or rate of cell loss could be too high for quantitatively significant replacement to occur. Second, the neurons produced may not convert into mature and fully functional neurons and thus become incapable of integration into the existing brain circuit. Third, the brain microenvironment in AD could be too toxic to facilitate survival of the newly generated neurons (Rapoport et al., 2002). Nevertheless, strategies aimed to increase and support neurogenesis could have a therapeutic value in AD.

Alterations in Ganglioside and Sialic Acid Metabolism

Amyloid precursor protein (APP) processing by membrane-associated α-, β-, and γ-secretase is strongly dependent on membrane fluidity (Eckert et al., 2010; Bhattarai et al., 2020). Aβ has been shown to upregulate APP amyloidogenic processing by binding and reducing membrane fluidity, which increases its own production in HEK293 and SH-SY5Y cells, and this effect was postulated to be mediated by gangliosides due to their strong affinity to Aβ (Ariga et al., 2011). Multiple studies have reported altered ganglioside metabolism in AD and this alteration is predominantly manifested as decreased ganglioside levels in several brain regions (Brooksbank and McGovern, 1989; Crino et al., 1989; Kalanj et al., 1991). In addition, the pattern of alteration varied depending on the age of AD onset (Ariga et al., 2008). The amount of ganglioside was reduced by 58–70% compared to controls in gray matter and by 81% in frontal white matter in the brains of early-onset AD patients. However, a significant reduction was seen in the temporal cortex, frontal white matter and hippocampus in the brains of LOAD cases (Svennerholm and Gottfries, 1994). Similarly, brain analysis of individuals suffering from dementia of the Alzheimer type (DAT) also showed significantly reduced gangliosides as compared to the control group (Crino et al., 1989). The affected brain regions were the entorhinal and posterior cingulate, prefrontal cortex, nucleus basalis of Meynert, and visual cortex. Furthermore, studies have also reported alterations in ganglioside composition in AD brains where b-series gangliosides, including GD1b and GT1b, were decreased, whereas GT1a, GM1, GM2, and GD3 were increased in the frontal cortex (Brooksbank and McGovern, 1989; Kracun et al., 1992). Consistent with these findings, examination of cerebrospinal fluid of AD patients revealed that the total amount of gangliosides was comparable between the control and "probable AD" group; however, the distribution of ganglioside species was significantly different between the groups (Blennow et al., 1992; Kracun et al., 1992). The "probable AD" group had a higher level of GM1 and GD1a along with lower GT1b and GD1b levels. Although the mechanism for the observed changes in ganglioside composition in AD is unclear, these findings re-emphasize a crucial role of sialic acid-containing GM1 in forming GAβ and facilitating Aβ-membrane interactions (section "Interaction of Gangliosides With Aβ"). GM1 has also been shown to reduce the fluidity of sphingolipid-enriched membranes, which thereby favors amyloidogensis (Eckert et al., 2010). Apart from human studies, findings from mouse models of AD have been controversial. Sawamura et al. (2000) reported a lack of notable differences in the major brain gangliosides of mutant presenilin-2 mice, despite their having a remarkably elevated Aβ1–42 level. Furthermore, Bernardo et al. did not observe a significant alteration in a- or b-series gangliosides in the AD mouse model (APP with Swedish mutation and presenilin-1 with exon 9 deletion) as compared to WT (Bernardo et al., 2009). On the contrary, elevated levels of GM2 and GM3 and reduced GQ1b, GD1b, GT1a, and GD3 have been reported in the cortices of mice expressing human presenilin-1 and human APP with Swedish and London mutations (APP^{SL}) (Barrier et al., 2007). Significantly increased levels of cortical GM2 and GM3 were also reported in APP^{SL} transgenic mice co-expressing a point mutation in presenilin-1 (Ariga et al., 2008). The disparities in these findings could be attributed to the different mouse models used for the studies (Grimm et al., 2013). Overall, the findings from human brains and selective mouse models of AD point to a prominent role of alteration in ganglioside metabolism in AD pathogenesis.

Untargeted metabolomics conducted on CSF of patients with MCI also revealed a higher sialic acid metabolism as compared to normal controls (Hajjar et al., 2020). Furthermore, association studies between discriminatory metabolites and disease phenotype using Spearman's correlation analyses revealed that altered sugar metabolism was associated with elevated levels of tau and phosphorylated tau and reduced cognitive performance, cortical thickness, and hippocampal volume. This finding indicates that an impaired sugar regulation including sialic acid may occur years before AD is clinically manifested. It has been postulated that the increased sugar metabolism may in part be explained by a reduced brain glucose uptake, for instance, secondary to impaired glucose uptake transporter thereby leading to a higher CSF sugar level. Alternatively,

central insulin resistance as reported in AD could lead to higher metabolic byproducts in the CSF (Hajjar et al., 2020). Based on these findings, another follow-up study hypothesized that glycan profile could also be altered in AD brains and therefore analyzed the N-linked glycan profile in the cortex and hippocampus in control and AD brains (Gaunitz et al., 2020). Two and four glycans in the cortex and hippocampus, respectively, showed different levels in AD brains as compared to controls. Strikingly, all the glycans that differed had similar structures: complex glycan with one sialic acid, a potential or confirmed bisecting N-acetylgluocasamine and at least one fucose. This finding provides support to previous propositions that N-glycans could serve as a relevant biomarker for AD and glycosylation is impacted in AD pathology, however, how this altered glycan profile affects AD pathology remains elusive (Gaunitz et al., 2020). Impaired neural glycosylation state has also been previously reported as a potential early event in neurodegenerative process in 1995, when it was found that the ST enzyme activity was significantly reduced in postmortem brain samples of AD patients as compared to age matched controls (Maguire and Breen, 1995). It was hypothesized that the altered neural ST activity could affect APP glycosylation state and the subsequent production of Aβ. This notion has since been supported by a study conducted by Annunziata et al. that investigated the functions of NEU1 and lysosomal exocytosis in amyloidogensis in mouse brains with NEU1-targeted deletion (Annunziata et al., 2013). As discussed in section "Sialic Acid Structure and Metabolism: An Overview," NEU1 induces sialyglycoconjugates catabolism by eliminating their terminal sialic acids. NEU1 also regulates lysosomal exocytosis by limiting the sialic acid content of lysosomal-associated membrane protein-1 (LAMP1). LAMP1 facilitates the recruitment of lysosomal pool to the plasma membrane and the subsequent release of luminal content extracellularly and without NEU1, hypersialylated LAMP1 increases lysosomal recruitment and lysosomal exocytosis. $NEU1^{-/-}$ mice was found to exhibit accumulation of oversialylated APP in endolysosomes, a novel substrate of NEU1. Furthermore, the endolysosomal APP was proteolytically cleaved to produce Aβ that was released extracellularly by exacerbated lysosomal exocytosis. Remarkably, an intracranial injection of NEU1 to the 5XFAD mouse model of AD reduced the number of Aβ plaques and Aβ peptide levels. Therefore, NEU1 was identified as a risk factor for developing AD-like amyloidosis (Annunziata et al., 2013).

SIALYLATION AS A THERAPEUTIC TARGET FOR AD

Sialic Acid to Reduce Aβ Toxicity

Binding of Aβ to sialic acid on gangliosides on neuronal membranes has been shown to promote amyloidosis and induce cytotoxicity in AD (section "Interaction of Gangliosides With Aβ" and **Figure 2A**). Furthermore, inhibition of synthesis or enzymatic removal of membrane-associated sialic acid was found to be neuroprotective against Aβ-induced toxicity (Patel et al., 2006). These findings prompted the development of strategies to disrupt the cytotoxic interaction between Aβ and sialic acid and by such strategies to reduce AD pathology (Dhavale and Henry, 2012). Kakio et al. (2001) reported that the affinity of Aβ to cell membrane increases when sialic acids are clustered on the cell surface. This finding led to the hypothesis that a membrane mimic with similar sialic acid structure could compete with physiological membrane for Aβ binding (Patel et al., 2006). In support of this hypothesis, a sialic acid-conjugated dendrimer was found to bind and sequester Aβ, thus rendering it unavailable for cell interaction, which led to improved cell viability (Patel et al., 2007). Furthermore, Yin et al. (2015) synthesized a selenium nanoparticle modified with sialic acid and alternative-B6 peptide conjugation (B6-SA-SeNPs). Selenium nanoparticles are widely used because they exhibit very low toxicity and possess anti-oxidative properties. B6 peptide allowed the compound to show high blood-brain barrier permeability (Liu et al., 2013). More importantly, sialic acid on the nanoparticle inhibited Aβ fibrillation and reduced Aβ toxicity in a dose-dependent manner in two different cell models. Moreover, the nanoparticle caused the preformed Aβ fibrils to disaggregate into non-toxic oligomers, making it a promising therapeutic agent to reduce AD pathogenesis. Similarly, inhibition of Aβ-mediated toxicity in SH-SY5Y cells was obtained by sialic acid-conjugated chitosan, a polysaccharide containing D-glucosamine and N-acetyl-D-glucosamine (Dhavale, 2009). Overall, these studies show that a biomimetic compound containing sialic acid structures similar to those expressed on neuronal membranes can compete and prevent cell surface Aβ binding and associated cellular damage, making it a promising strategy to reduce the development of Aβ pathology in AD (**Figure 2A**).

Inhibition of CD33–Sialic Acid Interaction

With failure of drugs targeting Aβ plaques and neurofibrillary tangles and identification of multiple AD risk genes exclusively expressed by microglia (Bellenguez et al., 2020), alternative microglia-based therapies have become a more recent focus of AD drug discovery (Spangenberg and Green, 2017). Such therapies, while facilitating phagocytotic clearance of Aβ plaques and hyperphosphorylated tau, could also restore microglial phenotype to a healthy and functional state. The protective CD33 allele (rs3865444A) causes reduced expression of CD33 and is associated with lower AD risk (Zhao, 2019). Consequently, strategies targeting CD33 have been aimed to reduce CD33 expression and prevent CD33-mediated inhibition of microglial Aβ phagocytosis (**Figure 2B**). Gemtuzumab ozagamicin and lintuzumab are two CD33 antibodies that have been extensively tested in patients suffering from acute myeloid leukemia (AML) (Jurcic, 2012). These antibodies were found to reduce the expression of CD33 on the cell surface of monocytes. Lintuzumab also reduced the expression of CD33 by 50 and 80% in non-differentiated U937 cells and differentiated U937 cells, respectively (Zhang et al., 2016). These studies, along with the well-established safety profile in clinical trials for AML, have positioned lintuzumab as one of the top therapeutic candidates to be repurposed for AD. Antibodies targeting CD33 are able to reduce cell surface CD33 expression by inducing internalization and degradation (Malik et al., 2015). It is important to note

that both of these antibodies act specifically against the domain encoded by exon 2 that mediates sialic acid binding to CD33 (Jurcic, 2012). Therefore, they work by specifically reducing the levels of sialic acid-binding CD33 isoforms (Malik et al., 2015). Alternatively, small molecular inhibitors of sialic acid–CD33 interaction have also been considered to be a suitable CD33 intervention strategy (Wes et al., 2016). However, targeting CD33 with small molecules has posed several challenges (Zhao, 2019). First, the sialic acid-binding region in CD33 is very flat, with no binding pockets. Second, this region has high polarity and thus requires a polar small molecule inhibitor (Varki and Angata, 2006). Polar molecules are very unlikely to permeate the blood-brain barrier, thus making the inhibition of sialic acid–CD33 interaction challenging. Therefore, a non-polar allosteric site that can be approached by small molecules to efficiently interrupt sialic acid binding to CD33 might be a better target (Zhao, 2019). Recent discovery of the 3D structure of CD33 and its binding domains will allow for identification of various allosteric modulators (Miles et al., 2019). In summary, CD33-based immunotherapy and sialic acid–CD33 small molecule inhibitors represent two promising avenues in the development of microglia-based AD therapeutics.

Monocyte-Derived Activating Siglecs

The presence of Aβ plaques in the brain induces microglial activation and triggers an inflammatory response (Koenigsknecht and Landreth, 2004). Acutely activated microglia can efficiently cause Aβ phagocytosis and prevent plaque formation. However, suppression of such microglial activation inhibits Aβ phagocytosis and leads to Aβ deposition in the brain. Although inhibitory Siglecs with ITIM and ITIM-like signaling motifs are the major form of Siglecs, activating Siglecs also exist (Siddiqui et al., 2019). Activating Siglecs possess immunoreceptor tyrosine-based activation motif (ITAM), facilitate MAPK signaling, and cause immune cell activation. In other words, activating Siglecs when bound by their ligands can promote microglial phagocytic functions. Studies have shown that, when transferred to the brain, the bone marrow-derived monocytes induced efficient Aβ phagocytosis (Malm et al., 2005; Simard et al., 2006). Monocytes express several activating Siglecs, such as Siglec 14 (Fong et al., 2015) and Siglec 15 (Takamiya et al., 2013) in humans. It has been postulated that, since the majority of microglial Siglecs are inhibitory, exposure of sialylated plaques to monocytes can allow sialic acid to bind to monocyte-resident activating Siglecs (Salminen and Kaarniranta, 2009). This in turn activates the immune response and induces phagocytosis and clearance of Aβ plaques. With promising results in mouse models of AD, this approach has the potential to limit Aβ plaque formation and deposition in AD.

GM1 as a Peripheral Sequester of Aβ

One of the therapeutic approaches tested for AD treatment is peripheral administration of anti-Aβ antibodies to reduce Aβ load in the brain (Matsuoka et al., 2003). These treatments have been shown to alter Aβ dynamics and lead to Aβ efflux from the brain to the circulation, a process that is referred to as the "peripheral sink" effect (DeMattos et al., 2002). However, due to adverse patient response associated with Aβ-based immunotherapy, clinical trials were suspended (Matsuoka et al., 2003). Nonetheless, this concept has encouraged researchers to identify alternative compounds that can bind to Aβ in the periphery. As discussed in section "Interaction of Gangliosides With Aβ," sialic acid-mediated binding between GM1 and Aβ forms GAβ and promotes Aβ deposition. Prompted by this finding, Matsuoka et al. (2003) examined the effects of peripheral administration of GM1 in APP/PSEN1 mouse model of AD. The treatment induced a significant decrease in both Aβ1–40 and Aβ1–42 aggregation in the brain with a parallel increase in plasma Aβ levels. Furthermore, the GM1-bound Aβ could no longer cross the blood-brain barrier and form plaques centrally. In summary, peripheral administration of GM1 can potentially sequester Aβ in the plasma and reduce brain amyloidosis. This approach has paved the way to develop novel therapeutics that are not limited by adverse immune response or brain permeability in AD.

In addition, a monoclonal antibody, 4396C, that targets GAβ was developed (Hayashi et al., 2004). This antibody was found to inhibit the aggregation of Aβ1–40 and Aβ1–42 *in vitro* by binding to GAβ. Furthermore, peripheral administration of the antibody was found to significantly reduce Aβ accumulation in the brain of transgenic mice expressing mutant human APP gene (Yamamoto et al., 2005). Overall, these findings support the development of GM-1-based therapeutics to inhibit Aβ aggregation in AD.

CONCLUSION AND FUTURE PERSPECTIVES

Sialic acids represent a diverse family of sugars that possess a 9-carbon backbone and are mostly found as terminal residues in glycans of glycoconjugates. Sialic acids have been shown to play a variety of roles in human physiology and pathophysiology, ranging from kidney filtration to airway lubrication to cancer progression. The highest levels of sialic acids are found in the brain, where they are expressed mainly in gangliosides and PSA-NCAM. These two sialic acid carriers have been shown to regulate important brain functions, including axon myelination, synapse development and transmission, and modulation of microglial homeostasis. Age-associated loss of sialic acid in the brain has been demonstrated to negatively affect the regenerative potential of neuronal fibers, neural plasticity and microglial phagocytosis. Moreover, a decrease in ganglioside levels has been linked to increased neuronal loss in aged brains. In addition to aging, sialic acid has also been indicated to play important roles in AD pathogenesis. While gangliosides primarily affect Aβ accumulation and deposition, PSA-NCAM deficiency has been associated with reduced brain repair capabilities in AD. Furthermore, the ability of sialic acid itself to serve as a ligand for Siglec enables it to alter microglial functions and axon myelination. Although a detailed mechanism for several sialic acid-mediated functions remains to be known, the existing knowledge has provided a foundation to develop sialic acid-based therapeutics in AD. Targeting sialic acid has so far shown promising results with its ability to downregulate Aβ

plaque formation. However, AD is a heterogeneous disease with complicated pathophysiology. Over the past two decades, extensive efforts have been made to reduce the levels of Aβ and its aggregation and increase its clearance from the brain (Long and Holtzman, 2019). Unfortunately, these efforts have failed to deliver a cognitive improvement in clinical trials. More recently, the presence of sustained inflammation mediated by microglia has been recognized as a core pathology in AD, leading to the focus of microglia-based strategies in AD drug discovery (Kinney et al., 2018). Despite the importance of Siglecs in mediating microglial activity and inflammation, the downstream ITIM and ITAM signaling is not fully understood (Linnartz et al., 2010). In addition, although the role of a small number of Siglecs has been defined, overall, the Siglec family remains underexplored in the context of neuroinflammation (Siddiqui et al., 2019). Species-specific expression and lack of monoclonal antibodies against Siglecs have further contributed to this gap. Moreover, confounding findings have been presented where Siglecs can play either a preventive or a causative role in AD, warranting further studies to bring clarity to these inconsistencies. There is also a pressing need for a better understanding of the neuroimmune pathways and responsible molecular players involved. Although research on gangliosides and PSA-NCAM in the brain has been extensive, approaches to explore novel functions of the sialo residues are necessary. Furthermore, examination of the biosynthesis pathway would allow us to understand how sialic acid levels are altered in different pathologies. Studies using mouse models with targeted deletion of NCAM or gangliosides have been crucial to understand the functional role of these sialoglycans. However, it should be noted that such deletions can remove both the sialylated and the unsialylated forms of the molecule, which can result in changes not specific to the sialylated form but instead caused by entirely wiping off the molecule. Inconsistent results could also arise with the use of cancer cell lines for sialic acid studies. As discussed in section "Sialic Acid Physiochemical and Biological Properties," cancer cells have a notorious reputation for utilizing sialic acids to their benefit such that hypersialylation is used to identify cancer stage and disease prognosis. Therefore, primary cell culture or tissue preparation could be better models for studying sialic acid–Siglec interactions. In addition, there are discrepancies in studies looking at Aβ and sialic acid binding and aggregation. Some of the studies have utilized Aβ1–40 peptide, whereas others have utilized Aβ1–42 peptide. Although they differ by only two amino acids, they have significant differences in metabolism, physiological functions, toxicities, and mechanism of aggregation (Qiu et al., 2015). This could be a causal factor for the discrepancy observed when trying to identify the role of sialic acid binding in neuronal toxicity.

In addition, studies on proteins relevant to AD pathogenesis could provide an insight into the role of sialylation in AD. Clusterin (CLU), also known as apolipoprotein J, is the third most prominent genetic risk factor for LOAD (Herring et al., 2019). Translation at exon 2 forms mature secreted CLU preprotein that is targeted to endoplasmic reticulum for glycosylation. CLU bearing six N-linked glycosylation is then further transported to Golgi for glycan modification and sialylation. Studies that sought to identify a novel biomarker for chronic ethanol consumption found CLU to be highly sialylated (26–28 moles of sialic acid residues per mole of CLU) as compared to 4 moles of sialic acid in the classic biomarker, carbohydrate-deficient transferrin (Torrente et al., 2012). Chronic ethanol exposure reduces the level of liver dolichol, a crucial mediator in the first step of N-linked glycosylation (Burda and Aebi, 1999). Reduced N-linked glycosylation causes CLU to lose its sialic acid and regain it upon alcohol abstinence (Lakshman et al., 2001; Ghosh et al., 2002; Javors and Johnson, 2003; Wurst et al., 2012). Therefore, the presence of sialic acid on CLU has been established as a reliable biomarker for chronic alcohol consumption. Despite these findings, the functional role of sialic acid present on CLU is unknown. In addition, ApoE ε4 and desmoglein-2, two other known LOAD risk genes, are also sialylated (Xu et al., 1999; Sugano et al., 2008; Giri et al., 2016; Debus et al., 2019). Additional studies are needed to understand the functional role of sialic acid residues on these proteins. Filling in these gaps would contribute to a better understanding of the role of sialylation and Siglec in AD. This could also allow us to explore the clinical potential of modulating sialic acid interactions in AD intervention.

AUTHOR CONTRIBUTIONS

Both authors wrote the manuscript.

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BRIEF RESEARCH REPORT
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ABCA7 Regulates Brain Fatty Acid Metabolism During LPS-Induced Acute Inflammation

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The ATP binding cassette subfamily A member 7 (*ABCA7*) gene is one of the significant susceptibility loci for Alzheimer's disease (AD). Furthermore, ABCA7 loss of function variants resulting from premature termination codon in the gene are associated with increased risk for AD. ABCA7 belongs to the ABC transporter family, which mediates the transport of diverse metabolites across the cell membrane. ABCA7 is also involved in modulating immune responses. Because the immune system and lipid metabolism causatively engage in the pathogenesis of AD, we investigated how ABCA7 haplodeficiency modulates the metabolic profile in mouse brains during acute immune response using a metabolomics approach through LC/Q-TOF-MS. Peripheral lipopolysaccharide (LPS) stimulation substantially influenced the metabolite content in the cortex, however, the effect on metabolic profiles in *Abca7* heterozygous knockout mice (*Abca7*$^{\pm}$) was modest compared to that in the control wild-type mice. Weighted gene co-expression network analysis (WGCNA) of the metabolomics dataset identified two modules influenced by LPS administration and ABCA7 haplodeficiency, in which glycerophospholipid metabolism, linoleic acid metabolism, and α-linolenic acid metabolism were identified as major pathways. Consistent with these findings, we also found that LPS stimulation increased the brain levels of eicosapentaenoic acid, oleic acid, and palmitic acid in *Abca7*$^{\pm}$ mice, but not control mice. Together, our results indicate that ABCA7 is involved in the crosstalk between fatty acid metabolism and inflammation in the brain, and disturbances in these pathways may contribute to the risk for AD.

Keywords: Alzheimer's disease, fatty acids, inflammation, lipopolysaccharide, metabolomics, ABCA7

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INTRODUCTION

ATP-binding cassette (ABC) transporters regulate the transport of a variety of substances across membranes as the importers, exporters, and extruders (Rees et al., 2009; Thomas and Tampe, 2018), thereby mediating various critical pathways to maintain systemic homeostasis (Thomas and Tampe, 2020). In particular, the subfamilies of ABCA, ABCB, ABCC, ABCD, and ABCG are predominantly

involved in the transport of lipids including cholesterol, bile acids, phospholipids, and sphingolipids (Tarling et al., 2013; Neumann et al., 2017). While those transporters are highly expressed in the brain and mediate lipid metabolism, their substantial contributions to inflammatory processes have been also implicated in neurodegenerative diseases (Kim et al., 2008; Kooij et al., 2012). Since lipid and lipoprotein metabolism in microglia is affected in pathological conditions such as Alzheimer's disease (AD) and multiple sclerosis (Loving and Bruce, 2020), the significance of defining crosstalk between lipid metabolism and the immune system in the brain has been increasingly made apparent.

Of note, accumulating genetic evidence collected through genome-wide studies (GWASs) has confirmed *ABCA7* variants to be late-onset AD susceptibility loci, which include rs3764650, rs3752246, and rs115550680 (Hollingworth et al., 2011; Naj et al., 2011; Guerreiro et al., 2013; Jonsson et al., 2013). Subsequent whole genome sequencing studies have shown that carrying one allele of *ABCA7* with a premature termination codon mutation that results in loss of function significantly increases the risk for AD (Cuyvers et al., 2015; Steinberg et al., 2015). ABCA7 and ABCA1 are the closest homologs, sharing 54% sequence identity (Kaminski et al., 2000). ABCA7 also shares functional attributes with ABCA1, such as mediating the efflux of cellular lipids including cholesterol and phospholipids (Wang et al., 2000, 2003; Fitzgerald et al., 2002; Abe-Dohmae et al., 2004; Tomioka et al., 2017). Indeed, lipid metabolism is substantially involved in AD pathogenesis through multiple pathways including amyloid precursor protein (APP) processing, immune responses, and energy balance (Chew et al., 2020). ABCA7 has been also shown to regulate phagocytosis in immune cells (Abe-Dohmae and Yokoyama, 2020). Furthermore, our previous work found that mRNA levels of inflammatory cytokines in the brain during acute immune responses induced by lipopolysaccharide (LPS) stimulation are compromised in both homozygous and heterozygous ABCA7 knockout mice compared to control mice (Aikawa et al., 2019). Therefore, to further investigate the crosstalk between inflammation and lipid metabolism through ABCA7, this study used a metabolomics approach to explore how ABCA7 haplodeficiency influences the LPS-induced changes in metabolite profiles in mouse brains. Our findings demonstrate that ABCA7 contributes to the metabolism of brain fatty acids during LPS-induced acute inflammation.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. *Abca7* knockout mice ($Abca7^{-/-}$) (Kim et al., 2005) were crossbred with wild-type C57BL/6 inbred mice. Littermate male $Abca7^{+/+}$ (control) and *Abca7* heterozygous knockout mice ($Abca7^{\pm}$) mice were intraperitoneally injected with LPS (5 mg/kg; *Escherichia coli* O26:B6; Sigma, L2654) or vehicle at the age of 2–3 months, and used for the experiments 3.5 h after the injection.

Qualitative Large-Scale Profiling for Metabolomics

The non-targeted metabolomics and non-esterified fatty acid analysis were conducted by the Mayo Clinic Metabolomics Core. Tissue homogenates were deproteinized with six times the volume of cold acetonitrile:methanol (1:1 ratio), kept on ice with intermittent vortexing for 30 min at 4°C, then centrifuged at 18,000 × *g*. 13C6-phenylalanine (3 μl at 250 ng/μl) was added as an internal standard to each sample before deproteinization. The supernatants were divided into two aliquots and dried down for analysis on a Quadrupole Time-of-Flight Mass Spectrometer (Agilent Technologies 6550 Q-TOF) coupled with an Ultra High-Pressure Liquid Chromatograph (1290 Infinity UHPLC Agilent Technologies). Profiling data were acquired under both positive and negative electrospray ionization conditions over a mass range of 100–1,200 m/z at a resolution of 10,000–35,000 (separate runs). Metabolite separation was achieved using two columns of differing polarity, a hydrophilic interaction column (HILIC, ethylene-bridged hybrid 2.1 mm × 150 mm, 1.7 mm; Waters) and a reversed-phase C18 column (high-strength silica 2.1 mm × 150 mm, 1.8 mm; Waters). For each column, the run time was 20 min using a flow rate of 400 μl/min. A total of four runs per sample were performed to give maximum coverage of metabolites. Samples were injected in duplicates, and a quality control sample made up of a subset of samples from the study will be injected several times during a run. All raw data files obtained were converted to compound exchange file format using Masshunter DA reprocessor software (Agilent). Mass Profiler Professional (Agilent) was used for data alignment and to convert each metabolite feature (m/z × intensity × time) into a matrix of detected peaks for compound identification. Each component was assigned a putative identification (ID) through the Metlin database or a mass (m/z) value. Mass accuracy of the Q-TOF method was <5 ppm with retention time precision better than 0.2%. Fold changes as >1.2 or <−1.2 were detected with a precision of 4%. Amounts of non-esterified fatty acids were measured against a standard curve on the Thermo TSQ Quantum Ultra mass spectrometer (West Palm Beach, FL, United States) coupled with a Waters Acquity UPLC system (Milford, MA, United States).

Weighted Gene Co-expression Network Analysis (WGCNA)

We performed WGCNA of the metabolomics dataset through R package (Langfelder and Horvath, 2008). To identify metabolites that are correlated with the 4 sample groups, we used a power of 5, a minimum module size of 40 metabolites, and a minimum height for merging modules at 0.25 to build an unsigned network. To assess the correlation of modules to the mouse groups, we defined control mice administrated with LPS as 1, $Abca7^{\pm}$ mice administrated with LPS as 2,

control mice without LPS administration as 3, and *Abca7*$^{\pm}$ mice without LPS administration as 4. Metabolites with high connectivity in their respective modules were considered hub metabolites. Intramodular metabolite-metabolite connections were visualized using VisANT. Pathway analysis was conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) in Metaboanalyst 4.0.

Statistics

In the metabolomics profiling, potential metabolic signatures with a false discovery rate (FDR) < 0.05 were identified. Statistical significance for the effects of LPS administration on individual metabolite was determined by Tukey *post hoc* analysis after analysis of variance (ANOVA). For the comparison of non-esterified fatty acid levels, statistical significance for the effects of LPS administration each fatty acid was determined by unpaired student-*t* test. A *p*-value less than 0.05 was considered as statistically significant.

RESULTS

ABCA7 Haplodeficiency Impacts the Metabolomic Changes Induced by LPS Administration in Mouse Brains

Non-targeted metabolomics was performed on the cortical samples from control and *Abca7*$^{\pm}$ mice administrated with or without intraperitoneal LPS injection. While a total of 5,593 metabolites were detected, principal component analysis (PCA) demonstrated the distinct clustering among the four groups (**Figure 1A**). The PCA plot showed the separation of samples with 44.96% variance captured by PC1 and 17.31% variance captured by PC2. In addition, 253 metabolites with FDR < 0.05 were identified in the 4 groups of mice (**Supplementary Data**). Among them, we identified 193 and 74 metabolites differentially induced by LPS administration in control and *Abca7*$^{\pm}$ mice (raw *p*-value < 0.05), respectively, with an overlap of 42 metabolites (**Figure 1B** and **Table 1**). While 119 metabolites were increased by LPS administration in control mice, 74 metabolites were decreased. In *Abca7*$^{\pm}$ mice, LPS administration induced the upregulation of 35 metabolites and the downregulation of 39 metabolites. Thus, these results indicate that peripheral LPS injection modulates the metabolome in mouse brains, in which ABCA7 haplodeficiency diminishes the changes in metabolic profiles.

Identification of Metabolite Modules Associated With ABCA7 Haplodeficiency and LPS Stimulation in Mouse Brains

WGCNA for the brain metabolites identified two modules that were changed among four mouse groups (control and *Abca7*$^{\pm}$ mice with or without LPS administration): lightgreen ($p = 0.02$) and paleturquoise ($p = 0.03$) (**Figure 2A**). In the analysis, the lightgreen module contains CAY10526, C6 H3 N O4 S, C9 H N S2, C4 H3 N4 O4 S, and C6 H5 N4 S3 as the top 5 ranked hub metabolites (**Figure 2B**), although most of them are classified as unknown metabolites. On the other hand, the paleturquoise module was relatively enriched with metabolites related to lipid metabolism, which include PC[20:4(5Z,8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)], Retinol acetate/All-*trans*-retinyl acetate, PC[22:6(4Z,7Z,10Z,13Z,16Z,19Z)/ 22:5(7Z,10Z,13Z,16Z,19Z)], C28 H34 N, and C13 H30 N3 O3 S as the top 5 ranked hub metabolites (**Figure 2C**). The KEGG pathway analysis demonstrated that "Glycerophospholipid metabolism" was enriched by metabolites in the paleturquoise module (FDR = 1.02E–02). A few other enriched pathways include "Linoleic acid metabolism," "α-Linolenic acid metabolism," and "Glycosylphosphatidylinositol (GPI)-anchor biosynthesis" with nominal significance (raw $p < 0.05$). Metabolites in the lightgreen module are suggested to be associated with "Pantothenate and CoA biosynthesis" with nominal significance (raw $p < 0.05$) (**Figure 2D**). Since glycerophospholipids and GPI are major components of the cell membrane, these results suggest that

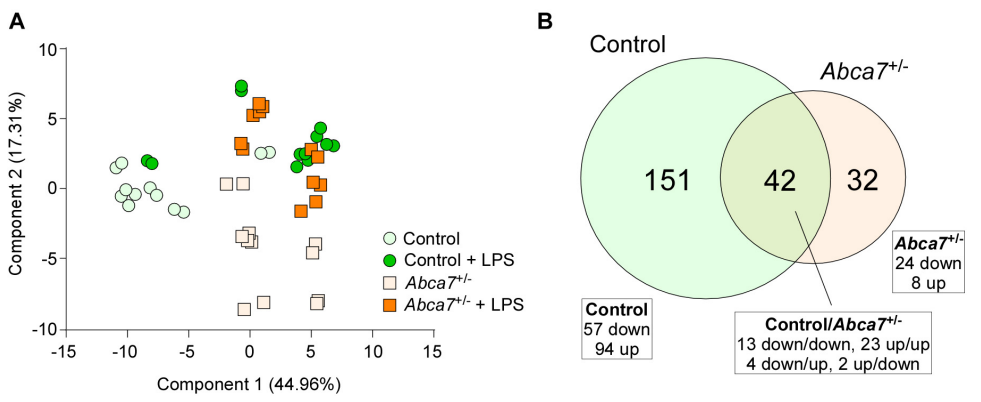

FIGURE 1 | Influence of ABCA7 haplodeficiency on LPS-induced metabolomic changes in mouse brains. Metabolomics was conducted in the cortex of control and *Abca7*$^{\pm}$ mice ($N = 6$/each) administrated with or without LPS. **(A)** Principal component analysis (PCA) of metabolites from control and *Abca7*$^{\pm}$ mice with or without LPS administration. **(B)** Venn diagram of the metabolites differently induced by LPS administration in control and/or *Abca7*$^{\pm}$ mice.

TABLE 1 | Metabolites affected by LPS administration in the mouse brains.

Compound	Control		*Abca7*$^{\pm}$		Ionization mode	MS1 composite spectrum (m/z)
	FC	*p*-value	FC	*p*-value		
C24 H9 N11 O4	−1229.987	1.74E–04	−556.729	2.24E–04	HILIC+	(516.0915, 2651.58) (517.0916, 692.18)
Quinacetol	703.429	1.30E–03	144.123	1.90E–02	HILIC+	(188.0709, 1615.56) (205.0975, 1986.9) (206.1013, 176.11)
C21 H43 N4 O4	138.129	9.79E–03	687.350	5.39E–04	HILIC+	(416.3347, 4091.88) (417.3392, 1173.53) (433.3659, 204.09)
His Ala	2.108	8.07E–04	1.985	2.00E–03	HILIC+	(249.0964, 555.13) (227.1139, 44894.72) (228.1171, 4758.09) (229.119, 466.04)
Carnosine	2.012	1.70E–04	1.753	2.79E–04	HILIC−	(225.0992, 13183.61) (226.1022, 1362.8)
PE [15:1(9Z)/22:0]	−4.176	2.91E–03	−2.244	N.S.	C18+	(760.5844, 113759.48) (761.5878, 50487.69)
POV-PC Esi + 12.015995	−3.239	1.15E–03	−1.130	N.S.	C18+	(616.3578, 50059.41) (617.3611, 15758.59) (594.3764, 170028.23) (595.3793, 52255.38) (596.3819, 11953.29) (597.3852, 1868.63) (611.4076, 297.2)
C34 H55 N12 O3	−2.491	2.13E–04	−1.067	N.S.	C18+	(702.4314, 4643.8) (703.4354, 1840.41) (680.4592, 6949.51) (681.4605, 3260.07) (682.4631, 882.31)
C32 H51 O10 Esi + 12.411007	−2.027	1.73E–03	−1.121	N.S.	C18+	(618.3381, 4348.9) (619.3419, 1693.53) (596.3551, 18953.5) (597.3587, 6100.73) (598.3611, 1816.91) (599.3599, 222.98)
C31 H49 O9	−2.004	3.21E–03	−1.100	N.S.	C18+	(588.3267, 17685.77) (589.3303, 5836.98) (590.3389, 1836.92) (1153.6628, 1279.33) (1154.6656, 840.86) (566.3452, 135593.83) (567.3482, 40496.61) (568.3516, 8898.48) (569.3551, 1504.54) (1131.6823, 1156.18) (1132.6847, 661.48)
PE [22:6(4Z,7Z,10Z,13Z, 16Z,19Z)/0:0] Esi + 10.902	−8.687	4.61E–03	1.258	N.S.	C18+	(548.2743, 95660.68) (549.2773, 29194.23) (526.2926, 565743.44) (527.2959, 167649.31) (528.2982, 29241.65)
PE [22:6(4Z,7Z, 10Z,13Z,16Z,19Z)/0:0]	−8.597	4.81E–03	1.258	N.S.	C18+	(548.2743, 95660.68) (549.2773, 29194.23) (526.2926, 565743.44) (527.2959, 167649.31) (528.2982, 29241.65)
C20 H16 N O6	−2.765	9.37E–04	1.059	N.S.	HILIC−	(365.0906, 6087.18) (366.094, 981.33)
PC [18:3(6Z,9Z,12Z)/ 20:4(8Z,11Z,14Z,17Z)]	139.405	2.04E–02	−13.074	N.S.	HILIC+	(826.5427, 424.52) (804.552, 2422.12) (805.556, 1161.39)
PE [19:0/22:6(4Z,7Z, 10Z,13Z,16Z,19Z)]	2.840	1.86E–03	−1.052	N.S.	C18+	(828.5514, 124966.67) (829.5544, 61987.04) (830.5574, 16763.68) (831.5606, 3728.36)
Barbituric acid, 5-ethyl-5-(2-hydroxyethyl)-	2.247	1.87E–03	−1.127	N.S.	HILIC+	(223.069, 7991.25) (224.0709, 1855.2) (201.0871, 12020.45) (202.091, 1167.57)
PC [17:1(9Z)/ 20:5(5Z,8Z,11Z,14Z,17Z)]	2.245	3.26E–03	−1.191	N.S.	C18+	(814.5356, 84342.96) (815.5387, 42522.77) (816.542, 11650.13) (817.5466, 2508.01) (1606.0784, 4863.3) (1607.0813, 5175.72) (1608.085, 2997.93) (1609.0872, 1133.71) (1610.0887, 307.46) (792.5542, 853601.8) (793.5578, 419070.66) (794.5605, 109935.91) (795.5629, 21364.05) (796.5681, 3906.1) (797.5746, 744.92) (1584.097, 9308.74) (1585.1006, 9151.15) (1586.1033, 4983.11) (1587.1051, 2043.59) (1588.1064, 571.09)
PC [18:3(9Z,12Z,15Z)/22:4 (7Z,10Z,13Z,16Z)]	2.149	1.91E–03	−1.043	N.S.	C18+	(854.5667, 23313.19) (855.5701, 12648.29) (856.5735, 3862.51) (857.578, 946.06) (832.5849, 101901.32) (833.5881, 51366.33) (834.5911, 15361.98) (835.5943, 3344.88) (836.6016, 790.0)
Ancitabine Esi + 3.470998	543.089	1.69E–04	6.087	N.S.	HILIC+	(226.0825, 5276.52) (227.0869, 802.4)
Anibine	286.991	2.55E–03	2.066	N.S.	HILIC+	(226.045, 2249.78) (227.0482, 103.85) (429.0144, 412.48) (204.0632, 922.09)
C24 H19 N19 O4	110.749	5.77E–03	4.714	N.S.	HILIC−	(636.1795, 2567.15) (637.1818, 682.69)
C25 H13 N6 O3	76.653	1.47E–02	2.014	N.S.	HILIC−	(444.0975, 2311.36) (445.1024, 561.37)
PC [22:6(4Z,7Z,10Z,13Z,16Z,19Z)/ 22:6(4Z,7Z,10Z,13Z,16Z,19Z)]	2.530	4.69E–04	1.157	N.S.	C18+	(900.5547, 1268.98) (901.551, 921.35) (902.5487, 329.27) (878.5694, 15051.85) (879.5725, 8619.16) (880.5738, 3269.69) (881.5759, 1019.28)
PE [22:6(4Z,7Z,10Z,13Z,16Z,19Z)/ 22:4(7Z,10Z,13Z,16Z)]	2.206	5.36E–04	1.106	N.S.	C18+	(862.5356, 3496.06) (863.5376, 1840.5) (864.5534, 666.24) (840.5538, 14033.28) (841.5571, 7698.2) (842.5743, 3260.31)

(Continued)

TABLE 1 | Continued

Compound	Control		*Abca7*$^{\pm}$		Ionization mode	MS1 composite spectrum (m/z)
	FC	*p*-value	FC	*p*-value		
PE [22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)]	2.503	6.34E–04	1.140	N.S.	C18+	(858.5046, 3908.22) (859.5076, 2355.39) (860.5147, 904.48) (861.513, 153.03) (836.5229, 15859.57) (837.5261, 8592.62) (838.5362, 4116.88) (839.5368, 919.5)
Quercetin 3,4′-dimethyl ether 7-glucoside	2.100	2.90E–03	1.303	N.S.	HILIC−	(491.1188, 9107.03) (492.1217, 2024.41)
C6 H10 N2 Esi + 3.0780017	−1.620	N.S.	−2.267	7.90E–03	C18+	(111.0915, 9416.54) (112.0944, 670.02)
C33 H39 N14 O	−2.807	N.S.	−1.585	1.09E–02	C18+	(670.3348, 81.0) (648.3504, 17316.61) (649.3547, 6659.75) (665.3842, 919.13)
C15 H39 N16	28.361	N.S.	598.849	2.73E–04	HILIC+	(444.3576, 4252.18) (445.3726, 746.13) (446.3756, 96.88)
C18 H39 N9	−1.073	N.S.	3.925	9.48E–04	HILIC+	(785.6224, 602.19) (382.3266, 5576.34) (383.3276, 2716.83) (399.3647, 8642.28) (400.3722, 2169.87) (780.6662, 3471.65) (781.6672, 1776.56) (764.6397, 5252.95)
Docusate	1.553	N.S.	3.088	1.69E–04	HILIC−	(421.2257, 12808.72) (422.2282, 3206.75) (423.2247, 955.26)

Metabolic signatures with raw p-value ≤ 0.05 and fold change (FC) > 2.0 or <−2.0 induced by LPS administration in control and/or Abca7$^{\pm}$ *mice are listed (N = 6/each). N.S., not significant.*

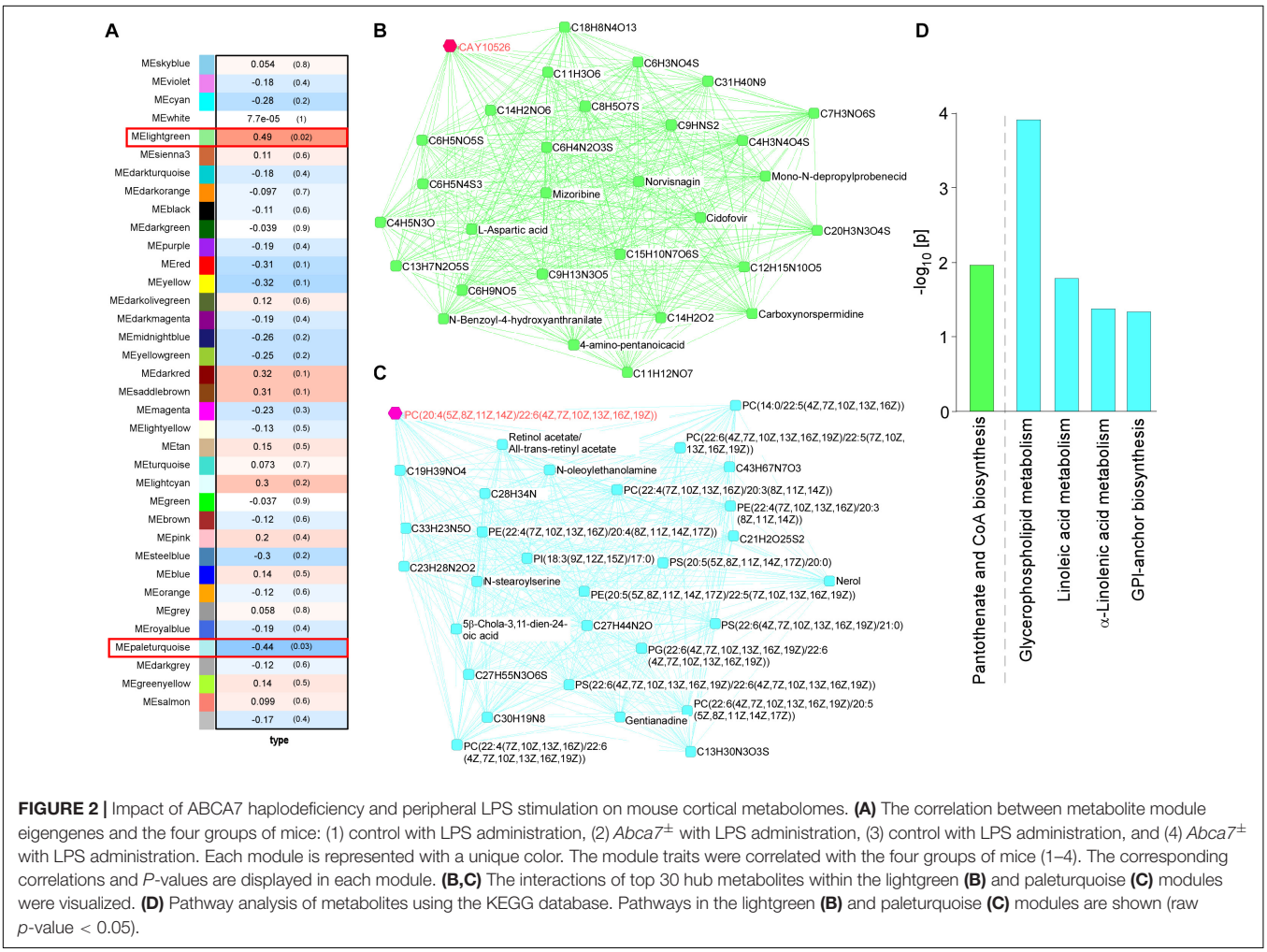

FIGURE 2 | Impact of ABCA7 haplodeficiency and peripheral LPS stimulation on mouse cortical metabolomes. **(A)** The correlation between metabolite module eigengenes and the four groups of mice: (1) control with LPS administration, (2) *Abca7*$^{\pm}$ with LPS administration, (3) control with LPS administration, and (4) *Abca7*$^{\pm}$ with LPS administration. Each module is represented with a unique color. The module traits were correlated with the four groups of mice (1–4). The corresponding correlations and *P*-values are displayed in each module. **(B,C)** The interactions of top 30 hub metabolites within the lightgreen **(B)** and paleturquoise **(C)** modules were visualized. **(D)** Pathway analysis of metabolites using the KEGG database. Pathways in the lightgreen **(B)** and paleturquoise **(C)** modules are shown (raw *p*-value < 0.05).

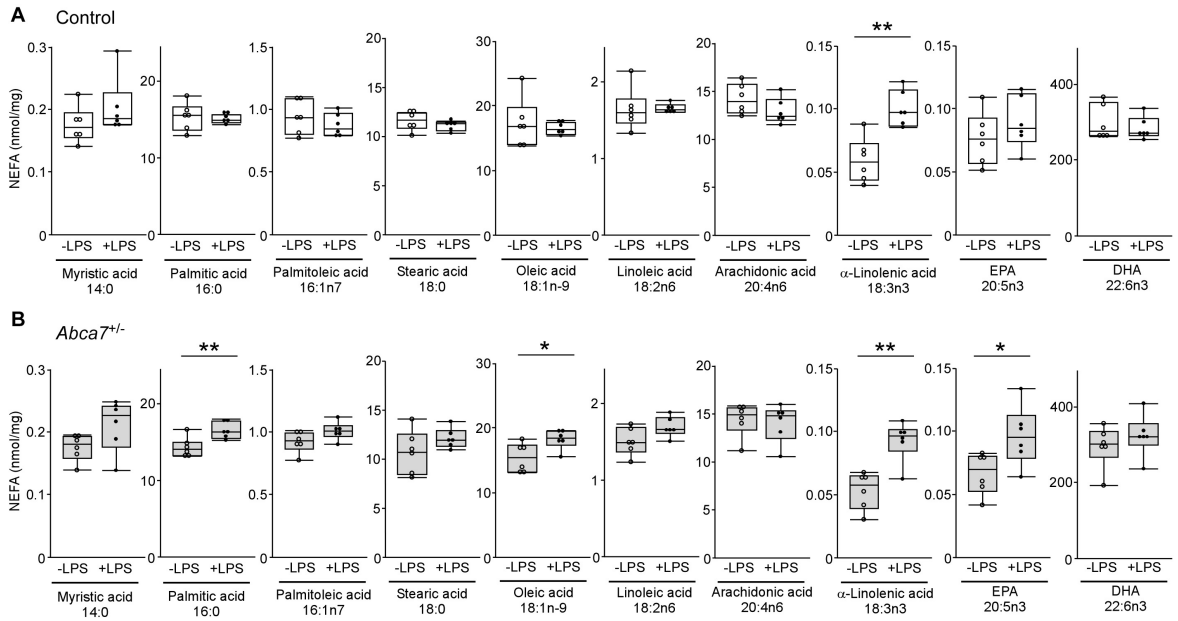

FIGURE 3 | Altered effects of LPS stimulation on brain non-esterified fatty acid levels in heterozygous ABCA7 knockout mice. The amounts of non-esterified fatty acids (NEFAs) were analyzed through mass spectrometry in the cortex of control (**A**; white) and *Abca7*$^{\pm}$ (**B**; gray) mice administrated with or without LPS. Concentration of myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidonic acid, α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were plotted. All NEFA concentrations were normalized to the protein levels. Horizontal lines, boxes, and whiskers correspond to median, interquartile range (IQR), and the furthest points within 1.5 × IQR from the box, respectively ($N = 6$/each). *$p < 0.05$, **$p < 0.01$, by student-*t* test.

ABCA7 is involved in the pathways related to polyunsaturated fatty acid conversion from membrane phospholipids during LPS-induced inflammation.

ABCA7 Haplodeficiency Modulates the Fatty Acid Metabolism in the Brain During LPS-Induced Inflammation

To further investigate how ABCA7 haplodeficiency influences the LPS-induced metabolism of polyunsaturated fatty acids in the brain, amounts of non-esterified fatty acid were measured by mass spectrometry in the cortex of control (**Figure 3A**) and *Abca7*$^{\pm}$ (**Figure 3B**) mice with or without the LPS administration. Among the 12 major analytes, 10 non-esterified fatty acids were detectable in the mouse cortical samples, which include myristic acid 14:0, palmitic acid 16:0, palmitoleic acid 16:1n7, stearic acid 18:0, oleic acid 18:1n-9, linoleic acid 18:2n6, α-linolenic acid 18:3n3, arachidonic acid 20:4n6, eicosapentaenoic acid (EPA) 20:5n3 and docosahexaenoic acid (DHA) 22:6n3, but not palmitelaidic acid and elaidic acid. The amount of α-linolenic acid increased upon LPS stimulation in the cortex of control mice, although there were no significant effects on other fatty acids (**Figure 3A**). In contrast, we found that LPS administration induced the upregulations of EPA, oleic acid and palmitic acid in addition to α-linolenic acid in the cortex of *Abca7*$^{\pm}$ mice (**Figure 3B**). These results indicate that ABCA7 plays a critical role in the brain metabolism of EPA, oleic acid and palmitic acid during LPS-induced acute inflammation.

DISCUSSION

ATP-binding cassette transporters play a critical role in the metabolism of inflammatory lipid mediators including leukotrienes and prostaglandins (Van De Ven et al., 2009). Indeed, our results revealed that ABCA7 haplodeficiency modulates metabolism of EPA, oleic acid and palmitic acid in the brain upon LPS stimulation. EPA, α-linolenic acid and DHA are major omega-3 fatty acids, which have strong biological functions in diverse pathways. Omega-3 and omega-6 polyunsaturated fatty acids are taken from various foods in the form of triglycerides and integrated into cellular lipids such as triacylglycerol, cholesterol esters and phospholipids (Kaur et al., 2014). Of note, those polyunsaturated fatty acids are abundantly localized in major immune cells including macrophages, neutrophils and lymphocytes (Gutierrez et al., 2019). Although proinflammatory eicosanoids are produced from omega-6 arachidonic acid in the acute phase of inflammation, omega-3 EPA and DHA mediates anti-inflammatory functions and are involved in the resolution phase of inflammation (Serhan, 2014; Astudillo et al., 2019). We found that brain EPA levels were upregulated in *Abca7*$^{\pm}$ mice 3.5 h after the LPS injection, whereas there was no significant increase observed in control mice. Thus, the compromised acute immune responses observed in the brain of *Abca7*$^{\pm}$ mice (Aikawa et al., 2019) may be partially due to the increase of EPA as a pro-resolving lipid mediator. There was no evident difference in the baseline levels of brain EPA between control and *Abca7*$^{\pm}$ mice, but α-linolenic acid levels were increased by LPS stimulation in both groups. Thus, ABCA7 haplodeficiency may increase

EPA production during acute inflammation by accelerating the interaction of phospholipids containing EPA with phospholipase A2 on the cell membrane, although further studies are needed for clarification.

In addition, increases of oleic acid and palmitic acid were also observed in the brains of *Abca7*$^{\pm}$ mice following LPS stimulation, but not in control mice. Given that phospholipids incorporated with oleic acid or palmitic acid are major components of the cell membrane (Lopez et al., 2014), ABCA7 may be involved in the pathways for the metabolism of those fatty acids in activated immune cells. Whereas oleic acid has likely both proinflammatory and anti-inflammatory effects depending on the cell type, it inhibits LPS-induced NF-κB transactivation in microglial cells (Oh et al., 2009). Thus, the increase of brain oleic acid is consistent with the observations of immune suppressive phenotypes of *Abca7*$^{\pm}$ mice. In contrast, palmitic acid has been shown to rather enhance the inflammatory responses and induce endoplasmic reticulum (ER) stress (Korbecki and Bajdak-Rusinek, 2019). The effect of palmitic acid on LPS-induced acute neuroinflammation may be compromised by other factors. Nonetheless, we previously found that aged *Abca7*$^{-/-}$ mice have higher brain levels of PERK and phosphorylated eIF2α compared to control mice (Sakae et al., 2016). Thus, ABCA7 deficiency may alter brain palmitic acid metabolism, which is involved in exacerbated ER stress under pathological conditions.

In summary, our results demonstrated that ABCA7 haplodeficiency substantially influences the metabolomics profile of mouse brains after LPS stimulation. Since fatty acids with anti-inflammatory effects including EPA and oleic acid were specifically increased in *Abca7*$^{\pm}$ mice upon LPS stimulation, ABCA7 haplodeficiency possibly facilitates the shift from pro-inflammatory phase to resolution phase during acute inflammation. Further studies should refine how ABCA7 deficiency influences the brain metabolism of specialized pro-resolving mediators at different time points after LPS stimulation. When activations of the immune system are excessively repeated and/or prolonged, it is possible that ABCA7 loss of function exacerbates the depletion of resources for anti-inflammatory fatty acids, thereby increasing the risk of AD due to insufficient immune resolutions. Indeed, levels of EPA and oleic acids have been noted to be lower in the inferior temporal gyri of AD cases compared to cognitively unimpaired subjects as early as at the asymptomatic stage (Snowden et al., 2017). Prospective epidemiological studies also show the strong causal association between low consumption of fish and/or low DHA intake and AD (Avallone et al., 2019). Therefore, determining the molecular mechanisms for which ABCA7 loss of function causes the dysregulation of fatty acid metabolism should provide important clues to understand the pathogenesis of AD and to develop novel therapeutic strategies for combatting the disease. Although some clinical trials involving the administration of omega-3 fatty acids (DHA + EPA) fail to show therapeutic effects in treating AD (Avallone et al., 2019), early interventions through nutritional approaches may be a potential strategy to prevent the development and progression of symptoms in AD patients, particularly those carrying *ABCA7* loss of function variants.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Mayo Clinic IACUC.

AUTHOR CONTRIBUTIONS

TA and TK designed the research studies. TA, YR, and M-LH conducted experiments and acquired data. TA, YR, YA, AA, MF, GB, and TK analyzed the data. TA and TK wrote the first draft. All authors contributed to writing the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.647974/full#supplementary-material

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frontiers in Neuroscience

REVIEW
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Glycolytic Metabolism, Brain Resilience, and Alzheimer's Disease

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Alzheimer's disease (AD) is the most common form of age-related dementia. Despite decades of research, the etiology and pathogenesis of AD are not well understood. Brain glucose hypometabolism has long been recognized as a prominent anomaly that occurs in the preclinical stage of AD. Recent studies suggest that glycolytic metabolism, the cytoplasmic pathway of the breakdown of glucose, may play a critical role in the development of AD. Glycolysis is essential for a variety of neural activities in the brain, including energy production, synaptic transmission, and redox homeostasis. Decreased glycolytic flux has been shown to correlate with the severity of amyloid and tau pathology in both preclinical and clinical AD patients. Moreover, increased glucose accumulation found in the brains of AD patients supports the hypothesis that glycolytic deficit may be a contributor to the development of this phenotype. Brain hyperglycemia also provides a plausible explanation for the well-documented link between AD and diabetes. Humans possess three primary variants of the apolipoprotein E (ApoE) gene – *ApoE**ϵ*2, ApoE**ϵ*3, and ApoE**ϵ*4* – that confer differential susceptibility to AD. Recent findings indicate that neuronal glycolysis is significantly affected by human ApoE isoforms and glycolytic robustness may serve as a major mechanism that renders an ApoE2-bearing brain more resistant against the neurodegenerative risks for AD. In addition to AD, glycolytic dysfunction has been observed in other neurodegenerative diseases, including Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, strengthening the concept of glycolytic dysfunction as a common pathway leading to neurodegeneration. Taken together, these advances highlight a promising translational opportunity that involves targeting glycolysis to bolster brain metabolic resilience and by such to alter the course of brain aging or disease development to prevent or reduce the risks for not only AD but also other neurodegenerative diseases.

Keywords: Alzheimer's disease, glycolysis, bioenergetics, biosynthesis, apolipoprotein E, diabetes, brain resilience

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ALZHEIMER'S AS A METABOLIC DISEASE

The human brain contains an average of more than two hundred billion cells, one quadrillion connections, 100 km of nerve fibers, and 600 km of blood vessels (Steiner, 2019). Therefore, it is not surprising that a brain demands an outstanding amount of energy and building blocks to maintain its highly dynamic homeostasis. An adult brain makes up only 2% of total body weight,

but it uses about 20% of total body energy (Costantini et al., 2008). The brain's significant energy-consuming quality is largely attributed to the extremely active and complex processes involved in neuronal transmission. Failures to maintain basal energy levels, such as those under hypoglycemia or hypoxia, can potentially induce synaptic loss and cognitive impairment within a few minutes, thus rendering the brain exceedingly vulnerable to energy deficit-mediated damage (Fleck et al., 1993; Takata and Okada, 1995; Yamane et al., 2000).

Accumulating evidence indicates that in the development of AD, pathophysiological changes can occur up to 20–30 years before clinical symptoms manifest. Metabolic dysfunction has been recognized as a prominent anomaly in the brain during this preclinical stage (Small et al., 1995; de Leon et al., 2001; Mosconi et al., 2006, 2008b; Langbaum et al., 2009). The cerebral metabolic rate of glucose (CMRglc) is a critical indicator of neuronal and synaptic activity (Malonek and Grinvald, 1996; Attwell and Iadecola, 2002; Rocher et al., 2003; Khatri and Man, 2013). By means of positron emission tomography (PET) imaging and using 2-[18F]fluoro-2-deoxy-D-glucose (FDG) as the tracer, studies have shown that nearly all clinical AD symptoms are accompanied by significant reduction of CMRglc, and the extent and topography are correlated closely with symptom severity (Mosconi, 2005). Among individuals with mild cognitive impairment (MCI), the prodrome of AD, significantly decreased glucose metabolism has also been observed in AD-vulnerable brain regions, such as hippocampus, posterior cingulate cortex, and temporal cortex (Mosconi et al., 2008a,b). This condition reportedly predicts the progression from MCI to AD with greater than 80% accuracy. Additionally, individuals carrying an ApoE4 allele without dementia were found to exhibit a mild but definite reduction in CMRglc comparable to the typical AD pattern when compared to non-carriers (Small et al., 1995; Reiman et al., 1996, 2001, 2004; Perry et al., 2002; Mosconi et al., 2008a). This cerebral metabolic deficit substantially predisposes neurons to energy perturbation and functional crisis (Sims et al., 1980; Lying-Tunell et al., 1981; Hoyer et al., 1988; Filosto et al., 2007; Esteves et al., 2008; Vlassenko and Raichle, 2015; An et al., 2018; Vlassenko et al., 2018).

A great deal of research has sought to understand the biological basis responsible for the impaired glucose metabolism observed in the brains of AD patients and high-risk individuals. Glycolytic deficit has long been suggested as a prominent metabolic abnormality in the early stage of AD, evidenced by a much greater decline in cerebral glucose utilization when compared to the decrease in cerebral blood flow and the cerebral metabolic rate of oxygen (Hoyer et al., 1991; Fukuyama et al., 1994). These early findings have been solidified by recent studies, demonstrating that reduced glycolytic flux correlates closely with the severity of the disease, with more plaques and tangles found in the brains of AD patients (An et al., 2018). Moreover, studies have shown that a number of glycolytic elements, including glycolytic enzymes, glycolytic metabolites, and amino acids produced in the glycolytic pathway, are altered in AD. This suggests that glycolytic impairment associated with AD may cause both bioenergetic and biosynthetic disturbances that ultimately disrupt the metabolic and synaptic homeostasis, leading to abnormal protein deposition and cognitive decline (Sims et al., 1987; Fisher et al., 1991; Bigl et al., 1999; Hashimoto et al., 2004; Katsouri et al., 2011; Madeira et al., 2015; Wu et al., 2018). In this review, we summarize the current understanding of the function of glycolysis in the maintenance of brain health, and the role of glycolytic dysfunction as a possible cause of neurodegeneration in AD and other neurodegenerative conditions. We further discuss the research evidence that supports the emerging opportunity of targeting glycolysis as a potential therapeutic strategy aimed to bolster brain metabolic resilience and by such to alter the course of brain aging or disease development in the fight against the neurodegenerative risks for AD.

GLYCOLYSIS OVERVIEW

Bioenergetic Function of Glycolysis

Many tissues can utilize fat or protein as a source of energy. Others, however, such as the brain, depend primarily on glucose to maintain normal functions (Maughan, 2009). Glycolysis is the cytosolic pathway in which one molecule of glucose is broken down into two molecules of pyruvate, along with a net production of two molecules of ATP and NADH (nicotinamide adenine dinucleotide). Pyruvates are then fully metabolized in mitochondrial respiration. The first five reactions of glycolysis constitute the preparatory or investment phase, where ATP is consumed, while the other five reactions form the payoff phase where ATP is produced (**Figure 1a**). Three key rate-limiting enzymes are utilized in glycolysis: hexokinase, phosphofructokinase-1 (PFK-1), and pyruvate kinase (PK). Each of them serves as a critical and tightly regulated site. In the initial step of glycolysis, hexokinase catalyzes the phosphorylation of glucose by ATP to produce glucose-6-phosphate (G-6-P), in which an ATP molecule is consumed. Hexokinase is feedback-inhibited by G-6-P as well as when the function of PFK-1 is suppressed, thus ensuring less hydrolysis of ATP and glycolytic intermediates. Notably, hexokinase possesses a low Km (high affinity, strong binding) for glucose which enables it to function actively even if the concentration of glucose is very low (Niemeyer et al., 1975; Massa et al., 2011).

Among the three key glycolytic enzymes, PFK-1 is regarded as the crucial point of regulation. PFK-1 converts fructose 6-phosphate to fructose 1,6-bisphosphate with consumption of another ATP molecule. This pathway is considered the commitment step to glycolysis and is allosterically regulated by the energy state of the cell. PFK-1 is inhibited by high levels of ATP and citrate, but activated when the ratio of ATP/AMP is low (Berg et al., 2007). When the body experiences acidosis (low pH), PFK-1 will also be suppressed to avoid excessive lactate production. In addition, fructose 2,6-bisphosphate acts as a potential activator for PFK-1 by enhancing its affinity for fructose 6-phosphate and inhibiting fructose-1,6-bisphosphatase, thus diminishing the inhibitory effect of ATP (Lunt and Vander Heiden, 2011).

The third, irreversible reaction is catalyzed by PK, which is also the final step of glycolysis, converting phosphoenolpyruvate

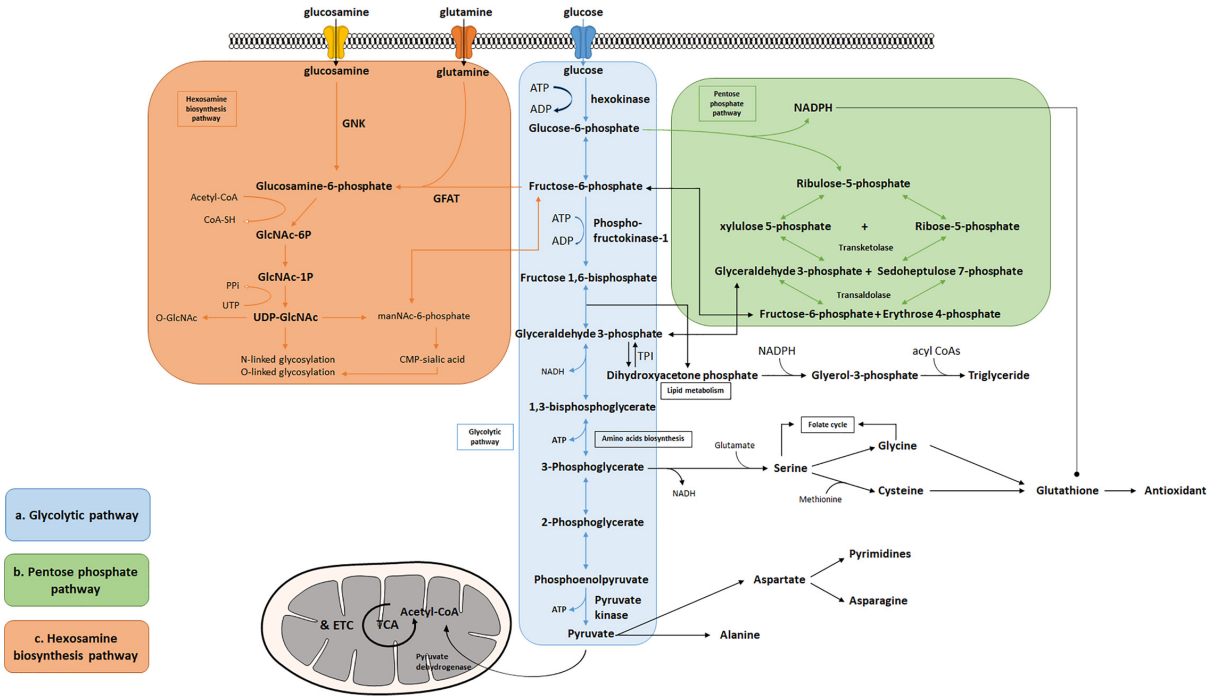

FIGURE 1 | Bioenergetic and biosynthetic pathways of glycolysis. **(a)** Glycolysis breaks down glucose into two molecules of pyruvate accompanied by a net production of two molecules of ATP and 2 NADH + 2 H^+. Metabolic intermediates derived from glycolysis serve as precursors in the biosynthesis of non-essential amino acids including serine, glycine, cysteine, and alanine. **(b)** The pentose phosphate pathway consists of two phases: oxidative and non-oxidative phases. Oxidation of G-6-P to pentose phosphates leads to the production of NADPH and five-carbon sugars, which are critical for reductive biosynthesis, antioxidant defense, and RNA/DNA synthesis. PPP is interconnected by transketolase and transaldolase with glycolysis. **(c)** Fructose-6-phosphate produced from G-6-P serves as the starting point that diverts 2–3% glucose to the hexosamine biosynthesis pathway, a branch derived from glycolysis, which generates the "sensing molecule" UDP-*N*-Acetylglucosamine (UDP-GlcNAc), the substrate of the enzymes involved in protein *N*- and *O*-glycosylation. The rate-limiting enzyme of the pathway is glutamine: fructose-6-phosphate amidotransferase (GFAT).

to pyruvate and yielding two molecules of ATP. This ATP-forming reaction occurs by substrate-level phosphorylation and is highly regulated by the energy state of the cell. When the cell undergoes a high rate of glycolysis, fructose 1,6-bisphosphate can activate PK to keep up with the rate of glycolytic flux. Increased concentrations of downstream, energy-yielding intermediates, such as ATP or citrate, will inhibit PK and decelerate glycolysis (Berg et al., 2007). However, when cells are under energy deficit conditions, such as low blood glucose levels, phosphorylation of PK reduces its enzymatic activity, thereby limiting the consumption of glucose by the liver to meet the urgent energy demand of vital organs such as the brain (Pilkis et al., 1982).

Reducing equivalents, in the form of NADH, produced by glycolysis, are used to facilitate ATP production in the mitochondria by donating electrons to the electron transport chain. Cells normally rely on oxidative phosphorylation as the main source of energy (Zheng, 2012), however, the rate of ATP generation in the glycolytic pathway has been observed at a much higher level than oxidative phosphorylation in a number of different types of cells and tissues, such as muscle cells, neurons, astrocytes, microglia, endothelia cells, activated lymphocytes, or tumor cells (Pfeiffer et al., 2001; Pellerin et al., 2007; Pearce et al., 2013; Schmitz et al., 2013; Volobueva et al., 2013; Ghesquiere et al., 2014). This phenomenon is essential to organisms when rapid ATP production is needed, such as muscle cells in heavy exercise or acute initiation of neuronal activities.

Biosynthetic Function of Glycolysis

Producing energy is not the sole purpose of glycolysis. A wide variety of metabolic intermediates generated in the glycolytic pathway flow into a range of biosynthetic processes, including gluconeogenesis, lipid metabolism, the pentose phosphate pathway (PPP) and the TCA cycle. The Warburg effect has long been observed in rapidly proliferating cells and firstly described in cancer, where growing cells use glycolysis as the predominant pathway for ATP production even when oxygen is abundant (Warburg, 1956). Studies have demonstrated that induced chemotherapy-resistant cell lines, such as human LoVo colon carcinoma cells and HeLa, have elevated aerobic glycolysis (AG), indicating a mechanistic link between resistance and glycolysis (Ganapathy-Kanniappan and Geschwind, 2013). The Warburg effect may also play a role in anti-apoptotic effects in Aβ resistant neural cell lines via hypoxia inducible factor 1, indicating its protective role in AD brains (Newington et al., 2011). Thus, the significance of glycolysis extends beyond rapid energy generation both to facilitate nutrient assembly into essential precursors of biosynthesis and to promote cellular homeostasis.

Several amino acids are directly derived from glycolytic intermediates and play important roles in maintaining normal cellular function (**Figure 1a**). The carbon backbone that originates from 3-phosphoglycerate serves as the structural unit in the biosynthesis of serine, glycine, and cysteine, whereas pyruvic acid functions as the carbon provider for biosynthesis of alanine. Serine serves as one of the major sources for generation of nicotinamide adenine dinucleotide phosphate (NADPH) in the cell via the tetrahydrofolate (THF) cycle, supports cell proliferation via the regulation of pyruvate kinase, and functions as a head group when supplied directly to the biosynthesis of phosphatidylserine, a component of cell membrane in the brain (Lunt and Vander Heiden, 2011; Ye et al., 2012; Tedeschi et al., 2013; Fan et al., 2014; Lewis et al., 2014; Maddocks et al., 2014; Ducker et al., 2016; Gao et al., 2018). Moreover, the production of D-serine, a co-agonist of NMDA glutamate receptors, has been shown to be negatively controlled by glycolytic flux in astrocytes, via the interaction of serine racemase (an enzyme that converts L-serine to D-serine) and glyceraldehyde 3-phosphate, suggesting that glycolysis may play an important role in modulating excitatory neurotransmission in the brain (Suzuki et al., 2015; Guercio and Panizzutti, 2018). Le Douce et al. (2020) recently reported that glycolysis-derived L-serine production in astrocytes is impaired in AD. As the precursor of D-serine, reduced L-serine can cause D-serine deficiency leading to impaired NMDA receptor activity and synaptic and cognitive deficits (Le Douce et al., 2020). Alanine and aspartate play key roles in body function as well. Alanine transaminase (ALT) catalyzes the reversible reaction of glutamate and pyruvate into alanine and α-ketoglutarate, while aspartate transaminase catalyzes glutamate and oxaloacetate into aspartate and α-ketoglutarate. The subsequent alanine is then shuttled into the liver and enters the urea cycle. Aspartate can give rise to asparagine and the first committed step of pyrimidine biosynthesis by providing four atoms of the ring. The transamination reaction can also produce intermediatepyruvate and oxaloacetate, for gluconeogenesis thereby exerting its important role in both facilitating nutrient cycling and maintaining energy homeostasis.

A branch of glycolysis that accounts for 2–3% of total glucose metabolism is the hexosamine biosynthesis pathway (HBP) (**Figure 1c**) (Marshall et al., 1991). Fructose 6-phosphate, together with glutamine, is diverted to generate UDP-*N*-acetylglucosamine (UDP-GlcNAc). This end product of HBP is then used to form glycosaminoglycans, proteoglycans, and glycolipids (Schleicher and Weigert, 2000; Milewski et al., 2006; Yang and Qian, 2017). UDP-GlcNAc also functions as the substrate for O-linked *N*-acetylglucosamine transferases (OGTs) in various species involved in protein *N*- and *O*-glycosylation (Wells et al., 2001; Wells and Hart, 2003; McLarty et al., 2013; Hwang and Rhim, 2018). Glutamine: fructose-6-phosphate amidotransferase (GFAT) catalyzes the rate-limiting step in HBP conversion of fructose-6-phospate and glutamine, thus serving as an important regulatory point (Marshall et al., 1991). The regulation of GFAT, however, is not fully understood. A previous study suggests that glucose-6-phosphate dehydrogenase (G6PD) *O*-GlcNAcylation promotes the pentose phosphate pathway as well as cell proliferation and survival through an increased binding affinity of $NADP^+$ to G6PD (Rao et al., 2015). *O*-GlcNAcylation is also reported to play a key role in regulating pyruvate kinase expression and activity leading to accumulation of upstream glycolytic metabolites (Chaiyawat et al., 2015; Wang et al., 2017). Overall, this evidence potentially suggests that glycolysis is an important mechanism underlying the regulation of the hexosamine biosynthetic pathway.

In addition to supporting UDP-GlcNAc biosynthesis, glycolysis also plays an important role in regulating triglyceride synthesis, by forming Glycerol-3-phosphate (G-3-P) through the reduction of dihydroxyacetone phosphate (DHAP) via G-3-P dehydrogenase (Zechner et al., 2012). G-3-P and fatty acyl CoAs are primary materials for *de novo* synthesis of glycerolipids, which are crucial for energy homeostasis, proper lipid transport, balancing glucose/lipid metabolism, and generation of metabolic signals (Prentki and Madiraju, 2008; Zechner et al., 2012). Research also finds that DHAP acts as an important precursor existing in the membranes of mitochondria and exhibits an important functional role in mitochondrial bioenergetics (Schlame et al., 1990, 1993; Paradies et al., 2014). Additionally, a deficiency of triose-phosphate isomerase (TPI or TIM), the enzyme catalyzing the rapid interconversion of DHAP and D-glyceraldehyde 3-phosphate (GAP), will lead to progressive neurological dysfunction and childhood mortality, thus highlighting its unique role in the process of glycolysis (Orosz et al., 2008).

GLYCOLYSIS FUNCTIONS IN THE BRAIN

The human brain depends mostly on glucose as the source of fuel, rendering it at great risk for neuronal dysfunction when glucose is in short supply. Apart from glucose, the brain can also utilize other energy substrates. Ketone bodies can provide energy (Owen, 2006; Zielke et al., 2009), but its fuel role is considered minor, except in times of starvation and glucose deprivation. Studies indicate that neurons predominantly undergo oxidative phosphorylation, whereas astrocytes are mainly responsible for lactate production via glycolysis (Hyder et al., 2006; Belanger et al., 2011). For the past 25 years, a prevalent viewpoint on neuroenergetics has been presented and developed: the astrocyte-neuron lactate shuttle (ANLS) (Pellerin and Magistretti, 1994). Upon intensified neuronal activity, glutamate is released into the synaptic cleft, followed by its action on postsynaptic receptors and uptake by astrocytes via excitatory amino acid transporters (EAAT). Glutamate in astrocytes is then converted by glutamine synthetase into glutamine, which is released by astrocytes, and taken up by neurons from the extracellular space. Within neurons, glutamine is converted to glutamate by glutaminase, packed in synaptic vesicles, and prepared to be released, thus the neurotransmitter pool of glutamate is replenished and the glutamate-glutamine cycle is completed (Mason, 2017). The uptake of glutamate by astrocytes is driven by a sodium gradient generated by Na^+/K^+ ATPase, of which ATP source is primarily from glycolysis (Pellerin and Magistretti, 1996). Theories suggest that neurons take up the extracellular lactate as the energy substrate from astrocytes in support of ATP

generation upon the intensified neuronal activity (Magistretti and Allaman, 2018). The increased demand of energy accompanied by the glutamate-glutamine cycle induces higher glucose uptake from the circulation and enhanced lactate release by astrocytes, which attenuates extracellular prostaglandin E2 uptake, thus providing a potential mechanism underlying vasodilation and increased cerebral blood flow (Gordon et al., 2008; Howarth, 2014; MacVicar and Newman, 2015). A few studies have also indicated a neuroprotective role of an elevated brain lactate level, as evidenced by its ability in preventing neuronal excitotoxicity and decreasing lesion size in animal stroke models, possibly via an ATP- and redox-dependent pathway (Ros et al., 2001; Berthet et al., 2012; Jourdain et al., 2016; Margineanu et al., 2018). However, controversies exist showing neurons are capable of maintaining glutamatergic activity independent of the glutamate-glutamine cycle (Kam and Nicoll, 2007). Studies also showed that GABAergic neurons may not necessarily rely on lactate (Magistretti and Allaman, 2018). In addition, studies have also shown that, in response to increased neuronal activities, rather than obtaining lactate from astrocytes, neurons are capable of increasing glycolysis to produce lactate themselves, underscoring the role of glycolysis in sustaining neuronal function and maintaining homeostasis in the brain (Yellen, 2018).

Glycolysis in Membrane Transport

It is a well-known fact that oxidative phosphorylation produces ATP in a much more efficient manner than does glycolysis. However, in acute neuronal events, glycolysis has been shown to become the dominant pathway for ATP generation (Fox et al., 1988). Proper functions of many ionic pumps such as Na^+/K^+-ATPase, H^+-ATPase, and Ca^{2+}-ATPase have been linked to membrane-bound glycolytic enzymes, both in the central nervous system and in other tissues, indicating that glycolysis-derived ATP may be an important source of energy for ion transport, which is critical for the conduct of action potential and synaptic transmission. V-type H^+-ATPase (V-ATPase) is an ATP-dependent pump that mediates transmembrane transport of protons, thereby maintaining pH gradients between intracellular compartments, and V-ATPase is also required for proton secretion from the plasma membrane of certain specialized cells. V-ATPase has the highest expression in the brain and is a crucial constituent of synaptic vesicles. On the membrane of synaptic vesicles, V-ATPase pumps protons through the membrane into the synaptic vesicle, creating a proton concentration gradient, which is then used as an energy source, driving the movement of neurotransmitters into the vesicle through their respective transporters. Neurotransmitter concentration in the vesicle is an essential step preceding neurotransmitter release, underscoring the extremely essential role of V-ATPase in synaptic transmission (Moriyama et al., 1992). A number of studies have shown close interactions of V-ATPase with glycolytic enzymes, including PFK-1, Pfk2p, aldolase, and hexokinase, indicating the functional dependence of V-ATPase on glycolysis (Moriyama and Futai, 1990; Lu et al., 2001, 2004; Su et al., 2003; Nakamura, 2004; Kohio and Adamson, 2013; Chan et al., 2016; Woody et al., 2016).

Moreover, evidence exists that, following synaptic transmission, glutamate is quickly removed from the synaptic cleft by astrocytic uptake. This is an extremely efficient process in part powered by increased glycolysis that further increases the activity of Na^+/K^+-ATPase and the Na^+-dependent cotransport uptake system in astrocytes (Pellerin and Magistretti, 1994). Furthermore, one study shows that when *Caenorhabditis elegans* neurons are under energy stress, a compensatory mechanism mediated by glycolysis maintains enough energy to support endocytosis and the synaptic vesicle cycle (Jang et al., 2016). Overall, despite the low yield of ATP produced by the glycolytic pathway, rapid ATP generation by glycolysis provides a significant advantage over oxidative phosphorylation, thereby playing a critical role for neuronal processes such as action potential, neurotransmitter release and uptake.

Glycolysis in Postsynaptic Activity

A further role of glycolysis has been associated with postsynaptic density (PSD). PSD is a dense protein complex localized on the cell surface of post synapses in dendritic spines, containing receptors for almost all glutamate neurotransmission at excitatory synapses (Chen et al., 2015). The PSD clusters also regulate ion flux for Na^+, K^+, and Ca^{2+}, adhesion proteins, scaffolding proteins, and protein kinases, including protein kinase A, protein kinase C, and Ca^{2+}/CaM-activated protein kinase, along with their substrates. The rapid turnover and the dynamic property of PSD95 implies a great need for energy supply, as well as a significant quantity of intermediates for anabolism in dendritic spines, where, however, mitochondria are seldom present (Li et al., 2004). Instead, glycolytic enzymes are abundantly expressed in the PSD, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), which may possibly be the source of ATP in the isolated PSD (Wu et al., 1997). Lactate is the end-product of anaerobic glycolysis functioning, and acts not only as a substrate for mitochondria, but also a second messenger to modulate the activity of neurons and astrocytes in neighboring regions. It is speculated that during acute neuronal activity, a significant interstitial lactate transient may be observed in areas where glutamate is released. However, when neuronal activity occurs, the diffusion and convection of lactate reaches much further than glutamate and the active zone, away from the area where glucose is predominantly consumed. Naïve neurons subsequently receive multiple signals, including lactate, which potentially augments inhibitory effects by adjacent GABAergic interneurons. This increased inhibition gives rise to limited glucose consumption in remote areas, thus recruiting more glucose to the active zone. The metabolic role of lactate in the brain is well discussed in a previous review (Barros, 2013). Monocarboxylate transporter 2 (MCT2), responsible for transporting lactate across the plasma membrane, has been reported to be present in PSD and co-localize with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor GluR2/3 (Nusser et al., 1994; Bergersen et al., 2005; Goncalves et al., 2020). It is suggested that dysregulation of MCT causes axonal damage, amnesia, and memory deficits (Barros, 2013). These research findings provide strong support for the role of glycolysis in the regulation of postsynaptic activities.

Glycolysis in Redox Homeostasis

Given that the brain is metabolically vulnerable, proper metabolic response of neurons is critical in defending the brain against injury, reactive oxygen species (ROS), and other neurodegenerative insults (Brand and Hermfisse, 1997; Rodriguez-Rodriguez et al., 2013; Butterfield and Halliwell, 2019). The pentose phosphate pathway (PPP) in glucose metabolism is a central source of pentoses and ribose 5-phosphate for cellular synthesis of nucleotides, as well as for reducing equivalents in the form of NADPH (**Figure 1b**). PPP accounts for about 60% of NADPH produced in a human body and is highly active in the liver, the adrenal cortex, and in red blood cells. NADPH is essential for cholesterol and steroid synthesis and respiratory bursts. In addition, NADPH is used by glutathione reductase to convert disulfide glutathione to reduced glutathione, which is in turn oxidized by glutathione peroxidase, coupled with the reduction of peroxides. Therefore, NADPH plays a critical role in maintaining the cellular redox state, which is tightly controlled by the rate-limiting enzyme of PPP, glucose-6- phosphate dehydrogenase (G6PDH). Coordination between glycolysis and PPP has been extensively described, including the rearrangement reactions in PPP that convert ribose-5-phosphate and xylulose-5-phosphate to fructose-6-P and glyceraldehyde-3-P under transketolase and transaldolase (Patra and Hay, 2014). Treatment with NADPH protects neurons against ROS and apoptosis, leading to increased ATP level, reduced long-term mortality, and improved functional recovery (Soucek et al., 2003; Mejías et al., 2006; Ying, 2008; Stanton, 2012; Li et al., 2016; Huang et al., 2018).

Glycolysis in Brain Development

The PPP is a branch pathway from glycolysis after the first rate-limiting step catalyzed by hexokinase. Apart from its important role in antioxidant defense and nucleic acid synthesis, PPP also provides NADPH for reductive biosynthesis, such as biosynthesis of fatty acids and sterols (**Figure 1b**). Ranking as the second highest in lipid content after adipose tissue, human brain is particularly enriched with lipid content for maintenance of brain functions, such as synaptic activity, which in turn, renders it highly vulnerable to fatty acid and lipid disorders (Poitelon et al., 2020). In contrast to the peripheral system where lipids are the major form of energy storage, the lipids in the brain are primarily used for membrane construction, such as phospholipids (Hamilton et al., 2007). Synapse formation and elimination dynamically exist throughout a person's entire life. A burst of synaptic formation occurs during early brain development, a phase known as exuberant synaptogenesis (Huttenlocher and Dabholkar, 1997). This synaptic dynamic is critical for normal synaptic connection and plasticity, proper neuronal network formation, task execution, learning process, and memory establishment. Moreover, studies show that during neuron differentiation, PFK-1 protein expression significantly increases, suggesting that glycolysis is necessary in supporting biosynthesis for neurite outgrowth and synaptic formation (Goyal et al., 2014; Agostini et al., 2016). In addition, a human brain represents only 2% of total body weight but contains 20% of the body's cholesterol. Membrane lipids, primarily phospholipids, together with lipid composition of the myelin, comprise more than 50% of the brain solid matter, with concentration of brain phosphoglycerides being age dependent (Svennerholm, 1968). Dysregulation of lipid composition in the brain reportedly contributes to deterioration of CNS functions and pathological alteration in AD (Svennerholm, 1968; Söderberg et al., 1991; Velasco and Tan, 2014; Kao et al., 2020). Therefore, an impaired glycolytic pathway, such as dysregulated hexokinase, may reduce the essential intermediates and undermine PPP, thus inhibiting proper brain function and development.

The energy used by a developing brain is striking. Studies indicate the newborn's brain is about 13% of body mass but consumes up to 60% of total body energy, and this soaring energy utilization lasts throughout one's entire childhood. Notably, AG comprises 30% of glucose metabolism in a developing brain, compared to about 10% in an adult brain (Magistretti and Allaman, 2015), indicating an important role of glycolysis in brain development. Moreover, during pregnancy and infancy, brain volume and weight sharply increase, with brain size reaching about 75% of an adult's brain by 2 years old, compared to 25% at birth (Steiner, 2019). Since neurogenesis mainly occurs prenatally—although some regions, such as the cerebellum, continue to generate after birth—rapid postnatal brain growth is mostly attributed to axon growth, dendritic morphogenesis, synaptic proliferation/elimination and axon myelination. This is a period when a brain meets both its highest energy demand and highest level of AG (Vlassenko and Raichle, 2015; Silbereis et al., 2016). Goyal et al. (2014) found that the elevated level of glycolysis during childhood correlates to the child's highest rate of synaptic growth. They also discovered that in adult brain regions with the highest AG, genes that are responsible for synapse formation and growth are significantly increased (Vaishnavi et al., 2010; Goyal et al., 2014). Glycolysis is important in synaptic plasticity and as a link between glycolytic function and motor adaptive learning (Shannon et al., 2016). Other research shows that, in early postnatal mice, the neurite architecture was significantly impaired when glycolysis was pharmacologically inhibited (Segarra-Mondejar et al., 2018). Additionally, multiple researchers have demonstrated that glycolysis has a predominant role in elevated neuritic and synaptic formation, as well as their turnover, a role that presumably remains throughout human lifespan (Marder and Goaillard, 2006; Goyal and Raichle, 2013; Magistretti, 2014). Taken together, even though there are a limited number of studies, the developing brain is considered to be predominantly glycolytic. This is largely due to its dependence on *de novo* biosynthesis of lipids, amino and nucleic acids in support of developmental processes such as synaptogenesis, which ultimately leads to proper neuronal network that underlies cognitive function (Bauernfeind et al., 2014; Goyal et al., 2014; Steiner, 2019).

GLYCOLYSIS IN AD

Before reaching adulthood, brain glucose consumption is slightly reduced. Overall glycolysis, however, exhibits a much steeper

decline from representing about 30% of glucose utilization to 8–10% (Vlassenko and Raichle, 2015; Steiner, 2019). Notably, AG appears to be spatially varied in the brain and is diminished topographically during normal aging. Regional variations are involved with reasoning, cognition, navigation, and executive motor control (Vaishnavi et al., 2010). Consequently, brain regions relying on high level of AG in young adults can be exceptionally vulnerable when approaching middle-age, given AG's bioenergetic, biosynthetic, and neuroprotective role in the brain (Goyal et al., 2017).

Correlation Between Aβ Deposition, Tauopathy, and Glycolysis

Accumulation of neurotoxic Aβ plaques and hyperphosphorylation of tau have long been considered the pathological hallmarks that contribute to synaptic disruption and neuronal loss in the brains of AD patients (Skovronsky et al., 2006; Querfurth and LaFerla, 2010; Serrano-Pozo et al., 2011; Krstic and Knuesel, 2013; Amtul, 2016; Harrison and Owen, 2016). It is reported that Aβ distributes variably among brain regions, with more deposition found in areas of high dependence on glycolysis. In a PET study of 33 neurologically healthy participants, Vaishnavi et al. (2010) discovered that AG was significantly elevated in the medial, lateral, and prefrontal cortices, whereas the cerebellum and medial temporal lobes exhibited lower glycolysis when compared to the mean value of the brain. Follow-up studies reported that the regions with increased glycolysis in the resting state of healthy young adults closely mirror the later regional pattern where Aβ accumulates in the brains of AD patients (Vaishnavi et al., 2010; Vlassenko et al., 2010; Goyal et al., 2020). The correlation observed in these studies is considered a compensatory mechanism in response to Aβ toxicity and mitochondrial dysfunction at a very early stage of AD. Another clinical study of 42 individuals aged 53–88 years at either preclinical or symptomatic stages of AD revealed close relationships among amyloid burden, AG, and tau deposition. Data showed that reduced synaptic plasticity and neuroprotection are related to the loss of AG, which may promote tauopathy in individuals with amyloid burden (Vlassenko et al., 2018). Studies using h-tau mice, which express all human tau isoforms, found that reduced glucose utilization, possibly via the downregulation of glycolysis, directly triggers tauopathy leading to synaptic dysfunction and behavior deficits (Lauretti et al., 2017). These results indicate the important role of brain glycolysis in the pathogenesis of AD.

Altered Glycolytic Metabolite and *O*-GlcNAcylation in AD

A prospective ongoing cohort study that began in 1958 by the Baltimore Longitudinal Study of Aging (BLSA) investigated whether AD pathogenesis is correlated with dysfunction of glucose homeostasis. Glucose concentration and ratios of glycolytic amino acids (serine, glycine, and alanine) to glucose, which represent the cerebral glycolytic function, were measured within the autopsy cohort. The results showed that elevated brain glucose levels and reduced glycolytic flux are associated with the severity of AD pathology and expression of AD symptoms. They concluded that impaired glycolytic function may be intrinsic to glucose metabolic dysfunction inherent in AD pathogenesis (An et al., 2018). Another clinical study that analyzed 122 metabolites in the CSF of AD and non-AD subjects showed that only intermediates of glycolysis, such as dihydroxyacetone phosphate (DHAP) and phosphoenolpyruvate (PEP), were significantly decreased in AD patients. The reduction of these glycolytic intermediates also exhibited positive correlation with $A\beta_{1-42}$ and $A\beta_{1-42}/A\beta_{1-40}$ (Bergau et al., 2019). A very recent study also reported that astrocytic glycolysis-derived L-serine exhibited a significant decrease in 3xTg-AD mice indicating a reduced glycolytic flux that led to impaired synaptic plasticity and memory (Le Douce et al., 2020).

A number of studies have looked at the roles of key glycolytic enzymes in glycolytic dysregulation of AD; the results, however, were mostly obtained from postmortem brain specimens of AD patients and are inconsistent. Hexokinase activity was reported to decrease significantly in brains, skin-cultured fibroblasts, and leukocytes of AD patients (Marcus et al., 1989; Sorbi et al., 1990). Whereas, in a paper of investigating hexokinase activity in a large Italian pedigree, the results showed no significant change of hexokinase activity (Mortilla and Sorbi, 1990). Studies of PFK showed that inhibition of fructose-2,6-biphosphatase (PFKFB3) led to Aβ accumulation in astrocytes and a higher risk of Aβ toxicity in cultures of human fetal astrocytes. In a study involving autopsy of AD patients, PFK activity was shown to decrease significantly to approximately 10% of the activity in the control group (Bowen et al., 1979). In another study, PFK activity in the frontal and temporal cortex of post-autopsy AD brains was found to be elevated when compared to age-matched non-AD individuals (Bigl et al., 1999). Furthermore, Sims et al. (1987) found no decrease of PFK activity in patients with primary degenerative dementia who are relatively young and at an early stage of the disease. The activity of the third key glycolytic enzyme, pyruvate kinase, is reported to significantly increase in the frontal and temporal cortex of AD brains (Bigl et al., 1999). However, other studies found a reduced level of pyruvate kinase activity in an age-dependent fashion in the frontal cortex of APP/PS1 mice (Harris et al., 2016). Factors that may have contributed to these inconsistencies include sample preparation procedure, age of the patients, AD study models, as well as the stage of AD.

Another key enzyme that has raised great interest in the past three decades regarding its role in neurodegenerative disease is GAPDH, a classic glycolytic enzyme that is primarily considered as the "housekeeping" protein and predominantly presents in the cytosol. GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in a NAD^+-dependent manner and mediates the production of NADH + H^+ and ATP. In a study measuring GAPDH activity in post-mortem brains of AD patients, the researchers discovered a ∼19% decline of GAPDH activity in temporal cortex (Kish et al., 1998). In line with the previous study, Mazzola and Sirover (2001) found a 27∼33% decline of GAPDH glycolytic activity in human skin fibroblast cell strains obtained from AD patients. However, controversies exist with studies showing that GAPDH

enzymatic activity exhibited a significant increase in the frontal cortex lysate of six AD patients compared to an age-matched healthy group (Soucek et al., 2003). A possible explanation of the observed elevated GAPDH activity which contradicts the hypometabolism theory of AD brain could be the small sample size studied. Moreover, a human microglial cell line, CHME-5, showed an increased level of GAPDH upon the treatment of plasma from AD subjects (Jayasena et al., 2015). Increased glycolysis, however, is considered as a compensatory mechanism of impaired mitochondrial function following the exposure of AD plasma and functions as the forerunner of the energy deficit status in the cell. Furthermore, competing theories suggested that overexpression and aggregation of GAPDH serve as the pro-apoptotic regulator in neurodegenerative diseases, which possibly interacts with a voltage-dependent anion channel and mediating permeability transition of the mitochondria, a mechanism recognized as the non-glycolytic function of GAPDH (Ishitani and Chuang, 1996; Kish et al., 1998; Tarze et al., 2007; Nakajima et al., 2009; Itakura et al., 2015; Lazarev et al., 2020). Moreover, a heightened level of the reversible *S*-glutathionylation of GAPDH was found in the AD brain compared to age-matched control groups, which leads to the inactivation of GAPDH. The studies also showed the impaired enzyme activity can be rescued under certain reducing conditions, such as glutathione (Newman et al., 2007). Considering *S*-glutathionylation is an important post-translational modification in preventing protein from irreversible oxidation, a proposed theory points to GAPDH as an oxidative stress sensor, further implying a correlation between reduced glycolysis and the disturbed redox state of the cell (Gallogly and Mieyal, 2007). Additionally, inactivation of GAPDH has been considered to play a protective role under oxidative stress, by diverting carbohydrate flux to PPP, thus producing more reducing power, NADPH, against oxidants (Ralser et al., 2007). However, inactivated GAPDH also results in reduced energy. Therefore, in the long term when cells become exhausted by massive oxidative stress, a disastrous cascade effect that ultimately leads to cell death will be initiated by the cell's inability to sustain energy demand and redox homeostasis. GAPDH was also found bonded on Inositol 1,4,5-trisphosphate receptors (IP3R) and proposed to mediate Ca^{2+} release via NADH (Patterson et al., 2005). Hence, GAPDH also plays a significant role in regulation of Ca^{2+} homeostasis, an important mechanism underlying AD pathogenesis, further linking perturbed glycolysis to cell death (Canzoniero and Snider, 2005; Bojarski et al., 2008).

Another area associated with glycolysis that has been examined in the context of AD is the HBP, also known as the nutrient sensing pathway (Zhu et al., 2014). Notably, O-linked *N*-acetylglucosamine (O-GlcNAc) is found greatly enriched in the brain, particularly at neuronal synapses, suggesting a role of the HBP in synaptic transmission (Zhang and Bennett, 1996; Vosseller et al., 2006; Skorobogatko et al., 2011; Trinidad et al., 2012). Moreover, increased *O*-GlcNAcylation has been demonstrated to promote neuroprotective outcomes such as reduced cerebral trauma, improved outcome of strokes, and alleviated stress (Groves et al., 2013; Gu et al., 2017). Using a human neuroblastoma cell model, upregulating *O*-GlcNAcylation led to an increased level of non-amyloidogenic sAPP α fragments and reduced Aβ secretion, suggesting *O*-GlcNAcylation of APP as an anti-Aβ target for AD (Jacobsen and Iverfeldt, 2011; Chun et al., 2017). *O*-GlcNAcylation has also been observed to inversely correlate with phosphorylation of tau, as demonstrated by fourfold less expression of *O*-GlcNAc in hyperphosphorylated tau than in non-hyperphosphorylated tau. Of particular note, rodent brains with impaired glucose metabolism exhibit changes in *O*-GlcNAcylation and tau phosphorylation that resemble those in the brains with inhibited HBP, suggesting the HBP is largely controlled by glucose metabolism (Liu et al., 2009). In sum, given that the HBP is a branch of glycolysis and that there is evidence that a decreased level of *O*-GlcNAcylation can result from impaired glucose metabolism, elevation of the HBP activity via boosting glycolysis may be a promising therapeutic approach in AD.

Redox State, Glycolysis, and AD

Oxidative stress is a common feature in AD (Perry et al., 2002; Butterfield and Halliwell, 2019). NAD^+ is a cofactor for redox reactions and plays an essential role in glycolysis to regulate cellular energy metabolism. Increasing evidence suggests that NAD^+ is also involved in many other biological processes such as cell death, calcium homeostasis, gene expression, carcinogenesis, immunological functions, as well as aging (Ying, 2008). The NAD^+/NADH ratio is an index of cellular reducing potential, dysregulation of which has been extensively indicated in AD. A DNA repair-deficient 3xTgAD/Polβ$^{\pm}$ mouse model with exacerbated AD features, including tauopathy, synaptic dysfunction, neuronal death, and impaired cognitive function, exhibited a reduced cerebral NAD^+/NADH ratio and indicated impaired energy metabolism. Treatment with nicotinamide riboside reversed the NAD^+/NADH ratio and produced improved phenotypes, indicating that bolstering NAD^+/NADH, potentially via glycolysis, may contribute to AD treatment (Hou et al., 2018). Another study using primary neuron culture showed that exposure to Aβ oligomers significantly reduced the NAD^+ level and the decreased NAD^+ level was rescued by nicotinamide treatment (Liu et al., 2013). Similar results were also observed in 3xTgAD mice (Liu et al., 2013). In a clinical study to evaluate the orally administrated NADH effect, 26 AD patients received NADH (10 mg/day) or a placebo. After 6 months of treatment, the NADH-treated group performed significantly better on the Mattis Dementia Rating Scale (MDRS) and the Mini Mental State Examination and showed no significant progression of AD symptoms, indicating the therapeutic value of NADH for AD (Demarin et al., 2004).

Moreover, the NADPH generated by PPP reacts with oxidized glutathione to form reduced glutathione (**Figure 1b**). The reduced glutathione further converts reactive H_2O_2 to H_2O by glutathione peroxidase, thus maintaining cellular redox homeostasis. The role of PPP, a branched pathway directing from the first key step of glycolysis, in maintaining proper NADPH level and protecting against oxidative stress is thereby highlighted. In a study that involved 45 AD patients and 28 age-matched control subjects, both GSH levels and the GSH/GSSG

ratio exhibited a profound reduction in the AD group (Bermejo et al., 2008). Additionally, Ivanov et al. (2014) reported that, during synaptic stimulation in hippocampal slices, a significant fraction of NAD(P)H response corresponded to glycolysis, suggesting that glucose serves as an effective energy substrate for both neurons and astrocytes in network activity. Considering the biosynthetic dependence of NADPH on glycolysis and its role in producing GSH from GSSG, proper function of glycolysis is vital not only for glucose metabolism, but also for maintaining redox homeostasis in AD.

ApoE Isoforms Differentially Modulate Neuronal Glycolysis

As in many other chronic diseases, AD risk can be influenced by multiple factors, such as age, gender, family history, brain injury, environment, and lifestyle. Notably, results from postmortem autopsy showed that about 30% of cognitively normal people present various signs of AD pathology in the brain (Arenaza-Urquijo and Vemuri, 2018; Dumitrescu et al., 2020), raising the question of why some people have AD-like pathology but remain cognitively intact. Individual differences in overcoming adverse factors and thus maintaining a better performance can be a reason. This is referred to as brain resilience. Age-dependent reduced glycolysis in the brain occurs independent of amyloid plaques and serves as a biomarker for aging (Goyal et al., 2017). Given that aging is the greatest risk factor for the development of AD, enhancing glycolysis can potentially increase neuronal metabolic strength to sustain a better cognition and slow down or prevent AD progress. For instance, research has shown that neuronal cells with a higher level of glycolysis are more resistant to Aβ toxicity, indicating a neuroprotective effect mediated by glycolysis (Newington et al., 2011, 2012).

ApoE is the primary cholesterol carrier in the brain and must be produced locally. It is predominantly synthesized by astrocytes and to a lesser extent by microglia, vascular smooth muscle cells, and the choroid plexus (Uchihara et al., 1995; Achariyar et al., 2016). In addition, studies showed that under normal conditions, neurons also produce ApoE. This may be responsible for ∼20% of total ApoE protein levels in the cortex (Xu et al., 2006; Knoferle et al., 2014). Particularly, the intense induction of ApoE expression has been observed in injured or stressed neurons, indicating a critical role of this neuron-specific source of ApoE expression in cellular repair and maintenance (Boschert et al., 1999; Aoki et al., 2003). Human ApoE exists in three major alleles (ε2, ε3, and ε4) and each of them conveys different susceptibility for development of AD. ApoE2 is considered as neuroprotective, whereas ApoE4 is the greatest genetic risk for AD. Recent research led by Tarja Malm showed that human iPSC-derived microglia carrying ApoE4 exhibited a reduced extracellular acidification when compared to those carrying ApoE3, indicating an ApoE4-induced glycolytic deficit (Konttinen et al., 2019). Studies by Zhao et al. (2017) demonstrated that compared to ApoE3, ApoE4 secreted by astrocytes failed to induce insulin-stimulated glycolysis in *ApoE*$^{-/-}$ neurons. In clinical studies, ApoE4 has widely been associated with early onset of AD, rapid progression of the disease, more severe impairment of cognitive function and altered response to AD treatment (Morris et al., 1995; Nagy et al., 1995; Wilson et al., 2002; Wu and Zhao, 2016). Moreover, studies showed that ApoE2 carriers without dementia do not display the typical age-related increase of an Aβ burden (Grothe et al., 2017). Functional connectivity in the amygdala and entorhinal cortex tends to be increased and remain stable in individuals with ApoE2 allele (Gong et al., 2017). Attenuated hippocampal atrophy and a lower level of age-related myelin breakdown have also been observed in individuals carrying ApoE2, compared to non-carriers (Bartzokis et al., 2006; Chiang et al., 2010). The underlying mechanism of ApoE2 as a neuroprotective variant, however, remains largely unknown. Recent studies found that ApoE2-bearing mouse brains exhibit the most robust bioenergetic profile as evidenced by the highest levels of hexokinase expression and activity, glycolytic function, and ATP production when compared to both ApoE3 and ApoE4 mouse brains (Keeney et al., 2015; Woody et al., 2016; Wu and Zhao, 2016; Wu et al., 2018). In addition, ApoE2-mediated glycolytic robustness via the upregulation of hexokinase appears to directly correlate to a healthier cell status, which could serve as a major mechanism that allows ApoE2-bearing brains to be more resilient against AD (Zhang, 2018). In summary, growing evidence supports the idea that introduction of ApoE2 into the ApoE4 brain, such as by a gene or protein/peptide therapy, can be a plausible strategy to rescue the glycolytic deficits, improve cognitive function, and ameliorate AD-related neurodegeneration, thus helping close the therapeutic gap for AD patients.

BRAIN HYPERGLYCEMIA AS A COMMON FEATURE IN DIABETES AND AD

Diabetes has been well established as a risk factor for AD (Profenno et al., 2010). A great number of epidemiological studies revealed that being diabetic results in a higher risk of developing AD in later life, and such a risk is heightened when diabetes coexists with other risk factors such as the ApoE4 genotype. For example, in a Taiwanese population-based study of 615,532 diabetic patients and 614,871 non-diabetic individuals, the diabetic group showed a higher rate for developing AD over a period of 9 years with a hazard ratio of 1.45 relative to non-diabetics (Wang et al., 2012). In a longitudinal cohort study that followed up on 824 individuals older than 55 years, diabetes was associated with 65% higher incidence of AD over an average period of 5.5 years (Arvanitakis et al., 2004). Moreover, the Hisayama study followed up on 11,017 individuals older than 60 years for an average of 10.9 years and showed that impaired glucose tolerance and diabetes were respectively associated with 46 and 94% higher risk for developing AD (Ohara et al., 2011). In a community-based study whose subjects were obtained randomly from the Mayo Clinic Alzheimer Disease Patient Registry, the frequency of type 2 diabetes and impaired fasting glucose (IFG) was compared in 100 AD patients and 138 non-AD age-matched individuals. Results showed greater spread within the AD patients. Type 2 diabetes was present in

34.6% of AD patients vs. 18.1% of non-AD patients, whereas IFG was present in 46.2% of AD patients vs. 23.8% of non-AD patients (Janson et al., 2004). This indicates a possible common pathological link between the two diseases. Furthermore, the Honolulu-Asia aging study revealed that diabetes elevates the risk of developing AD in ApoE4 carriers compared to an isolated effect of ApoE4 without a diabetic condition (5.5 vs. 1.7 folds) (Peila et al., 2002). In light of the close association between AD and diabetes, the term "Type 3 Diabetes" has been used by some researchers as a label for AD (Steen et al., 2005; de la Monte and Wands, 2008). The underlying molecular mechanism, however, is not well understood. Research efforts directed toward better understanding of this relationship can ultimately pave the way toward better understanding of AD causative factors and thus to identification of key molecular targets that can be focused upon in future therapeutics.

Multiple studies have explored the relationship between blood plasma and brain glucose levels. An approach that has often been used is to alter plasma glucose levels within a predetermined range by dextrose injections, which is followed by measuring the levels of brain glucose that are achieved at different blood glucose levels. In a study performed on 18 healthy participants with mean age of 41 years, a linear relationship for glucose levels was shown between the cerebral and vascular compartments after stabilizing plasma glucose in a range of 4–30 mM: brain glucose levels proved to be 20–30% of plasma levels in the tested range (Gruetter et al., 1998). This plasma-to-brain glucose ratio and hyperglycemia-associated elevation of brain glucose are comparable to the data obtained in a study performed on white Wistar rats under normo- and hyperglycemia (Silver and Erecinska, 1994). These findings clearly indicate that peripheral hyperglycemia induces a corresponding elevation of brain glucose levels. As hyperglycemia is one of the well-defined pathologies of diabetes, it has been hypothesized that the blood-brain barrier might adapt to chronic hyperglycemic states and thus limit the uptake of glucose into the brain as a protective mechanism. This hypothesis, however, was refuted in multiple studies which revealed that brain glucose levels are elevated in states of chronic diabetic hyperglycemia. For instance, in a study performed on 14 healthy individuals with mean age of 37, and 14 poorly controlled diabetic patients with mean age of 43 (type 1 = 8 patients, type 2 = 6 patients), hyperglycemia was induced in participants to an approximate blood glucose level of 300 mg/dL. This level produced brain glucose levels of 4.7 and 5.3 mM respectively in diabetics and non-diabetics (Seaquist et al., 2005). Another study performed on male Sprague Dawley rats showed similar results. Levels of glucose in brain extracellular fluid (ECF) under a hyperglycemic condition were compared to chronically hyperglycemic diabetic rats and non-diabetic rats. High plasma glucose (22 and 28 mM) led to comparable levels of brain ECF glucose in the two groups (mean brain ECF glucose: 7.5 and 8.7 mM respectively), while the mean of control-group normoglycemic rat brains ECF glucose was 2.1 mM at plasma glucose of 8 mM (Jacob et al., 2002). These data strongly indicate that diabetic hyperglycemia can directly result in brain hyperglycemia, regardless of the state of the disease.

As discussed earlier, a decreased glucose metabolic rate has been widely established as one of the main features of AD. However, the actual brain tissue glucose levels did not receive similar attention and the existence of a correlation between developing AD and alteration of brain tissue glucose levels was not explored until recent evidence from BLSA linked Alzheimer's with accumulation of glucose in the brain (An et al., 2018). The study revealed that the brains of AD and asymptomatic AD patients, as opposed to brains of healthy participants, had higher levels of glucose—an alteration particularly prominent in brain regions of the cerebral cortex that are vulnerable to AD pathogenesis. Furthermore, brain tissue glucose concentrations were positively correlated with the severity of AD brain pathology. This and previous evidence lead to the conclusion that brain hyperglycemia is a shared feature of both diabetic and Alzheimer's brains.

A considerable amount of evidence is available concerning the pathological impact of diabetes and hyperglycemia on the brain. Animal models of hyperglycemic diabetes have been shown to exhibit brain abnormalities that are comparable to dysfunction of Alzheimer's brains, such as synaptic impairment (Malone et al., 2008; Liu et al., 2015), brain atrophy, and mitochondrial impairment (Carvalho et al., 2015). Furthermore, animal models having both diabetes and AD manifest an exacerbated level of brain pathology and cognitive impairment compared to models having AD only. A study that compared the effects of diabetes versus AD on the brain showed similar phenotypes in the brains of both a sucrose-induced mouse model of diabetes and a triple transgenic AD mouse model (3xTg-AD), including reduced brain weight, mitochondrial dysfunction, and reduced levels of synaptic and autophagy-related proteins when compared to WT animals (Carvalho et al., 2015). Activity of Na+/K+ ATPase, an essential pump that maintains neuronal resting membrane potential and its activity, was shown to be impaired by hyperglycemia in isolated synaptic terminals of aged Wistar rats' brains (Torlinska et al., 2006). In a streptozotocin-induced hyperglycemic rat model of diabetes, neuronal spine density and dendritic branches were reduced compared to WT rats. The reduction was accompanied by memory impairment in the Morris water maze cognitive test (Malone et al., 2008). Synaptic plasticity as measured by long-term potentiation (LTP) was decreased in a high-fat diet-induced hyperglycemic mouse model (Liu et al., 2015). Animals with both diabetes and AD attained worse outcomes in the Morris water maze test, LTP, and mitochondrial respiration and enzymatic activities of complexes I and IV, when compared to animals with only diabetes or AD (Wang et al., 2015). In a similar study, comorbidities of diabetes and AD led to increased formation of Aβ oligomers and deposition of Aβ plaques, increased tau pathology, exacerbated neuroinflammation, and worsened performance on the Morris water maze test when compared to animals having only diabetes or AD (Guo et al., 2016). Overall, considering the hyperglycemic phenotype and other associated pathological features shared in brains of animals having both diabetes and AD, it is probable that brain hyperglycemia serves as a

mechanistic link between the two diseases and contributes to AD development. Glycolytic deficit could be a major cause of increased glucose accumulation and ultimately hyperglycemia in non-diabetic AD brains.

GLYCOLYTIC DYSFUNCTION IN OTHER NEURODEGENERATIVE DISEASES

Glycolytic dysfunction has been associated with other neurodegenerative diseases, including Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). In the glycolytic pathway, phosphoglycerate kinase (PGK) is a major enzyme that catalyzes the first ATP-generating step in which a phosphate group in 1,3-biphosphoglycerate is transferred to ADP, producing 3-phosphoglycerate and one molecule of ATP. Deficiency of PGK activity caused by genetic mutations (e.g., c.649G > A) that results in impaired ATP production had been established as a major cause of medical conditions such as hemolytic anemia, myopathy, and neurological deficits (Matsumaru et al., 2017). Multiple studies have reported that patients suffering from these disorders exhibited PD-like symptoms, pointing to the role of PGK deficiency in the development of idiopathic PD (Sotiriou et al., 2010; Sakaue et al., 2017; Cai et al., 2019; Le Bras, 2019; Shimizu et al., 2020). For example, in one case report, a child with PGK1 deficiency developed parkinsonism at 9 years of age, whereas the mother, a heterozygous carrier of the mutation, developed parkinsonism at 36 years of age, suggesting a gene dose-dependent effect of PGK1 deficiency in conferring susceptibility to PD (Sakaue et al., 2017). Similarly, a 25-year-old male carrier of a PGK1 mutation that caused a marked decrease in PGK activity presented both exertional myoglobinuria and severe parkinsonism that was responsive to levodopa treatment (Sotiriou et al., 2010). These clinical findings have been further validated in molecular studies conducted in preclinical models. In a *Drosophila* model, dopaminergic (DA) neuron-specific PGK knockdown led to locomotive defects accompanied by significant reductions in ATP and dopamine levels, and progressive loss of DA neurons (Shimizu et al., 2020). Furthermore, in a variety of either toxin-induced or genetic PD models as well as in iPSC, treatment with terazosin, a PGK agonist, increased brain ATP and dopamine levels and restored motor function, providing support for the therapeutic approach of enhancing PGK and glycolytic activity in the treatment of PD (Cai et al., 2019).

Altered glycolytic metabolism has been observed in HD as well, although discrepancies exist. In an iPSC-based model of the disease, it was found that when compared to control cells, HD cells had decreased ATP levels, lowered expression of glycolytic enzymes, and decreased spare glycolytic capacity. In contrast, both mitochondrial messenger levels and protein levels, as well as respiratory capacities driven by oxidative phosphorylation, were largely unchanged. Moreover, ATP levels in HD cells were restored by treatment with pyruvate or late glycolytic intermediates, but not earlier glycolytic metabolites, providing further evidence for glycolytic and not mitochondrial deficits associated with HD (The HD iPSC Consortium, 2019). In agreement with these studies, Powers et al. reported a significant increase in the molar ratio of cerebral oxygen metabolism to cerebral glucose metabolism [CMRO(2)/CMRglc] in the striatum of HD patients, and the group postulated that glycolytic reduction in striatal metabolism could be involved in the pathogenies of HD (Powers et al., 2007). On the contrary, in HEK293 cell lines and transgenic *Drosophila* expressing polyglutamine (polyQ) in exon 1 of the huntingtin (HTT) protein, Sameni et al. (2016) observed an increased glycolytic rate, as indicated by an increased production of free NADH, in cells and tissues that expressed the expanded HTT-polyQ, when compared to controls that expressed unexpanding HTT-polyQ. In another study, it was found that the WNT/β-catenin pathway was increased in both HD and ALS, which increased activation of several glycolytic enzymes, which in turn resulted in increased glycolysis (Alexandre et al., 2018). An independent investigation conducted by Manzo et al. (2019) in a *Drosophila* model of TDP-43 proteinopathy demonstrated that increased glycolysis may serve as a compensatory mechanism that neurons attempt to use to fight against metabolic deficits in ALS. Further studies are certainly required to resolve these inconsistencies. Nevertheless, these findings clearly indicate a prominent involvement of glycolytic metabolism in the development of these neurodegenerative conditions.

CONCLUSION

Alzheimer's disease has long been recognized as a metabolic disease (Drzezga et al., 2003; Chen and Zhong, 2013; de la Monte and Tong, 2014). In particular, glucose hypometabolism has been established as a prominent anomaly in the very early development of the disease (Mosconi et al., 2008c). As the primary substrate of energy in the brain, glucose is first metabolized in the cytoplasmic glycolytic pathway, followed by oxidative phosphorylation in the mitochondria. Beyond its bioenergetic function, glycolysis plays crucial roles in many biosynthetic processes, for example, in the production of certain amino acids, ribose phosphate, and reduced glutathione, as well as in the glycosylation modification of proteins and lipids. Specifically, glycolysis has been extensively described for its essential roles in brain development and fast-occurring neuronal activities such as ion transport in neurotransmission. Despite its essential neural functions, glycolysis in the context of AD has not been explored much until recently (Bergau et al., 2019; Butterfield and Halliwell, 2019; Theurey et al., 2019; Yan et al., 2020). Several clinical studies have indicated the involvement of glycolytic dysfunction in the development of AD pathologies (Vlassenko et al., 2018). Moreover, increased brain glucose accumulation has been validated in AD patients, supporting the hypothesis that glycolytic deficit as an important contributor to the development of this phenotype (An et al., 2018). Brain hyperglycemia also provides a plausible explanation for the well-documented link between AD and diabetes. Human ApoE exists as three isoforms,

ApoE2, ApoE3, and ApoE4. Carrying ApoE4 is the greatest genetic risk factor for sporadic AD, whereas ApoE2 carriers are resistant to AD. Historically, extensive research has focused on the neurotoxic effect of ApoE4, leaving ApoE2 largely unexplored (Wu and Zhao, 2016). Recent studies have provided several lines of evidence supporting the hypothesis that differential regulation of neuronal glycolysis could serve as one significant mechanism that underlies the different AD risk of ApoE isoforms (Wu et al., 2018). Glycolytic robustness, in large part via upregulation of hexokinase, could play a critical role in conferring ApoE2-bearing brains their resilience to AD. Besides AD, glycolytic dysfunction has been observed in other neurodegenerative diseases, including PD, HD, and ALS, strengthening the concept of glycolytic dysfunction as a common pathway leading to neurodegeneration. Taken together, these advances highlight an exciting translational opportunity not only for the AD field but also for research fields of other neurodegenerative diseases.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GLOSSARY

AD, Alzheimer's disease; ApoE, apolipoprotein E; CMRglc, cerebral metabolic rate of glucose; PET, positron emission tomography; FDG, 2-[18F]fluoro-2-deoxy-D-glucose; MCI, mild cognitive impairment; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide; PFK-1, phosphofructokinase-1; PK, pyruvate kinase; G-6-P, glucose-6-phosphate; AMP, adenosine monophosphate; PPP, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle; AG, aerobic glycolysis; Aβ, amyloid beta; NADP, nicotinamide adenine dinucleotide phosphate; THF, tetrahydrofolate; NMDA, *N*-methyl-D-aspartate; ALT, alanine transaminase; HBP, hexosamine biosynthesis pathway; UDP-GlcNAc, UDP-*N*-acetylglucosamine; OGTs, O-linked *N*-acetylglucosamine transferases; GFAT, glutamine: fructose-6-phosphate amidotransferase; G6PD, glucose-6-phosphate dehydrogenase; G-3-P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; TPI or TIM, triose-phosphate isomerase; ANLS, astrocyte-neuron lactate shuttle; EAAT, excitatory amino acid transporters; V-ATPase, V-type H^+-ATPase; Pfk2p, phosphofructokinase-2 subunit β; PSD, postsynaptic density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; MCT2, monocarboxylate transporter 2; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ROS, reactive oxygen species; G6PDH, glucose-6- phosphate dehydrogenase; BLSA, baltimore longitudinal study of aging; CSF, cerebrospinal fluid; PFKFB3, fructose-2,6-biphosphatase; IP3R, inositol 1,4,5-trisphosphate receptors; O-GlcNAc, O-linked *N*-acetylglucosamine; GSH/GSSG, reduced glutathione/oxidized glutathione; iPSC, induced pluripotent stem cell; IFG, impaired fasting glucose; ECF, extracellular fluid; WT, wild type; LTP, long-term potentiation; PD, Parkinson's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis; DA, dopaminergic; CMRO(2)/CMRglc, ratio of cerebral oxygen metabolism to cerebral glucose metabolism; polyQ, polyglutamine; HTT, huntingtin.

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A Systematic Review of Glucose Transport Alterations in Alzheimer's Disease

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Introduction: Alzheimer's disease (AD) is characterized by cerebral glucose hypometabolism. Hypometabolism may be partly due to reduced glucose transport at the blood-brain barrier (BBB) and across astrocytic and neuronal cell membranes. Glucose transporters (GLUTs) are integral membrane proteins responsible for moving glucose from the bloodstream to parenchymal cells where it is metabolized, and evidence indicates vascular and non-vascular GLUTs are altered in AD brains, a process which could starve the brain of glucose and accelerate cognitive decline. Here we review the literature on glucose transport alterations in AD from human and rodent studies.

Methods: Literature published between 1st January 1946 and 1st November 2020 within EMBASE and MEDLINE databases was searched for the terms "glucose transporters" AND "Alzheimer's disease". Human and rodent studies were included while reviews, letters, and *in-vitro* studies were excluded.

Results: Forty-three studies fitting the inclusion criteria were identified, covering human (23 studies) and rodent (20 studies). Post-mortem studies showed consistent reductions in GLUT1 and GLUT3 in the hippocampus and cortex of AD brains, areas of the brain closely associated with AD pathology. Tracer studies in rodent models of AD and human AD also exhibit reduced uptake of glucose and glucose-analogs into the brain, supporting these findings. Longitudinal rodent studies clearly indicate that changes in GLUT1 and GLUT3 only occur after amyloid-β pathology is present, and several studies indicate amyloid-β itself may be responsible for GLUT changes. Furthermore, evidence from human and rodent studies suggest GLUT depletion has severe effects on brain function. A small number of studies show GLUT2 and GLUT12 are increased in AD. Anti-diabetic medications improved glucose transport capacity in AD subjects.

Conclusions: GLUT1 and GLUT3 are reduced in hippocampal and cortical regions in patients and rodent models of AD, and may be caused by high levels of amyloid-β in these

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regions. GLUT3 reductions appear to precede the onset of clinical symptoms. GLUT2 and GLUT12 appear to increase and may have a compensatory role. Repurposing anti-diabetic drugs to modify glucose transport shows promising results in human studies of AD.

Keywords: GLUT 3, GLUT 1, blood-brain barrier, glucose transporters, Alzheimer's disease

INTRODUCTION

Background

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by the presence of β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) (Grundke-Iqbal et al., 1986; Scheltens et al., 2016). The majority of AD is sporadic, with <5% being classified as genetic (Reitz et al., 2011). Several mechanisms have been proposed for its pathophysiology. According to the amyloid cascade hypothesis, the Aβ precursor protein (APP) is abnormally cleaved, leading to an imbalance between Aβ production and clearance, favoring the accumulation of Aβ. This, in turn, forms clusters in the brain which induce oxidative stress, leading to synaptic dysfunction, neuronal death, and subsequent cerebral atrophy (Chételat et al., 2010). These clusters, called oligomers, form fibrils, then beta-sheets and eventually develop into plaques which are considered a hallmark of AD (Grundke-Iqbal et al., 1986). While Aβ accumulation has a critical role in AD, it is becoming increasingly recognized that brain Aβ burden does not correlate with the severity of cognitive impairment (Games et al., 1995; Price et al., 2009). Aβ accumulation also occurs in aging individuals without cognitive impairment (Castello and Soriano, 2014; Morris et al., 2014; Herrup, 2015), indicating the limitations of the amyloid hypothesis (Kametani and Hasegawa, 2018). The tau hypothesis conjectures that tau is the main causative protein for AD (Kosik et al., 1986). Tau is a protein normally associated with microtubules which serve to stabilize tubulin assemblies. In AD, tau is abnormally hyperphosphorylated and forms pathological inclusions known as NFTs, which are widely identified in AD brains. Tau is more strongly associated with cognitive impairment than Aβ (Hanseeuw et al., 2019). However, attempts to stabilize cognitive function through modification of Aβ and tau in the clinical setting have been unsuccessful to date (Congdon and Sigurdsson, 2018; Yiannopoulou and Papageorgiou, 2020).

Hypometabolism in AD

In addition to Aβ and tau, AD is considered a metabolic disorder, which relates to reduced cerebral glucose metabolism, brain insulin resistance, and age-induced mitochondrial dysfunction (Van Der Velpen et al., 2019). The conventional view is that reduced brain metabolism is secondary to brain atrophy and neuronal loss (Bokde et al., 2001). However, there is accumulating evidence that hypometabolism occurs before the onset of brain atrophy and clinical symptoms, indicating that changes in metabolism may occur prior to reduced glucose demand by tissues (De Leon et al., 2001; Jagust et al., 2006; Mosconi et al., 2006, 2008, 2009; Masdeu, 2008).

Abnormal cerebral glucose metabolism was observed using FDG-PET in 1983 by de Leon et al. (1983), who observed a 17–24% reduction in the regional cerebral metabolic rate of glucose (CMR_{glu}) in a cohort of 24 AD patients. This correlated with a reduction in cognitive performance compared to age matched controls. Later studies confirmed these results, building support for regional declines in CMR_{glu} as a hallmark of AD, including in frontal white matter, caudate, thalamus, temporal, and parietal regions (Small et al., 2000; Mosconi et al., 2004, 2007a). Masdeu (2008) examined seven pre-symptomatic, at-risk subjects with familial AD using MRI and FDG-PET imaging. Compared to seven matched healthy controls, the familial AD subjects showed a reduction in glucose metabolism in most brain regions examined, including the whole brain, right, and left inferior parietal lobules, superior temporal gyrus, left entorhinal cortex, posterior cingulate cortex, and hippocampus. De Leon et al. (2001), Mosconi et al. (2008), and Jagust et al. (2006) examined CMR_{glu} in multiple brain regions of healthy elderly individuals using FDG-PET and MRI scans to differentially predict cognitive decline from normal aging. In all three studies, reductions in glucose metabolism predicted cognitive decline in those participants who went on to develop cognitive impairment or a clinical diagnosis of AD.

In a longitudinal study, Mosconi et al. (2009) examined four cognitively normal elderly subjects and three patients with mild AD. Their pathological diagnosis was verified through post-mortem studies 6 ± 3 years after the subjects' last FDG-PET scan. All participants who were cognitively intact at baseline developed mild cognitive impairment (MCI) 2–7 years after their baseline assessment. On post-mortem studies, two of these subjects had definite AD, one had probable AD and the last had pathological findings consistent with Parkinson's disease with mild AD-related pathology. In all four patients, CMR_{glu} was reduced in areas involving the hippocampus, up to 7 years before their diagnosis of MCI. Follow up FDG-PET scans showed progressive reductions in CMR_{glu} with wider brain involvement including the temporal and parietal lobes, and to varying degrees the anterior and posterior cingulate regions. A more recent study by Ou et al. (2019) examining 551 participants with AD observed reduced metabolism as measured using FDG-PET as an independent biomarker for AD. Their findings demonstrate faster cognitive decline and brain atrophy in participants with reduced metabolism.

Glucose Transport as the Rate-Limiting Step in Glucose Metabolism

While these studies included a small number of participants, their results suggest that hypometabolism precedes a clinical diagnosis

TABLE 1 | GLUTs expressed in the central nervous system.

Protein (*gene*)	References	Insulin sensitive (Yes/No)	Site expressed in the CNS
GLUT1 (*SLC2A1*)	Kumari and Heese, 2010	No	Endothelial cells (55 kDa isoforms), astrocytes (45 kDa isoforms)
GLUT2 (*SLC2A2*)	Kumari and Heese, 2010	No	Astrocytes
GLUT3 (*SLC2A3*)	Kumari and Heese, 2010	No	Endothelial cells, astrocytes, hippocampal neurons
GLUT4 (*SLC2A4*)	Leloup et al., 1996; Vannucci et al., 1998; Reagan, 2002; Kumari and Heese, 2010	Yes	Hypothalamic neurons, hippocampal neurons, cerebellar neurons, sensorimotor cortex, pituitary
GLUT8 (*SLC2A8*)	Kumari and Heese, 2010	Yes	Hypothalamic neurons, hippocampal neurons
GLUT12 (*SLC2A12*)	Zhang et al., 2014 (details extracted from https://www.brainrnaseq.org)	Yes	Cortical astrocytes

of AD. Glucose metabolism requires both delivery of glucose to cells from the bloodstream, and phosphorylation by hexokinase at the site of mitochondria. One possible explanation for early changes to glucose metabolism observed via FDG-PET may be due to abnormal delivery of glucose to the brain. Glucose is a hydrophilic molecule and requires transporters to cross cell membranes. Glucose uptake into the brain occurs predominantly via the sodium-independent facilitative transporters GLUT1 and GLUT3, encoded by the SLC2A1 and SLC2A3 genes, respectively. GLUT1 is responsible for glucose uptake across the BBB endothelial cells, where the higher density isoforms are located (55 kDa), and into astrocytes, where the lower density isoforms are located (45 kDa). Glucose uptake into the brain appears to correlate with the number of GLUT1 transporters at the BBB (Zeller et al., 1997). Neurons do not express GLUT1 (Zlokovic, 2011). The main glucose transporter that facilitates uptake of glucose into neurons is GLUT3, which is encoded by the SLC2A3 gene. GLUT3 is also detected at lower levels on astroglial and endothelial cells (Kumari and Heese, 2010; Patching, 2017). Low levels of GLUT2, encoded by the SLC2A2 gene, are present on astrocytes (Magistretti and Pellerin, 1999; Larrabee, 2002). Reduced supply of glucose to the brain via loss of these major glucose transporters may lead to a brain glucose deficit, halting metabolism and other processes dependent on ATP production (Iadecola, 2015).

Several insulin-sensitive transporters are present in the brain at low levels, including GLUT4, a transporter mainly present in non-cerebral fat and muscle tissue, GLUT8, which has been suggested to contribute toward glucose homeostasis in hippocampal neurons (Piroli et al., 2002), and GLUT12, a newly discovered glucose transporter found primarily in insulin-sensitive tissues (Szablewski, 2017). The location of these transporters within the central nervous system (CNS) and their presence on different cell types are shown in **Table 1**, **Figure 1**.

Detailed descriptions of glucose transporters and their respective functions are not described here—these aspects have been reviewed extensively elsewhere by Szablewski (2017) and Koepsell (2020). Szablewski (2017) also reviewed glucose transporter alterations in AD with particular focus on links between AD and insulin resistance, but a systematic review of human and animal data was not performed. Furthermore, Szablewski did not cover in detail results from tracer studies of glucose uptake into the brain, the effects of AD pathologies such as amyloid-β and tau on GLUTs, the timing of effects, or how GLUT alterations affect the brain. Here we perform a systematic review to capture all human and rodent studies of glucose transport alterations in AD to date and aim to evaluate the evidence to support (i) which transporters are affected, if any, (ii) how glucose uptake into the brain is altered, (iii) which brain regions are most affected, (iv) when changes occur relative to other AD pathologies, and (v) how GLUT changes affect brain function.

METHODS

Literature published between 1st January 1946 and 1st November 2020 was searched using the PubMed search engine. Exploded headings were used for "glucose transporters" and "Alzheimer's disease" with the Boolean operator "AND." Searches were performed in EMBASE and MEDLINE databases and duplicates were removed. Only studies investigating cerebral glucose transporters were included. Titles and abstracts were scanned to identify relevant papers and articles, and those which did not clearly examine glucose transport specific to Alzheimer's disease were discussed with the research team and excluded if not considered relevant. Human and rodent studies (post-mortem and *in-vivo*) were included and results are summarized in **Tables 2**, **3**. Reviews, letters and *in-vitro* studies were excluded. **Figure 2** shows the PRISMA flow chart detailing the search results.

RESULTS

Twenty-three human studies and twenty rodent studies met the inclusion criteria. The transporter GLUT1 was most commonly investigated ($n = 23$), followed by GLUT3 ($n = 13$). A total of 10 human studies and 12 rodent studies observed reductions in GLUT1 expression, primarily in cortical and hippocampal regions. One study found increased GLUT1 expression. A total of 5 human studies and 6 rodent studies found reductions to GLUT3 expression. One study found no change, and one study

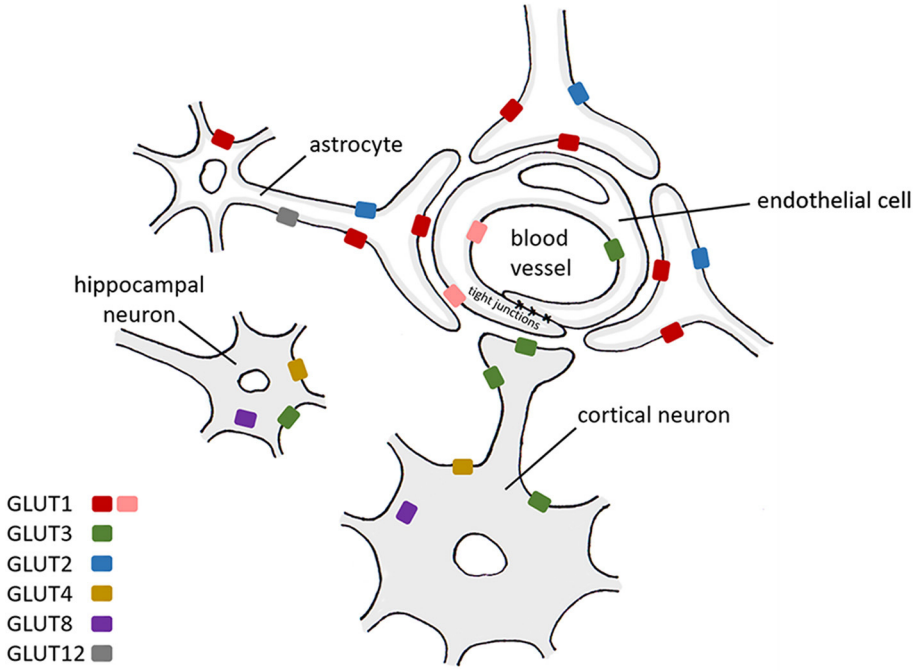

FIGURE 1 | A schematic diagram representing the expression of glucose transporters within CNS cells. Red: GLUT1 55 kDa isoform, pink: GLUT1 45 kDa isoform. GLUT1 (Kumari and Heese, 2010), GLUT2 (Kumari and Heese, 2010), GLUT3 (Kumari and Heese, 2010), GLUT4 (Leloup et al., 1996; Vannucci et al., 1998; Reagan, 2002; Kumari and Heese, 2010), GLUT8 (Kumari and Heese, 2010), GLUT12 (Zhang et al., 2014) (details extracted from https://www.brainrnaseq.org on 19/04/2021).

found increased GLUT3 expression. There were fewer studies investigating GLUT2 ($n = 2$, both showing increased expression), GLUT4 ($n = 6$; no changes observed in 4 studies, one study showing increased GLUT4), GLUT12 ($n = 1$ showing increased expression), and GLUT14 ($n = 1$). Tracer-based methods were used to measure glucose uptake into brain tissue in 9 studies. All tracer studies except one reported reduced glucose uptake into the brain with AD, although in rodent studies reduced uptake was not observed until later stages of disease. A detailed review of all studies is given below.

Evidence of Glucose Transporter Alterations in AD From Post-Mortem Human and Rodent Studies

Early work by Kalaria and Harik (1989) showed a significant reduction in hexose transporters, primarily GLUTs, in the neocortex and hippocampus of post-mortem AD brain tissue. In a subsequent study, Harik (1992) showed a significant reduction in the density of GLUT1 in the cerebral microvessels in the AD brain compared to age-matched controls, with no change in the density of GLUT1 in erythrocyte membranes. Simpson et al. (1994) showed that AD patients display reduced density of vascular and non-vascular forms of glucose transporters, GLUT1 and neuronal GLUT3. After correcting for synaptic loss, which is a prominent feature of AD (Masliah et al., 1990, 1991; Terry et al., 1991; Honer et al., 1992), the authors confirmed that the reduction in GLUT density persisted. Similar results were shown by Horwood and Davies (1994) who observed GLUT1 reductions in hippocampal tissue of the AD brain. Harik and Kalaria (1991) observed a decrease of ∼50% in the density of glucose transporters in cerebral microvessels of patients with AD. They identified glucose transporters by reversible and irreversible binding to the ligand [3H] cytochalasin B. The type of glucose transporter identified using this method was not stated, however, cytochalasin B is an inhibitor of glucose transport in erythrocytes (May, 1988; Carruthers and Helgerson, 1991) and is likely to reflect GLUT1 levels.

Mooradian et al. (1997) observed a reduction in GLUT1 in the frontal and parietal cortex of AD brain, albeit with unchanged GLUT1 mRNA concentrations. In a study of hippocampal microvasculature, Burke et al. (2014) examined GLUT1 as a marker of capillary density in post mortem brain tissue of patients with AD, stroke, vascular dementia, and mixed-type dementia. Contrary to previous studies, they observed increased GLUT1 density in AD brain tissue compared to controls. By contrast, a very recent study investigating white matter tissue from the AD brain demonstrated collapsed string microvessels along with loss of GLUT1 immunoreactivity in the white matter of the frontal lobe compared to overlying cortex (Hase et al., 2019).

Liu et al. (2009) studied AD patients with and without type-2 diabetes mellitus (T2DM), and patients with T2DM alone. They showed that GLUT1 was significantly reduced in AD

TABLE 2 | A summary of results from human post-mortem and tracer studies.

References	Methods	Findings
Kalaria and Harik, 1989	Immunohistochemistry study of brain tissue obtained from the frontal and temporal neocortex, hippocampus, putamen, cerebellum, and cerebral microvessels in AD subjects and controls to determine levels of hexose transporter (likely GLUT1, although not specified).	Significant reduction in hexose expression (likely GLUT1) transporter in brain microvessels, cerebral neocortex and hippocampus of AD brain.
Kawai et al., 1990	Immunohistochemistry study of the relationship between Aβ plaques, capillary density (collagen-4), and glucose transporters (GLUT1).	Collagen-4 and GLUT1 expression was reduced within Aβ plaques and increased in the immediate surroundings of Aβ plaques relative to gray matter.
Harik and Kalaria, 1991	Immunohistochemistry study of GLUT1 in cerebral microvessels of subjects with AD and age matched controls using irreversible binding to the ligand [3H] cytochalasin B.	GLUT1 expression was decreased by ∼50% in cerebral microvessels of patients with AD compared to age-matched controls.
Harik, 1992	Immunohistochemistry study of GLUT1 in AD brain tissue and controls.	Reduction in the expression of GLUT1 in the cerebral microvessels in AD brain compared to age-matched controls, with no change in the density of GLUT1 in the erythrocyte membranes.
Horwood and Davies, 1994	Immunohistochemistry study of AD brain tissue obtained from the central part of the hippocampal formations (dentate gyrus, cornu ammonis, and subicular complex) to determine levels of microvascular GLUT1.	GLUT1 expression was significantly reduced in the microvessel endothelium in hippocampi of AD subjects compared to controls.
Simpson et al., 1994	Immunoblotting study of brain tissue obtained from AD subjects and controls.	Reduced glucose metabolism in the temporal and parietal regions of AD subjects. Reduced expression of GLUT1 and GLUT3 in the cerebral cortex of AD brains compared to controls, with greater and more significant reductions in GLUT3.
Harr et al., 1995	Immunohistochemistry study of brain tissue obtained from the dentate gyrus of AD subjects' to determine levels of GLUT3.	Significant reduction (49.5%) in GLUT3 expression in the outer portion of the molecular layer of the dentate gyrus in AD brains.
Mooradian et al., 1997	Western-blot study of GLUT1 in brain tissue from AD subjects and controls.	GLUT1 expression was reduced in AD but GLUT1 mRNA concentrations were not significantly different.
Liu et al., 2008	Western-blots and immune-dot-blot study of GLUT1-4 levels in the frontal cortex of frozen human brain tissue in subjects with AD and controls.	Decreased expression of GLUT1 and GLUT3 in AD brain tissue which correlated to hyperphosphorylation of tau and neurofibrillary tangle density. Downregulation of hypoxia-inducible factor 1 (HIF-1) in AD brain.
Liu et al., 2009	Western-blots and immune-dot-blot study of brain tissue obtained by autopsy from AD patients, subjects who had T2DM and subjects who had both AD and T2DM.	GLUT1 expression was significantly lower in AD brains. GLUT2 expression was significantly higher in AD brain and brain of subjects with both AD and T2DM. GLUT3 expression was significantly lower in all three groups with the lowest levels in T2DM brain. O-GlcNAcylation of global proteins and tau was downregulated in T2DM brain and AD brain. Tau phosphorylation is higher in T2DM brain and AD brain.
Wang et al., 2012	A case-control study was performed in a Chinese population of 597 patients with AD and 605 healthy controls examining the role of *SLC2A14*, the gene encoding GLUT14, in developing late-onset AD. Results were stratified by ApoEε4-carrying status.	The rs10845990 polymorphism within the gene coding for GLUT14 was significantly associated with late onset AD in non-ApoEε4 allele carriers ($p < 0.001$).
Pujol-Gimenez et al., 2014	Western blot study of brain tissue obtained from the frontal cortex of AD subjects and age-matched controls measuring the expression of GLUT12.	GLUT12 expression was significantly increased in AD compared to age-matched controls.
Burke et al., 2014	Immunohistochemistry study of GLUT1 in patients with AD, vascular dementia and patients who had suffered from stroke. The cumulative vessel length and diameter of hippocampal microvessels was measured using stereological spherical probe software.	Increases in percentage per area were found in GLUT1 density in AD cases in the: • CA1 of the hippocampus compared with post-stroke non-demented subjects ($p = 0.011$). • CA1 of the hippocampus compared with post-stroke demented subjects ($p = 0.037$). • CA2 of the hippocampus compared to vascular dementia subjects ($p = 0.04$). • Entorhinal cortex compared with post-stroke non-demented subjects ($p = 0.004$). Post-stroke demented cases had significantly lower vascular length than AD ($p = 0.016$). Post-stroke non-demented cases had significantly lower vascular length compared with controls ($p = 0.015$).

(Continued)

TABLE 2 | Continued

References	Methods	Findings
Mullins et al., 2017	Immunohistochemistry study into the relationship between brain insulin resistance and glucose transporter expression and the propensity to develop plaques and NFT.	Regional expression of GLUT1 showed a negative correlation with NFT density. Regional expression of GLUT4 showed a positive correlation with NFT density. Areas with reduced insulin signaling proteins (including IRS-1) showed higher NFT load.
An et al., 2018	LC-MS/MS study to measure GLUT1 and GLUT3 levels in brain tissue obtained from the middle frontal gyrus of 14 participants with AD, 14 controls, and 15 with asymptomatic AD (ASYMAD).	GLUT3 levels were lower in both AD and ASYMAD groups compared to controls, before and after adjusting sex, age at death and neuronal nuclear protein levels. Lower levels of GLUT3 correlated with the severity of both Aβ and NFT pathology.
Hase et al., 2019	Immunohistochemistry study of endothelial GLUT1. Microvascular pathology, capillary width and densities were measured using histopathological methods in the frontal lobe white matter across several dementia types including 18 participants with AD.	Collapsed string microvessels along with loss of GLUT1 immunoreactivity was detected in AD frontal lobe white matter compared to overlying cortex.
Friedland et al., 1989	Dynamic FDG-PET study in patients with probable Alzheimer's disease (AD) and healthy age-matched controls.	There was no significant difference in rate constants for glucose transport (k_1 and k_2) or phosphorylation (k_3).
Jagust et al., 1991	Dynamic FDG-PET study in six subjects with clinical criteria for probable AD and normal controls.	Decrease in K_1 in frontal and temporal cortex in AD subjects compared to controls, minimal differences in occipital cortex and white matter and decreased $rCMR_{glc}$ in all cortical regions. Non-significant decrease in k_3 in all brain regions of AD subjects.
Kimura and Naganawa, 2005	Dynamic FDG-PET study in three subjects; a 45-year-old normal subject, a 65-year-old subject with mild AD, and a 70-year-old subject with severe AD.	Glucose transport was globally reduced in both AD cases compared to the normal subject. Glucose phosphorylation was diminished in gray matter of the severe case of AD, excluding the sensory, motor, and visual cortices. In the mild case, phosphorylation was reduced in the right parieto-temporal area.
Piert et al., 1996	Dynamic FDG-PET study in AD subjects and normal controls.	Significant reductions in glucose transport (K_1) and phosphorylation (k_3) in patients with AD compared to healthy age-matched controls in multiple cortical and subcortical regions of the brain with the greatest significant difference in the parietal and temporal cortex.
Mosconi et al., 2007b	Dynamic FDG-PET study with arterial blood sampling in 7 AD patients and 6 age matched controls and CMR_{glc} was calculated.	AD patients showed significant CMR_{glc} reductions in the hippocampus and posterior cingulate cortex. K_1 was reduced in the hippocampus and k_3 was reduced in the hippocampus, PCC and amygdala.
Gejl et al., 2017	FDG-PET study as part of a randomized control trial using 6 month treatment of GLP-1 analog or placebo in AD subjects, measuring blood-brain glucose transfer capacity (T_{max}) and cerebral metabolic rate of glucose (CMR_{glc}) in the AD patients and controls.	CMR_{glc} estimates were positively correlated with cognition while T_{max} and CMR_{glc} estimates were inversely correlated with AD duration. GLP-1 analog treatment significantly raised T_{max} estimates of cerebral cortex from 0.72 to 1.1 umol/g/min, matching T_{max} estimates in healthy volunteers.

but not significantly reduced in either T2DM or T2DM-AD groups. GLUT3 was significantly reduced in all three groups with the lowest levels in T2DM brain. Interestingly, GLUT2 was significantly increased in the AD brain and brains of subjects with both AD and T2DM, possibly due to astrocyte overactivation (Liu et al., 2009). Harr et al. (1995) observed reductions in GLUT3 levels in the outer portion of the molecular layer of the dentate gyrus in AD brains.

A small number of studies have investigated the link between glucose transporters and AD pathologies in human tissue. Kawai et al. (1990) investigated the relationship between Aβ plaques and capillary glucose transporter density. Capillary glucose transporter density was reduced within Aβ plaques but increased in the immediate surroundings of Aβ plaques. Liu et al. (2008) found decreased levels of GLUT1 and GLUT3 in hippocampus and entorhinal cortex of AD brain tissue correlated with hyperphosphorylation of tau and NFT density. A recent study using participants from the Baltimore Longitudinal Study of Aging cohort measured GLUT1 and GLUT3 levels in the middle frontal gyrus of 14 participants with AD, 14 controls, and 15 with asymptomatic AD pathology, i.e., participants who exhibited significant AD pathology at post-mortem (including Aβ plaques, NFTs, and neuropil threads) but without evidence of cognitive dysfunction as assessed shortly before death. GLUT3 levels were significantly lower in both AD and asymptomatic AD groups relative to controls, before and after adjusting for sex, age at death, and neuronal nuclear protein levels. Lower levels of GLUT3 correlated with the severity of both Aβ and NFT pathology (An et al., 2018). GLUT1 levels were not significantly different in any of the groups.

Pujol-Gimenez et al. (2014) and Wang et al. (2012) investigated glucose transporters other than GLUT1, GLUT2,

TABLE 3 | A summary of results from rodent post-mortem and tracer studies.

References	Methods	Findings
Ding et al., 2013	Longitudinal immunohistochemical and imaging study investigating hippocampal GLUT1, GLUT3, and GLUT4, glucose transport into the brain in female 3xTgAD mice (aged between 3 and 15 months).	Both 3xTgAD and wild-types underwent significant age-related reductions in glucose transport as detected using FDG-microPET, beginning at 6-9 months of age, but mechanisms were different. Reductions in GLUT1 (55 kDa isoform), increases in GLUT1 (45 kDa) and reductions in GLUT3 were observed in 3xTgAD mice, and non-monotonic changes in GLUT1 (55 kDa), decreases in GLUT3, and increases in membrane GLUT4 were observed in wild-types.
Do et al., 2014	Immunohistochemistry study investigating hippocampal GLUT1 expression in 3xTgAD mice aged between 3 and 18 months and APP/PS1 mice aged 8 months. Vascular volume fraction and uptake of D-glucose were measured using radionuclide-based brain perfusion tracers.	GLUT1 expression and D-glucose uptake were reduced in 18 month old 3xTgAD mice, but no differences were found in glucose transport or GLUT1 in either strain at 8 months. Reduced vascular volume fractions were observed at 6 months in 3xTg mice and in 8 month old APP/PS1 mice.
Griffith et al., 2019	Longitudinal analysis of 3xTg mice studied at ages 1–3, 6–8, and 16–18 months. Glucose tolerance was assessed alongside Western blot analysis of hippocampal insulin pathways PI3K/AKT and MAPK/ERK, and glucose transporters GLUT3 and GLUT4. GLUT1 was not studied.	Glucose tolerance and plasma insulin levels were found to be reduced as early as 1 month, well before detection of plaques (14 months). GLUT3 reductions but not GLUT4 were observed later at 18–20 months.
Hooijmans et al., 2007	Immunohistochemistry study into the causal relationship between GLUT1 reductions in the hippocampus and cortex and Aβ. Computer-assisted analysis of capillary density, and Aβ in young (8 months) and old (18 months) APP/PS1 mice. GLUT1 expression was normalized to capillary density to correct for potential loss of vascular volume.	At 8 months, GLUT1, capillary density or GLUT1 amount per capillary density were not different between APP/PS1 and wild-types. At 18 months, GLUT1 was reduced in the hippocampus of 18 month APP/PS1 mice relative to wild-types, while capillary density was not significantly different. The ratio of GLUT1 amount per capillary density was decreased in the dendate gyrus only. Aging produced significant reductions in GLUT1 and capillary density, but the ratio of GLUT1 amount per density was unchanged with age. No cortical changes were observed.
Kouznetsova et al., 2006	Immunohistochemistry study of Tg2576 mice aged 4–18 months to stain cortical GLUT1 and Aβ plaques and to assess impact of size and load of Aβ plaques on GLUT1 expression.	GLUT1 was reduced in cortical regions with high plaque load was associated with greater reductions compared to areas with low plaque load. Around large plaques, the capillary density was lower than around diffuse smaller plaques.
Kuznetsova and Schliebs, 2013	Immunohistochemistry study investigating GLUT1 and Aβ in somatosensory cortex of Tg2576 mice aged between 4 and 18 months.	No changes in GLUT1 at 10 months, when plaque deposition is beginning, but reductions found in 18 month mice when plaque load is considerable indicating Aβ may contribute to reductions in GLUT1.
Gil-Iturbe et al., 2020	Western blot study investigating GLUT1, GLUT3, GLUT4, and GLUT12 in the frontal cortex of two amyloidogenic mouse models Tg2576 (16 months old) and APP/PS1 (16 months old). Age effects assessed in C57/6/SJL wild-type mice aged 2–3 and 18 months old. To assess effects of Aβ directly, Aβ1–42 was injected intra-cerebroventricularly in 3 month old C47BL/6J mice.	Tg2576 and APP/PS1 mice exhibited decreased GLUT1 and GLUT3 and increased GLUT12. No changes were found in GLUT4. No age-dependent effects were found in GLUT1 and GLUT3 in C57/6/SJL mice. In Aβ1–42 injected mice, the same patterns in up- and downregulation of GLUTs were observed indicating a direct link between GLUTs and amyloid toxicity.
Merlini et al., 2011	Immunohistochemistry study into the morphology, biochemistry and functionality of cortical and hippocampal blood vessels in arcAβ mice at 6, 9–12, and 16–22 months. GLUT1 and GLUT3 measured by immunoblot, corrected for vascular volume using CD31. Brain glucose levels dynamically measured following i.v. administration of glucose using microdialysis.	Reductions in BBB and astrocytic GLUT1, but not GLUT3, reduced from mid-stage pathology onwards. Glucose uptake as measured using microdialysis confirmed reduced glucose transport. IgG extravasation observed at late-stages.
Ahn et al., 2018	Immunohistochemistry study investigating GLUT1 and tight junction protein ZO-1 expression in 4.5 and 9 month old 5xFAD mice.	Reductions in GLUT1 and ZO-1 observed in the hippocampus and cortex at both ages, which got worse with age and correlated with worsening of astrocyte activation (GFAP) and amyloid deposition.
Shang et al., 2019	Immunohistochemistry study assessing the impact of chronic cerebral hypo-perfusion (CCH) and AD pathology on cortical GLUT1 expression in 12 month old APP23 mice.	GLUT1 was reduced in cortex of APP23 mice compared to wild-types, which was further reduced in APP23 mice subject to CCH, indicating that AD and cerebrovascular pathologies may interact to exacerbate GLUT1 changes.
Lee et al., 2013	Western blot study of whole-brain expression of GLUT1, GLUT3, and GLUT4 and insulin markers in 12 month old NSE/hPS2m mice.	Reductions in GLUT1 and GLUT3, but not GLUT4 observed. Blood glucose found to be higher in AD mice.

(Continued)

TABLE 3 | Continued

References	Methods	Findings
Chua et al., 2012	Longitudinal western blot study of GLUT3 and GLUT4 AβPPsw/PS1ΔE9 mice	Reduced brain glucose and insulin content in 12 and 15 month old brains accompanied by increased GLUT3 and GLUT4, which preceded significant upregulation of Aβ42 in brain. GLUT1 not measured.
Deng et al., 2009	Western blotting study of insulin signaling and glucose transporters in intracerebroventricular streptozotocin (STZ) rat model of AD (6 months old, 21 days after STZ injections).	Reduced GLUT1 and GLUT3 in the cerebrum, and reduced GLUT3 in the cerebellum. Reduced pERK1 and pPI3K, pGSK-3β(S9) markers, and increased phosphorylation of tau.
Salkovic-Petrisic et al., 2014	Study investigating effect of long-term galactose administration on brain metabolism and glucose transporters via Western blot in intra-cerebroventricular streptozotocin (STZ) rat model of AD. Adult rats were used, and galactose administered for 1 month.	STZ rat exhibited significantly reduced GLUT3 expression in the hippocampus, which was normalized with galactose administration.
Knezovic et al., 2017	Western blot study investigating expression of GLUT2, insulin receptor, and neuroinflammatory marker GFAP in the hippocampus and cortex in intra-cerebroventricular streptozotocin (STZ) rat model of AD (adult males). Measurements were taken 1 h after STZ administration.	GLUT2 increased in the hippocampus, but unchanged in cortical regions. Insulin receptor decreased in parietal and temporal cortex, but not the hippocampus.
Biswas et al., 2018	Western blot and immunohistochemistry study investigating the expression of cortical and hippocampal GLUT1 and GLUT3, and brain glucose levels in intra-cerebroventricular streptozotocin (STZ) rat model of AD (adult males), and correlation with markers of endoplasmic reticulum stress, and astrocyte/microglia activation (Cd11b).	Glucose transporters GLUT1 and GLUT3 and brain glucose concentration were reduced in STZ rats. These changes were accompanied by reduced mitochondrial activity, increased endoplasmic reticulum stress, and increased microglial activation.

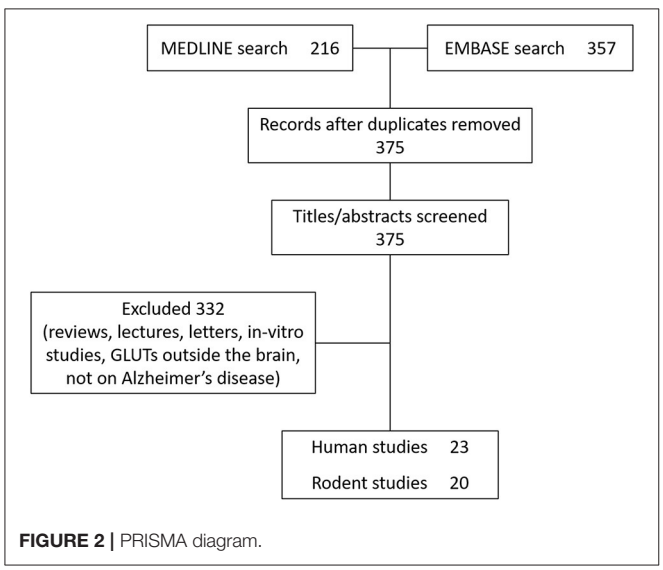

FIGURE 2 | PRISMA diagram.

and GLUT3. Pujol-Gimenez et al. (2014) identified that the expression of GLUT12, a newly discovered glucose transporter found primarily in insulin-sensitive tissues (Stuart et al., 2009), was significantly increased in the frontal cortices of AD subjects compared to age-matched controls. Wang et al. (2012) examined the role of SLC2A14, the gene encoding GLUT14, in blood samples of patients with developing late onset AD. They performed a case-control study in a Chinese population of 597 patients with AD and 605 healthy controls, showing that SLC2A14 polymorphisms appear to confer increased risk of developing AD.

Alterations in GLUTs have also been observed in rodent models of AD. In a longitudinal study of 3xTgAD mice between 3 and 15 months of age, glucose transporters GLUT1 (55 and 45 kDa), and GLUT3 were found to change in both AD and wild-type animals but with differing temporal trajectories (Ding et al., 2013). In AD mice, GLUT1 (55 kDa) and GLUT3 were found to decrease with age, whereas GLUT1 (45 kDa) was found to increase with age. In wild-types, non-monotonic changes with age were observed for GLUT1 (55 kDa), whereas GLUT1 (45 kDa) was unchanged. GLUT3 decreased, and GLUT4 increased with age. Unfortunately, a formal comparison between glucose transporter expression between 3xTgAD mice and wild-types was not performed. In another longitudinal study, Do et al. (2014) studied vascular volume fraction and GLUT1 expression in the hippocampus of 3xTgAD and APP/PS1 mice and found no change in GLUT1 compared to wild-types at 8 months, but significantly reduced GLUT1 expression in 3xTgAD mice at 18 months after substantial amyloid-pathology had developed. APP/PS1 mice were not studied at this later timepoint. Griffith et al. (2019) investigated GLUT3 and GLUT4, but not GLUT1, longitudinally in 3xTg mice. They found similar timing of effects on GLUT3 as observed for GLUT1 by Do et al. (2014). Young rats exhibited reduced glucose tolerance (as early as 1 month), but GLUT3 did not change relative to wild-types until at least 18–20 months. GLUT4 was unaltered at all ages. Similar results were found in other AD models. Hooijmans et al. (2007) did not find any changes in GLUT1 expression between APP/PS1 mice and wild-types at 8 months, but found significantly reduced total GLUT1, reduced capillary density, and reduced GLUT1 per vascular volume fraction in 18 month old mice. Changes were found in the hippocampus, but not the cortex. In the Tg2576 model, Kuznetsova and Schliebs (2013) showed that cortical

GLUT1 was unaltered at 10 months compared to wild-types, but at 18 months after development of amyloid pathology, AD mice had significantly lower cortical GLUT1.

A study by Kouznetsova et al. (2006) aimed to investigate if the degree of GLUT1 changes were related to amyloid load. In cortical regions with high amyloid load, GLUT1 staining was reduced compared to regions with low amyloid load. The authors also showed that GLUT1 staining was reduced nearer large senile plaques, relative to changes observed near smaller diffuse plaques. Gil-Iturbe et al. (2020) performed a more thorough investigation of GLUTs, measuring expression of GLUT1, GLUT3, GLUT4, and GLUT12 in two amyloidogenic models (Tg2576 and APP/PS1) aged 16 months old. They also investigated effects of aging in C57/6/SJL mice and the effects of amyloid on GLUTs via intracerebral injection of $A\beta_{1-42}$. The authors found reduced GLUT1 and GLUT3, and increased GLUT12 in both strains. No age dependent effects on GLUTs were observed, conflicting with results from Ding et al. (2013). Injection of $A\beta_{1-42}$ produced similar reductions in GLUT1 and GLUT3 as found in the Tg2576 and APP/PS1 mice, indicating a direct link between GLUT1 decreases and amyloid. Merlini et al. (2011) investigated GLUT changes in the arcAβ model. They observed reductions in BBB and astrocytic GLUT1 in the cortex and hippocampus from 9 to 12 months onward, which coincided with changes in glucose uptake as measured using microdialysis. However, GLUT3 was unaltered. By 16–22 months, IgG extravasation was observed, indicating loss of BBB integrity. In 5xFAD aged 4.5 and 9 months, reductions in GLUT1 and tight junction protein ZO-1 were found at both timepoints (Ahn et al., 2018). GLUT3 was not studied. Another study in 12 month old NSE/hPS2m mice also showed reductions in GLUT1 and GLUT3, but not GLUT4 (Lee et al., 2013). A study by Chua et al. (2012) in AβPPsw/PS1ΔE9 mice did not agree with changes observed in other models. Brain glucose levels were measured to be lower than wildtypes at 12 and 15 months of age, but GLUT3 and GLUT4 were found to be upregulated, not decreased (Chua et al., 2012). Unfortunately, changes to GLUT1 were not studied.

Shang et al. (2019) investigated the additional effects of chronic cerebral hypoperfusion on GLUT1 expression in APP23 mice. In normal APP23 mice, GLUT1 reductions were observed at 12 months, which were further reduced in APP23 mice with chronic cerebral hypoperfusion (Shang et al., 2019).

A number of studies have investigated GLUT expression in a rat model of AD produced by administering streptozotocin via intracerebroventricular injection. These studies found reduced expression of GLUT1 (Deng et al., 2009; Biswas et al., 2018), reduced expression of GLUT3 (Deng et al., 2009; Salkovic-Petrisic et al., 2014; Biswas et al., 2018), and increased expression of GLUT2 (Knezovic et al., 2017).

Evidence of Altered Glucose Transport in AD From *in-vivo* Tracer Studies

Friedland et al. (1989) used FDG-PET to measure cerebral transport and phosphorylation rates of glucose in patients with probable AD and healthy age-matched controls. No difference in the transport rate constants, K_1 and k_2, or the utilization rate k_3, were observed between groups. Two later FDG-PET studies in AD patients showed different results; Jagust et al. (1991) showed a reduction in the transport rate constant K_1 in the frontal and temporal cortex with minimal differences in occipital cortex and total brain white matter, and a reduction in regional CMR_{glu} in all cortical regions compared to controls; Piert et al. (1996) found significant reductions in K_1 and k_3 in multiple cortical and subcortical regions of the brain in people with AD compared to controls with the most significant reductions being seen in the parietal and temporal cortices. Kimura and Naganawa (2005) performed dynamic PET studies in three subjects, a 45-year-old normal subject, a 65-year-old subject with mild AD, and a 70-year-old subject with severe AD. Glucose transport was globally reduced in both AD cases compared to the normal subject. Glucose phosphorylation was diminished in gray matter of the severe case of AD, excluding the sensory, motor, and visual cortices. In the mild case, phosphorylation was reduced in the right parieto-temporal area. In another dynamic FDG-PET study of seven patients with mild AD and six normal age-matched controls, Mosconi et al. (2007b) showed significant reductions in K_1 in the hippocampus in AD compared to controls, although relative CMR_{glu} was better able to identify AD from controls, likely due to the additional contribution of reduced glucose phosphorylation.

A small number of tracer studies have been performed in rodents, which all support reduced transport of glucose in AD. Do et al. (2014) perfused brains of 3xTgAD and wild-type mice with [3H]-D-glucose (0.3 mCi/ml) immediately prior to decapitation and measurement of tissue radioactivity using a scintillation counter (Do et al., 2014). No difference in [3H]-D-glucose uptake was found in 3xTgAD mice compared to wild-types aged 6 or 8 months, but a significant decrease was found in 3xTgAD mice at 18 months. The reduced uptake at 18 month was associated with reduced expression of GLUT1 at the same timepoint. Ding et al. (2013) used micro-FDG-PET to study glucose uptake in 3xTgAD mice and wild-types at various ages. FDG-PET signals were measured at 40 min post injection of FDG, and are likely to reflect both transport and utilization. Reductions in the FDG-PET signal were found in both AD and wild-type animals with age, but while no formal comparison was made, FDG-PET signals did not appear to differ across genotype. Last, Merlini et al. (2011) performed microdialysis in arcAβ following intravenous injection of glucose and found reduced uptake compared to wild-types, despite increases in IgG extravasation in AD mice.

Effects of GLUT Alterations on Brain Physiology

The effects of GLUT disruptions on the brain have been studied in rodent models. Abdul Muneer et al. (2011) induced GLUT1 disruption in a mouse model through the administration of methamphetamine. This led to a reduction in BBB tight junction proteins, indicating that GLUTs may play a role in regulating BBB integrity. Winkler et al. (2015) later demonstrated that GLUT1 deficient mice overexpressing APP showed changes characteristic of AD. These included reduced brain capillary

levels of low-density lipoprotein receptor-related protein 1 (LRP1), a transporter at the BBB which clears Aβ from the brain (Zlokovic, 2008, 2011), diminished cerebral blood flow, early BBB breakdown, accelerated Aβ deposition in the hippocampus and cortex, neuronal dysfunction and cognitive impairment. They also observed that vascular changes preceded neuronal dysfunction in these mice. Decreased levels of GLUT1 and GLUT3 were found in a rat model of sporadic AD, achieved through the intracerebroventricular injection of streptozotocin, alongside impaired insulin signaling and abnormalities in phosphorylation and microtubule binding activity of tau (Deng et al., 2009).

Effects of severe glucose transporter depletion on early brain development can be observed in human GLUT1 deficiency syndrome, a rare genetic disorder characterized by impaired glucose metabolism due to a deficiency in GLUT1. Clinical features include intellectual disability, movement disorders and epileptic seizures refractory to treatment. Late-onset GLUT1 deficiency syndrome affects children at an older age, with evidence showing mild to moderate intellectual disability (Leen et al., 2010). A later study by the same group followed up patients with GLUT1 deficiency syndrome between 18 and 41 years old. Their results showed that while the prominent feature during childhood is epilepsy, this diminishes later in life and new movement disorders become apparent during adolescence. Cognitive function, however, did not appear to worsen with age (Leen et al., 2014). There is no evidence of GLUT1 deficiency syndrome manifesting in late adulthood.

Links Between Insulin Resistance and GLUT Changes

Deficits in transport and metabolism in AD may result from impaired insulin signaling, particularly due to alterations in the function of insulin-sensitive transporters. Mullins et al. (2017) investigated the relationship between the pathological hallmarks of AD and genes related to insulin resistance by converting histological and gene expression data into 3D spatial maps. Their findings showed that GLUT1 was positively correlated, whereas GLUT4 was negatively correlated, with insulin signaling proteins (including IRS-1).

In a mouse model of AD, Chua et al. (2012) found that insulin signaling molecules were increased, alongside increases in GLUT3 and GLUT4 expression and decreases in brain insulin and glucose content, and that these changes occurred earlier than pathological accumulation of Aβ. In the STZ rat model of AD, increases in GLUT2 were accompanied by decreases in insulin receptors (Knezovic et al., 2017). In the same model, Deng et al. (2009) found decreases in insulin signaling alongside decreases in GLUT1 and GLUT3 (Deng et al., 2009).

Insulin resistance has been targeted as a means to restore glucose transport by number of groups. In a small randomized control trial, Gejl et al. (2017) examined the effects of GLP-1 analog treatment in patients with a clinical diagnosis of AD. In their study, 18 participants received liraglutide and 20 received placebo in a 26-week period. Their results showed that treatment with liraglutide significantly raised the average blood-brain glucose transport capacity estimate ($p < 0.0001$) from 0.72 to 1.1 (μmol/g/min) compared to placebo, which positiviely correlated with measures of cognition. Salkovic-Petrisic et al. (2014) performed long-term dosing of STZ rats with galactose as an alternative energy source to glucose and found upregulation of GLUT3 transporters back to levels observed in healthy rats and improved cognitive deficit.

DISCUSSION

This systematic review examines the evidence for alterations in glucose transport in AD and the effect of these alterations on the brain. The majority of studies investigating GLUT1 and GLUT3 suggest that both transporters are reduced in the hippocampus and cortex of AD brains. Longitudinal rodent studies did not find changes at early timepoints, but consistently observed reductions in transporter expression after Aβ pathology had developed, indicating that Aβ itself may be responsible.

There were fewer studies investigating other GLUTs including GLUT2, GLUT4, GLUT12, and GLUT14. A rise in GLUT2 in AD brain tissue was observed by Liu et al. (2009) and Knezovic et al. (2017). In the study by Liu et al. (2009), GLUT2 changes were exacerbated in patients who also had T2DM. It is possible that GLUT2 increases to serve as a compensatory mechanism of GLUT1 and GLUT3 loss. The role of GLUT2, therefore, requires further investigation. No changes in GLUT4, an insulin sensitive transporter, were found in aged NSE/hPS2m (Lee et al., 2013) or 3xTg mice (Griffith et al., 2019), whereas Chua et al. (2012) found increases in AβPPsw/PS1ΔE9 mice. Pujol-Gimenez et al. (2014) showed GLUT12, also an insulin-sensitive transporter, was increased in AD brain tissue. A study by Purcell et al. (2011) showed that overexpression of GLUT12 in healthy mice improved whole-body insulin sensitivity, indicating GLUT12 may play a compensatory role when insulin resistance develops in AD. The findings by Wang et al. (2012) on GLUT14 demonstrate that SLC2A14 polymorphisms may increase susceptibility to late-onset AD. Further work is required to determine the role of these lesser-studied GLUTs in AD and gauge their therapeutic potential.

The cause of glucose transporter expression in AD remains unclear. One suggestion for the reduction of GLUT1 in the AD brain is an abnormality in the translation process of the transporter. Brains of AD patients have been found to express low levels of transcription factor hypoxia-inducible factor 1α, a protein complex which regulates GLUT1 and GLUT3 expression (Liu et al., 2008; Cohen et al., 2014). However, Mooradian et al. (1997) did not observe any changes in GLUT1 mRNA concentrations, which suggests a post-translational abnormality. Another study proposed that the activation of calpain I, a calcium sensitive protease, is a potential cause of GLUT3 under-expression (Jin et al., 2013) due to its damaging effect through proteolysis of GLUT3 promoters. Calpain I has also been associated with downregulation of protective mechanisms against tau phosphorylation (Gu et al., 2018).

AD pathologies such as Aβ or tau may directly reduce GLUT expression. The low capillary density found within Aβ plaques in the study by Kawai et al. (1990) may suggest that Aβ plaques lead to the degeneration of capillaries, which may in turn affect GLUT expression. Findings by An et al. (2018) suggest that a reduction of GLUT3 but not GLUT1 is more closely correlated with Aβ and NFT abnormalities. Studies using amyloidogenic mouse models support a link between spatial proximity of Aβ to glucose transporter alterations (Kouznetsova et al., 2006; Kuznetsova and Schliebs, 2013), and indicate that GLUT changes occur after development of Aβ pathology (Hooijmans et al., 2007; Kuznetsova and Schliebs, 2013; Do et al., 2014; Griffith et al., 2019). Injection of $A\beta_{1-42}$ into the brain of healthy mice produced very similar effects on GLUT expression as observed in AD mice, indicating Aβ plays a direct role in modulating GLUT expression (Gil-Iturbe et al., 2020). Mark et al. (1997) examined the relationship between Aβ and glucose transport, by examining cultured rat hippocampal and cortical neurons. Their results demonstrate that Aβ deposition impairs glucose transport through a mechanism involving membrane lipid peroxidation. The authors suggest that this mechanism may further lead to neurodegeneration in AD. In a drosophila study, Niccoli et al. (2016) demonstrated a protective effect against Aβ toxicity by genetically upregulating glucose transporters. Similar results were demonstrated when using metformin, an anti-diabetic drug designed to stimulate glucose uptake into cells. Human studies are yet to confirm these findings.

Without measuring glucose uptake into brain tissue, it is difficult to know how changes in GLUTs affect glucose availability in parenchymal cells. Tracer studies that measure uptake of glucose and glucose-analogs into brain are therefore crucial for determining the impact of GLUT changes on glucose transport. Uptake of glucose can be measured using a range of methods from invasive perfusion-based approaches, to non-invasive imaging techniques such as FDG-PET. Generally, tracer-based studies performed to date show that transport of glucose into the brains of AD patients and rodent models of AD is reduced. Unfortunately, these studies cannot distinguish between glucose transport through the BBB or through cell membranes. The affinity of glucose for GLUT3 ($K_t = 1.4$ mM, measured using 2DG) is much higher than that of GLUT1 ($K_t = 6.9$ mM, also measured using 2DG; lower K_t values relate to higher affinity), and therefore glucose transport across the BBB is likely to be rate limiting under most conditions (Simpson et al., 2008). Furthermore, Lund-Andersen (1979) and Pardridge (1994) argue that because the total surface area and mass of GLUTs on parenchymal cells far exceeds that of endothelial cells, glucose uptake into the intracellular space is limited by GLUT1 at the BBB, not by astrocyte GLUT1 or neuronal GLUT3. Therefore, it is likely that reductions in uptake of glucose or glucose-analogs into brain tissue reflect mainly reductions to GLUT1 at the BBB.

GLUT1 and GLUT3 are considered insulin-independent glucose transporters, and therefore are assumed not to be affected by insulin or insulin-like growth factors. It is therefore expected that insulin-resistance does not alter uptake of glucose through these transporters. However, there is some evidence that GLUT1 and GLUT3 may be moderately affected by insulin. Hernandez-Garzón et al. (2016) observed that astrocytic insulin-like growth factor receptor modulates the expression of GLUT1, but the authors did not determine if this affected uptake of glucose. Muhič et al. (2015) did not observe any effects of insulin or insulin growth factor on glucose uptake into astrocytes, suggesting that GLUT1, and even the insulin-sensitive transporter GLUT4 present on astrocytes, are not particularly sensitive to insulin. The effects of insulin on endothelial GLUT1 has not been studied. Hypoglycemia is known to cause upregulation of BBB GLUT1 and neuronal GLUT3, an adaptive mechanism to ensure sufficient glucose is delivered to the brain (Kumagai et al., 1995; Simpson et al., 1999; Yun et al., 2018). It is possible these changes occur due to hyperinsulinemia—a study in man showed that insulin increases uptake of glucose across the BBB, however the dose of insulin required to detect an effect was non-physiological. It is likely the dominant driver of GLUT1 changes at the BBB is glucose itself; *in-vitro* studies show hyperglycemia increases GLUT1 and glucose uptake in the absence of insulin (Takakura et al., 1991; Sajja et al., 2014). Despite this, the studies of Deng et al. (2009), Chua et al. (2012), and Mullins et al. (2017) support an association between insulin resistance and altered expression of insulin-insensitive transporters GLUT1 and GLUT3.

It is possible the link between insulin resistance and alterations to insulin-insensitive GLUTs occurs via a joint relationship with AD pathologies Aβ and tau. Mullins et al. (2017) found areas exhibiting lower expression of glucose transporters and insulin signaling genes had higher levels of Aβ and tau pathology. Insulin is known to promote Aβ clearance (Watson et al., 2003) while insulin resistance promotes the formation of Aβ oligomers (Yamamoto et al., 2012). It is suggested that a positive feedback loop subsequently occurs as Aβ oligomers lead to increased phosphorylation of insulin signaling proteins (Yoon et al., 2012) which, in turn, leads to further insulin resistance. The results from the randomized controlled trial on the use of liraglutide in AD provides further insight into the possible link between insulin resistance, glucose transporters and AD (Gejl et al., 2016). Their results show an improvement in glucose transport capacity with liraglutide. It is not clear whether this improvement reflects an increase in glucose transporters or an increase in postprandial insulin levels, however, as liraglutide does not cross the BBB, it is possible that it causes a direct effect on the BBB itself.

CLINICAL SIGNIFICANCE AND FUTURE PERSPECTIVE

Altered glucose metabolism occurs several years before evidence of cognitive impairment in AD (Chen and Zhong, 2013). Altered glucose transport has also been observed in mild cognitive impairment (MCI) (Mosconi et al., 2013), and deletion of glucose transporters has been shown to cause substantial neurodegenerative effects in animal models (Nicholson et al., 2010; Ding et al., 2013; Winkler et al., 2015). This suggests that

drugs targeting the restoration of normal GLUT expression may be highly effective at reducing cognitive decline and transition from MCI to AD. Only a small proportion of drugs are able to cross the BBB, mainly due to the characteristic properties of desolvation, lipophilicity, molecular volume and dipole moment required for molecules to cross the BBB (Fong, 2015). Additionally, the BBB has tight control over what molecules leave the brain, making drug development in neurodegenerative disease more challenging (Pardridge, 2009). GLUT1 therefore becomes a highly attractive therapeutic target since it is present on the BBB itself.

GLUTs play a significant role in AD pathology with substantial evidence suggesting that GLUT1 and GLUT3 reductions occur following amyloid accumulation, but may precede the onset of clinical symptoms, while GLUT2 and GLUT12 appear to increase and may have a compensatory role. FDG-PET imaging could provide a means to detect reduced glucose transport in a clinical setting. Repurposing anti-diabetic drugs shows promising results in human studies of AD (Gejl et al., 2016, 2017). With evidence suggesting that metabolic changes can accurately predict subsequent cognitive decline (De Leon et al., 2001; Jagust et al., 2006; Mosconi et al., 2008), therapeutic strategies aiming to modify glucose transport could have a significant impact in clinical management of AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

NK, BD, HE, LP, and OS contributed to the conception and design of the study. NK wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Reassessment of Pioglitazone for Alzheimer's Disease

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Alzheimer's disease is a quintessential 'unmet medical need', accounting for ∼65% of progressive cognitive impairment among the elderly, and 700,000 deaths in the United States in 2020. In 2019, the cost of caring for Alzheimer's sufferers was $244B, not including the emotional and physical toll on caregivers. In spite of this dismal reality, no treatments are available that reduce the risk of developing AD or that offer prolonged mitiagation of its most devestating symptoms. This review summarizes key aspects of the biology and genetics of Alzheimer's disease, and we describe how pioglitazone improves many of the pathophysiological determinants of AD. We also summarize the results of pre-clinical experiments, longitudinal observational studies, and clinical trials. The results of animal testing suggest that pioglitazone can be corrective as well as protective, and that its efficacy is enhanced in a time- and dose-dependent manner, but the dose-effect relations are not monotonic or sigmoid. Longitudinal cohort studies suggests that it delays the onset of dementia in individuals with pre-existing type 2 diabetes mellitus, which small scale, unblinded pilot studies seem to confirm. However, the results of placebo-controlled, blinded clinical trials have not borne this out, and we discuss possible explanations for these discrepancies.

Keywords: Alzheimer's disease, pioglitazone, preclinical models, observational studies, clinical trials

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ALZHEIMER'S DISEASE AND DEFINING THE NEED

Alzheimer's disease is a progressive, irreversible neurodegenerative disease whose most fearsome clinical manifestation, and the target of most treatment-oriented human clinical trials, is dementia. Dementia does not respect ethnicities or socioeconomic groups, and the fear of descending into mindlessness is a haunting prospect.

Alzheimer's disease is, by any definition, an unmet medical need. It is the most common cause of dementia and currently is the third leading cause of death, behind cancer and heart disease. Between 2000 and 2018, the number of deaths attributable to Alzheimer's disease increased by more than 145%, while the number of deaths attributable to heart disease declined by nearly 8% (Longhe, 2020). In 2018, an excess of 33 million people worldwide lived with AD. Without the development of preventative treatments, this number will soar to 132 million people globally by 2050 (Patterson, 2018). The global annual burden of caring for patients is ∼ 1 trillion USD currently, and is forecast to double by 2030 (Patterson, 2018).

Given this reality, even a small change in the pathophysiological trajectory of an individual with AD would substantially affect both the individual and society. A 1-year delay in the onset of AD could reduce the economic impact in 2030 by $113 billion. By 2050, that 1-year delay would save

$219 billion, and 3- and 5-year delays would result in savings of $415 billion and $599 billion, respectively (Zissimopoulos et al., 2015). Because of the high failure rate of treatment studies over the past decade, and in line with the FDA guidelines, the focus of AD clinical research has shifted to early intervention, during the asymptomatic phase of Alzheimer's disease, rather than initiating treatments after symptoms have emerged (Sperling et al., 2014).

CHALLENGES TO DRUG DISCOVERY FOR ALZHEIMER'S DISEASE

Alzheimer's disease is a heterogeneous disorder that develops over an extended preclinical phase (Sperling et al., 2011; Beason-Held et al., 2013; Thambisetty et al., 2013; Neff et al., 2021). An 'early onset' form (EOAD) typically appears before the age of ∼65 years, and is associated with more severe clinical manifestations than the 'late-onset' form typically associated with aging. Roughly half of the early onset cases are due to dominantly inherited mutations in any of three genes, presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*), or amyloid precursor protein (*APP*). *PSEN1* and *PSEN2* modulate the activity of γ-secretase, which processes *APP*. Defects in all three genes result in the accumulation of extracellular deposits of β-amyloid peptides, which are proteolytic products of APP.

Late-onset (or sporadic) Alzheimer's disease (LOAD) usually appears after the age of 65, and is not associated with the dominant inheritance of any single gene. A number of risk factors, including several genetic risk factors, predispose for Alzheimer's disease. The most important genetic risk factor is allelic variation in the apolipoprotein E (*APOE*) gene, followed by the rs75932628 (R47H) variant in the Triggering Receptor Expressed in Myeloid cell 2 (*TREM2)* gene. Trem2 is a myeloid cell receptor that binds both ApoE and β-amyloid peptides, and regulates microglial activation. A number of non-genetic determinants also predispose for Alzheimer's disease, including lack of early life education, hypertension, smoking, obesity, alcohol consumption, and diabetes (Zhang et al., 2021). Biological sex at birth is a significant risk factor for all cause dementia, and women are at greater risk of developing AD than men. Recent genetics findings, including that *APOE* is a risk factor for EOAD (Genin et al., 2011), belie the underlying similarities between EOAD and Alzheimer's disease (Jansen et al., 2019; Kunkle et al., 2019; Neuner et al., 2020). In the remainder of this review, we will use the term AD to denote late Alzheimer's disease, and distinguish between EOAD and LOAD when the situation calls.

Multiple hallmarks characterize AD. In addition to extracellular β-amyloid deposits, which is not detected in all cases (Terry et al., 1999; Monsell et al., 2015; Jack et al., 2019; Sperling et al., 2020), and intracellular neurofibrillary tangles (NFT, insoluble deposits of misfolded, hyperphosphorylated tau), AD is characterized by neuronal oxidative stress (Nunomura et al., 2001), neuroinflammation (Heneka et al., 2015a), cerebral insulin resistance (Talbot et al., 2012), and glucose hypometabolism (Mosconi et al., 2008a), calcium overload (Alzheimer's Association Calcium Hypothesis Workgroup, 2017), mitochondrial malfunction (Swerdlow, 2018) and redistribution (Flannery and Trushina, 2019), synaptic loss (Price et al., 2001), and brain atrophy (Jack et al., 2018). The extent to which any of these factors contributes to AD risk or to manifestations of disease reflects individual variations in biological flexibility and susceptibility to stressors (Neff et al., 2021).

As of 2019, the failure rate of AD drug trials exceeded 99% (Cummings et al., 2014), including the highly 'validated' targets amyloid and BACE. These failures reflect knowledge gaps about processes that promote and sustain AD, and how susceptibility to pathogenic determinants varies among individuals. Pan-omics approaches could stratify patient subpopulations according to their underlying pathologies and/or their responses to specific therapies, and identify potential safety issues regarding particular drugs (Roses, 2008). Cancer already uses 'precision medicine' approaches (Begum, 2019), and initiatives are underway for other complex diseases (Loscalzo, 2019; Prasad and Groop, 2019; Aletaha, 2020). O'Bryant et al. used similar tools to identify AD subjects who respond to NASID therapy (O'Bryant et al., 2018), but otherwise this approach is not in wide use by the AD clinical research community. Identification of the molecular basis for the heterogeneous nature of AD (Neff et al., 2021), may provide conceptual impetus for adopting it.

The complex pathophysiology of AD means the most successful strategies for lowering AD risk likely will require simultaneous pursuit of multiple targets, as for other multifactorial diseases (American Diabetes Association, 2020; Heidenreich et al., 2020; Unger et al., 2020). However, there are well-known drawbacks to typical polypharmacological approaches, including the appearance of new side effects not seen with the individual drugs, or additive side effects, or diminished efficacy. Adherence to multiple drug regimens may be challenging for prodromal patients and patients with mild or moderate dementia. An alternative approach is to develop a single drug entity that targets multiple disease determinants. PPARγ agonists fulfill this desideratum (**Figure 1**). After providing an overview of PPAR biology, we will describe AD risk factors and pathophysiological determinants contributing to AD, and the salutary effects of PPARγ agonists. Other agents, such as GLP-1 agonists, also may affect multiple targets in the AD pathogenic pathway. These are outside the scope of the current review, but have been discussed elsewhere (Grieco et al., 2019; Cheng et al., 2020; Yoon et al., 2020).

PPARγ AS A DRUG TARGET FOR AD

The PPARγ receptor is widely distributed the brain (Braissant et al., 1996; Moreno et al., 2004; Gofflot et al., 2007; Sarruf et al., 2009; Morales-Garcia et al., 2011) and is crucial for learning (He et al., 2009; Jahrling et al., 2014). Activation of the receptor enhances astrocyte/neuron metabolic coupling (Dello Russo et al., 2003; Izawa et al., 2009; Cowley et al., 2012), promotes formation of dendritic spines (Brodbeck et al., 2008), repairs synaptic failure (Chen et al., 2015; Moosecker et al., 2019), corrects LTP impairment (Cowley et al., 2012; Chen et al., 2015), and overcomes the pro-inflammatory, pro-oxidant milieu in the

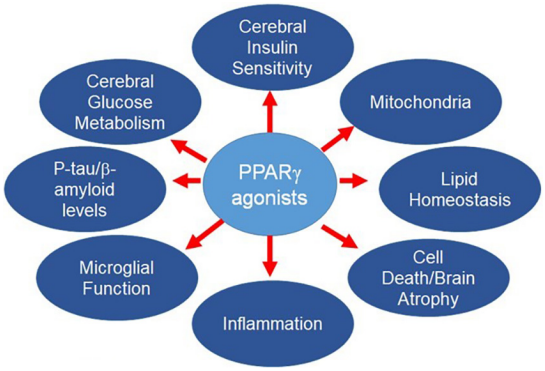

FIGURE 1 | PPARγ regulates many pathways that contribute to AD risk.

CNS that is central to the pathogenesis of AD. This topic has been reviewed previously (Galimberti and Scarpini, 2017; Cai et al., 2018; Villapol, 2018; Khan et al., 2019).

PPARs constitute a family of three ligand-dependent transcription factors, PPARα, PPARδ and PPARγ, encoded by separate genes and displaying wide, but subtype specific, tissue distribution. PPARs have broad metabolic and anti-inflammatory activities, and are attractive pharmacological targets for treating dyslipidemias (PPARα, Gemfibrozil), type 2 diabetes (PPARγ, pioglitazone, rosiglitazone), and obesity (PPARδ). Pioglitazone and rosiglitazone are high affinity ligands for both PPARγ and PPARα, but are distinguishable in that rosiglitazone is more selective for PPARγ, each agonist regulates bespoke down-stream genes (Verschuren et al., 2014), and pioglitazone enters the brain (Maeshiba et al., 1997; Grommes et al., 2013) to a greater extent than rosiglitazone (Festuccia et al., 2008).

PPARs recruit and/or enhance the activity of the general transcription machinery of target genes, or repress the expression of others. The PPAR family members share similar structural and mechanistic features (**Figure 2**). The N-terminal domain contains a ligand-independent transcriptional activation function, AF-1, which is the main determinant of PPAR subtype-selective gene expression. The DNA-binding domain (DBD) binds the receptor to the Peroxisome Proliferator Response Elements (PPRE) of the target genes. It contains the two zinc fingers, which distinguish PPARs from other DNA-binding proteins. PPREs are located either in the gene promoter or in the proximal sequence and contain one or two copies of the consensus sequence 5′-AGAACA-3′. Adjacent to the DBD are the transcriptional cofactor-binding domain (the D site), and the ligand-binding domain (LBD), which mediates binding of the receptor to the PPRE. All three PPARs form obligate heterodimers with RXR receptors. The PPAR and RXR partners bind to the 5′ and 3′ halves of direct repeats of the consensus binding sequence in the PPRE.

PPARγ signaling is non-linear and the net effect depends on fluctuations of PPARγ ligands, on the temporal sequences and durations of post-translational modifications (**Figure 2**), and on the nature of downstream gene expression networks that interact with the PPARγ transcriptional programs.

PPARγ AND AD-RELATED RISK FACTORS

Introductory Comments

The most significant risk factors for developing Alzheimer's dementia are potentially non-modifiable and include age, biological sex, a history of AD in first-degree relatives and genetics (Gaugler et al., 2019). The risk for developing AD increases with age (Qiu et al., 2009), and females are at greater risk of developing AD than males (Plassman et al., 2007). Approximately 30 genetic risk loci have been identified (Jansen et al., 2019; Kunkle et al., 2019), which account for only about 65% of the over-all population attributable risk (Livingston et al., 2017). The remainder of the risk is associated with co-morbidities that potentially are modifiable (Livingston et al., 2020). Not surprisingly, there is an underlying connection between biological sex and genetic risk factors for AD. In the first instance, *APOE ε4* affects females more severely than men (Farrer et al., 1997; Altmann et al., 2014; Neu et al., 2017). Secondly, recent investigations revealed sex-specific autosomal genetic effects (Zhou et al., 2019; Fan C.C. et al., 2020; Prokopenko et al., 2020). In several instances, risk genes for one sex are not risk genes for the other. For example, the risk haplotype of *PVRL2* was significantly associated with AD in females but not males (Zhou et al., 2019), and *ZBTB7Z*, which encodes a zinc-finger transcription factor, is a risk gene in females but is protective in males (Prokopenko et al., 2020).

Genetic Risk Factors

The genetic landscape of AD consists of about 30 genomic loci (Lambert et al., 2013; Jansen et al., 2019; Kunkle et al., 2019). PPARγ might be considered a 'master regulator' of this genetic landscape because it regulates the expression of at least seven of these genes (Barrera et al., 2018).

Early-Onset AD

The histopathological hallmarks of amyloid deposits and NFTs characterize both the 'Early' and 'Late' onset forms of AD. Causal mutations in three genes, *APP*, *PSEN1* and *PSEN2* contribute to amyloid deposits in the early-onset form (Neuner et al., 2020). *APP* is a cell-surface molecule that is widely distributed throughout the body, and is the precursor molecule of Aβ peptides in the CNS. APP knockout mice do not exhibit a phenotype and its exact role is unknown (O'Brien and Wong, 2011). *PSEN1* and *PSEN2* are catalytic components of the γ-secretase complex, which cooperate with BACE1 (β-site amyloid precursor protein cleaving enzyme) to process amyloid precursor protein and generate the aggregation-prone Aβ peptides found in plaques.

Pioglitazone regulates BACE1–mediated production of Aβ peptides at several levels. The BACE1 gene contains a PPRE and PPARγ controls BACE1 expression. Additionally, CDK5 regulates BACE1 at both the transcriptional and post-transcriptional levels by increasing BACE1 expression (Wen et al., 2008), and regulating β-secretase activity via phosphorylation (Song et al., 2015). Through mechanisms

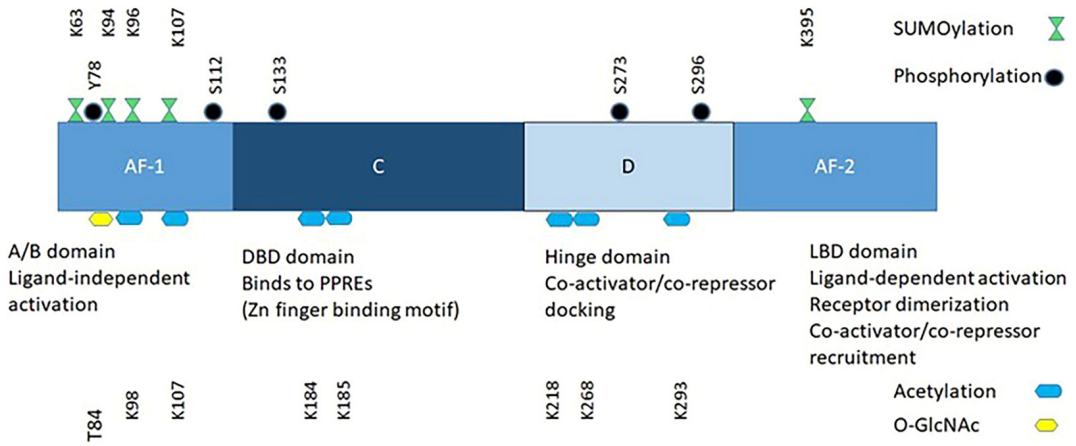

FIGURE 2 | PPARγ covalent modifications.

described below, pioglitazone inhibits these CDK5 effects. In both cell-based and *in vivo* models, PPARγ, but not PPARα or PPARδ (Camacho et al., 2004), blocked the generation and release of Aβ peptides (Sastre et al., 2003, 2006; Liu et al., 2013; Gad et al., 2016; Quan et al., 2019b) by blocking BACE1 mRNA and protein expression, and promoting Aβ peptide clearance (Camacho et al., 2004). *In vivo*, PPARγ activation resulted in significantly reduced β-amyloid plaques (Heneka et al., 2005; Escribano et al., 2010; O'Reilly and Lynch, 2012; Searcy et al., 2012; Liu et al., 2013; Quan et al., 2019b). *In vitro*, the RXR ligand *cis*-retinoic acid alone was as effective as PPARγ agonists alone, including pioglitazone (Camacho et al., 2004). In the cell culture, PPARγ agonists blocked increased BACE1 expression and synthesis and release of Aβ peptides elicited by pro-inflammatory cytokines (Sastre et al., 2003). Conversely, PPARγ knock-down increased BACE1 expression (Sastre et al., 2006). Aβ peptides and fibrils are pro-inflammatory and increase CDK5 activation (Quan et al., 2019a), and astrogliosis, microglial damage and neuronal apoptosis (Sastre et al., 2003). Pioglitazone down-regulated CDK5 expression and PPARγ phosphorylation, and increased PPARγ expression, inhibiting BACE1 expression and Aβ production. The PPARγ antagonist GW9662 blocked these pioglitazone effects (Quan et al., 2019b), affirming they were mediated by the PPARγ receptor.

PPARγ effects are dependent on the co-activator PGC-1α. Over-expression of PGC-1α in a cell line stably expressing APP inhibited Aβ production, concomitantly with decreasing BACE1 expression (Katsouri et al., 2011). Knocking out PPARγ expression abrogated the PGC-1α effect (Katsouri et al., 2011). The levels of both PPARγ and PGC-1α are reduced in brain extracts from Alzheimer's cases compared with cognitively normal controls (Sastre et al., 2006; Qin et al., 2009; Katsouri et al., 2011). This is associated with reduced PPARγ binding to the BACE1 PPRE, and elevated Aβ production. By over-coming counter-regulatory effects of CDK5 and other signaling kinases, PPARγ agonist pioglitazone increases PPARγ expression, inhibiting BACE1 expression and blocking amyloid plaque formation.

We discuss NFTs here because of their ubiquitous association with amyloid plaques. Neurofibrillary tangles are correlated with neuronal dysfunction and brain atrophy more directly than are amyloid deposits (Brion, 1998; Jack et al., 2018). Pioglitazone inhibited tau phosphorylation (Cho et al., 2013; Hamano et al., 2016; Moosecker et al., 2019) and oligomerization (Hamano et al., 2016) in cell-based tauopathy models, and in pre-clinical mouse models (Escribano et al., 2010; Searcy et al., 2012). It also blocked misrouting of tau to dendritic spines *in vitro* (Moosecker et al., 2019). The PPARγ-specific antagonist GW9662 blocked these effects, confirming they were PPARγ receptor-dependent. Rosiglitazone was similarly effective in mice (Escribano et al., 2010). PPARγ preserves synapses, which may be due to the correction of tau's mis-sorting (Moosecker et al., 2019). Pioglitazone also reduced tau phosphorylation in the 3xTg mouse AD model (Searcy et al., 2012). The effects on tau phosphorylation and aggregation may be a consequence of pioglitazone-mediated direct inhibition of CDK5 (Hanger et al., 1998). Additionally, PPARγ may regulate CDK5 indirectly through its effects on the inflammatory response. p35 is a regulatory protein that activates CDK5, and calpain-catalyzed cleavage of p35 in response to elevated cytosolic Ca2+ that occurs in neurons during the pathogenesis of AD cleaves p35 to form p25, which hyperactivates CDK5 and causes increased tau phosphorylation (Kimura et al., 2014; Seo et al., 2017). IL-6 enhances CDK5 activity (Quintanilla et al., 2004) by promoting the p35 – to – p25 conversion, and PPARγ suppresses IL-6 release (Jiang et al., 1998).

Late-Onset AD

Roughly 50% of the genes associated with late-onset Alzheimer's encode proteins involved in the innate immune system, and many of the remaining genes encode proteins involved in lipid metabolism (Jones et al., 2010). Both Apolipoprotein E ε4 (*APOE ε4*), which is the most significant and highly replicated genetic risk factor for AD (Corder et al., 1993; Saunders et al., 1993; Lambert et al., 2013), and the *TREM2* R47H polymorphism, which has the second largest effect size (Guerreiro et al., 2013;

Jonsson et al., 2013), affect innate immunity and lipid metabolism (Shi and Holtzman, 2018; Nugent et al., 2020). Metabolomics studies consistently point to pronounced alteration of lipid metabolism as an early marker of AD (Han, 2005, 2010).

APOE is one of a cluster of genes in the Chr 19q13.32 genomic region that affect AD risk, that also includes *PVRL2, TOMM40,* and *APOC1*. There are three common forms of *APOE*, distinguishable by the identity of amino acids at positions 112 and 158 that are determined by two closely linked SNPs in the *APOE* gene: rs429358 and rs7412, that result in the expression of three alternative protein isoforms: APOE ε2, which possesses cysteine residues at both positions, APOE ε3, which possess cysteine-112 and arginine-158, and APOE ε4, which possesses arginine residues at both positions. *APOE ε4* increases the risk for developing AD dose-dependently and also decreases the age of disease onset (Corder et al., 1993; Roses, 1994; Frisoni et al., 1995; Farrer et al., 1997). By contrast, *APOE ε2* is protective against AD, and *APOE ε3* has intermediate risk (Corder et al., 1994; Farrer et al., 1997). The brain produces all of its ApoE locally; the liver and macrophages produce peripheral ApoE. Glial cells account for most of the ApoE production in the brain. It mediates cholesterol and phospholipid transfer between astrocytes and microglia and neurons, on HDL-like lipoprotein particles. It is the main lipoprotein component of these particles, which are taken up by members of the low-density lipoprotein receptor family (Holtzman et al., 2012). Under conditions of stress, neurons also express *APOE* (Han et al., 1994a,b). Since the association between *APOE* ε4 and LOAD was first reported (Saunders et al., 1993), a variety of potential mechanisms underlying the contribution of *APOE ε4* to the pathogenesis of LOAD have been uncovered, from impaired neurite outgrowth (Holtzman et al., 1995), plasticity (Weeber et al., 2002) and repair (Ignatius et al., 1987), to defective Aβ clearance (Verghese et al., 2013; Kanekiyo et al., 2014; Mouchard et al., 2019), to mitochondrial dysfunction (Chen et al., 2011) and impaired endosome-lysosome trafficking (Nuriel et al., 2017; Zhao et al., 2017).

Although *APOE* is the most significant genetic risk factor for AD, it does not fully explain the risk attributable to the chr 19q13.32 genomic region. At least three additional genes in close proximity to *APOE*, *PVLR2, APOC1*, *TOMM40* (Takei et al., 2009; Roses, 2010; Zhou et al., 2019; Bussies et al., 2020; Fan K.H. et al., 2020; Squillario et al., 2020), make independent contributions.

TOMM40 encodes the mitochondrial protein import channel, and is indispensable for maintaining mitochondrial homeostasis (Baker et al., 1990; Taylor et al., 2003) and for life (Zeh, 2013). Multiple SNPs in *TOMM40* are associated with AD risk independently of the *APOE* gene., including rs7259620 (Takei et al., 2009; Nazarian et al., 2019), rs760136 (Marioni et al., 2018), rs2075650 (He et al., 2016; Huang et al., 2016; Bussies et al., 2020; Soyal et al., 2020; Squillario et al., 2020), and rs10524523 (Roses, 2010; Li et al., 2013; Yu et al., 2017a,b). Both *APOC1* and *PVRL2* fit the pattern of being lipid- or immune-related. ApoC1 interferes with ApoE-mediated cholesterol and phospholipid uptake in the CNS by blocking the binding of ApoE-enriched lipoprotein particles to the low-density lipoprotein receptor (Kowal et al., 1990; Weisgraber et al., 1990; Sehayek and Eisenberg, 1991), and it blocks binding of APOE ε3- and APOE ε4-enriched particles equally well (Kowal et al., 1990). The *APOC1* risk haplotype was associated with plasma levels of Aβ40 (Zhou et al., 2019). *PVRL2* mediates the uptake of herpesvirus (Warner et al., 1998). There is enduring speculation that Herpes virus contributes to the etiology of AD (Itzhaki et al., 2016; Readhead et al., 2018), but this theory is controversial (Rizzo, 2020). The risk haplotype of *PCRL2* was associated with worsening cognitive performance, reduced total brain and hippocampal volume, and total serum Aβ42 (Zhou et al., 2019). Additionally, *PVRL2, APOE* and *APOC1* have regulatory roles on the expression of genes in this linkage disequilibrium region: *APOE ε4* suppresses the transcription of *TOMM40*, *APOE* and *APOC1* in the brain, while risk haplotypes of *PVRL2* and *APOC1* increase brain *APOE* expression, regardless of *APOE* genotype (Zhou et al., 2019). Methylation of the *TOMM40* promoter decreases expression of *TOMM40* and increases *APOE* expression (Shao et al., 2018). Together, these studies indicate that four genes in linkage disequilibrium on chromosome 19, *PVRL2, TOMM40, APOE* and *APOC1* independently affect brain structure, neuroenergetics and cognitive performance, and the risk for AD.

The chr 19q13.32 genomic region is enriched in PPARγ binding sites (Subramanian et al., 2017), which is not surprising since most endogenous PPAR ligands are lipids or lipid derivatives and the region is enriched in lipoproteins or proteins that interact with them (Zhou et al., 2019). PPARγ agonists affect the expression of three of the four genes in the region: *TOMM40*, *APOE* and *APOC1*; their effects on *PVRL2* expression have not been studied to date. Pioglitazone increases *APOE* expression in macrophages (reviewed in Ricote et al., 2004), and in the brain (Mandrekar-Colucci et al., 2012). By contrast, Subramanian et al. showed that reducing PPARγ expression in the human hepatoma line HepG2 paradoxically increased *TOMM40*, *APOE* and *APOC1* expression. Consistent with these results, low (nM) concentrations of pioglitazone suppressed expression of both *APOE* and *APOC1*, without detectable effects *TOMM40* expression. Other workers reported that high (μM)concentrations of the PPARγ agonists ciglitazone and 15d-PGJ2, elicited a robust increase in *APOE* expression and a modest suppression of *APOC1* expression (Dahabreh and Medh, 2012). These contrasting results may reflect the respective drug concentrations used, since bi-phasic PPARγ dose-effect curves have been reported (Wada et al., 2006; Miglio et al., 2009; Moon et al., 2012). Using the SKNMC cell line that is more pertinent to AD, we found that pioglitazone increased Tom40 protein expression (Charalambous et al., 2016; **Figure 3**).

Lipid Metabolism

The pathology of AD is interwoven with extensive alterations in lipid metabolism (Foley, 2010), which are detectable in the CSF and plasma as well as the brain (Wood, 2012; Trushina et al., 2013; Varma et al., 2018). This topic has been reviewed recently (Penke et al., 2018; Kao et al., 2020), and we will limit our discussion to selected topics.

Ethanolamine plasmalogen (PIsEtn), comprises between 60 and 90 mol% of the total phospholipids of neuronal cell membrane fraction in human gray and white matter,

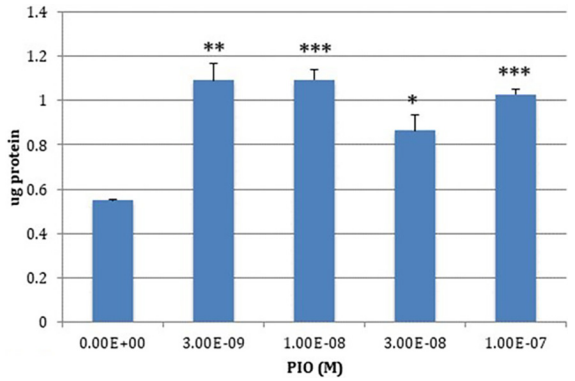

FIGURE 3 | PIO-elicited TOM40 expression in SKNMC neuroblastoma cells. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

respectively (Han, 2010), and over 60 mol% of all phosphatidylethanolamine in synaptic vesicles (Han, 2010). Plasmalogens are glycerophospholipids in which the substituent at the sn-1 position is a vinyl ether fatty alcohol (-O-CH = CH-R). They are protective against oxidative damage to polyunsaturated diacylphospholipids (Reiss et al., 1997), and they facilitate membrane fusion (Glaser and Gross, 1994). Plasmalogen deficiency is detectable at early stages of AD (Han et al., 2001). It is not detected in Huntington's disease or Parkinson's disease (Ginsberg et al., 1995; Farooqui et al., 1997). Although the deficiency is detected in both gray and white matter, it only worsens as AD pathology progresses in white matter (Han et al., 2001). Circulating levels of PIsEtn positively correlate with the extent of functional state deterioration (Wood et al., 2010). Very long chain fatty acids increase in late stages of AD, causing lipotoxicity (Schönfeld and Reiser, 2016) and exacerbating neuronal damage. Peroxisomes host both the synthesis of PIsEtn and the oxidation of very long-chain fatty acids, but they are deficient or dysfunctional in AD (Grimm et al., 2011; Kou et al., 2011). Pioglitazone corrects these defects, through several different mechanisms. As a partial PPARα agonist (Sakamoto et al., 2000; Orasanu et al., 2008), it promote peroxisome biosynthesis (Hoivik et al., 2004) and related lipid metabolism (Kersten, 2014). Pioglitazone also enhances PIsEtn synthesis by facilitating uptake of precursor fatty acids, including docosahexaenoic acid via fatty acid binding protein 5 (Pan et al., 2015). FABP5 knock-out mice exhibit impaired working memory and short-term memory (Pan et al., 2016), and pioglitazone increases FABP5 expression (Low et al., 2020). Pioglitazone also enhances PIsEtn synthesis through by inhibiting amyloidogenic processing of APP. The APP intracellular domain promotes expression of alkyl-dihydroxyacetone phosphate synthase (Grimm et al., 2011), the rate-limiting enzyme in PIsEtn synthesis. This fails when APP processing is diverted to the Aβ pathway, but pioglitazone blocks this and rescues PlsEtn synthesis.

Sphingolipids are major components of the myelin sheath, and MRI shows that demyelination occurs during the MCI phase (Bouhrara et al., 2018). Similarly, at early stages of pathogenesis, sulfatide sphingolipid levels are reduced by ∼ 92 mol% in gray matter, regardless of brain region, by 35 mol% in the cerebellum, and by 58 mol% in the temporal cortex. Rosiglitazone reversed myelin structural damage in a rodent model (Cowley et al., 2012).

Ceramide levels are ∼ 3-fold higher in white matter across all brain regions in MCI subjects vs. age-matched controls. The expression of an extensive gene network underlying ceramide synthesis also is increased at early stages of AD (Katsel et al., 2007). Increased availability of ceramides contributes to the pathogenesis of AD by causing mitochondrial damage and increasing apoptosis (Yu et al., 2000), and contributes to the depletion of PIsEtn via the stimulation of PIsEtn-PLA_2 (Farooqui, 2010; Ong et al., 2010). Sphingosine-1-phosphates (S-1-P), metabolic products of ceramides, generally counteract ceramide effects (see Wang and Bieberich (2018) and Czubowicz et al. (2019) for excellent reviews). PPARγ (Parham et al., 2015) is one of several surface and intracellular receptors that mediate S-1-P effects and maintains the homeostatic phenotype in T-lymphocytes; this role has not been investigated in microglia.

INNATE IMMUNITY

In significant measure, AD is a disease of the innate immune system (Zhang et al., 2013; Jones et al., 2015; Kan et al., 2015). Most (ca. 60%) of the AD GWAS at-risk genetic polymorphisms are in Sims et al. (2017) or near genes or their regulatory elements, that are enriched in microglia (Tansey et al., 2018; Jansen et al., 2019; Kunkle et al., 2019; Nott et al., 2019). *APOE ε4* expression and the expression of immune regulatory genes are positively correlated (Keren-Shaul et al., 2017; Mathys et al., 2019). Moreover, a haplotype associated with reduced expression of *PU.1*, a pivotal gene for microglial development (Turkistany and DeKoter, 2011), delays the age of onset of AD (Huang et al., 2017).

Microglia are the CNS' resident innate immune system cells (Ginhoux et al., 2010; Schulz C. et al., 2012). Their primary function is to insure the health and connectivity of the neurons [Streit and Kincaid-Colton (1995) and Nayak et al. (2014) and references therein]. In their 'quiescent' state, microglia survey their local environments, including direct communication with neighboring neurons and astrocytes, through ramified extensions. Detection of specific signals generated by injury to the surrounding cells triggers activation of microglia, involving morphological transformations and triggering specific biochemical and genetic programs. To sustain the activated state, bioenergetic metabolism is switched from reliance on oxidative phosphorylation to glycolysis, which supplies not only ATP but also important metabolic intermediates including NADPH and other intermediates of the pentose phosphate shunt (Lauro and Limatola, 2020).

Microglia are exquisitely sensitive to deviations in their local environments and changes in microglial transcriptomics, morphology or behavior (phagocytosis) are often the first signs of pathology (Boza-Serrano et al., 2018). Their programmed transcriptional responses are bespoke for different stimuli, and support increased phagocytosis, the production of interferon

and cytotoxic cytokines, chemokines, extracellular proteases and reactive oxygen species, as well as anti-inflammatory cytokines and factors that promote tissue repair and remodeling of the extracellular matrix (Porcheray et al., 2005; Stout et al., 2005). These inflammatory and immunosuppressive phenotypes represent the extremes of a spectrum of responses (Colton et al., 2006; Colton, 2009; Gray et al., 2020). Longitudinal gene transcription profiles of microglia isolated from mouse AD models reveal there are multiple discrete populations of activated microglia in the AD brain, reflecting interferon-related, proliferation-related and neurodegeneration-related phenotypes (Keren-Shaul et al., 2017; Friedman et al., 2018; Mathys et al., 2019).

Activated microglia play two distinct roles in amyloid metabolism. On the one hand, they promote the generation of Aβ peptides via interferon-mediated induction of IFITM3 (interferon-induced transmembrane protein 3). IFITM3, which previously had been recognized for its antiviral activity (Bailey et al., 2014), associates with the γ-secretase complex and promotes amyloidogenic APP processing (Hur et al., 2020). This role is consistent with the theory that Aβ peptides are part of the innate immune system's anti-infection repertoire (Eimer et al., 2018). Activated microglia also participate in the clearance of amyloid deposits, forming clusters adjacent to – and sometimes surrounding – β-amyloid plaques (Condello et al., 2015). Some microglia associated with plaques become dysmorphic as the disease advances, through exhaustion or via collateral damage from unrestrained proinflammatory activity of adjacent microglia, and the plaques engulf some (Streit et al., 2009, 2018).

Increased expression of inflammation-associated genes, including *APOE*, and reduced expression of homeostatic genes characterize activated microglia. *APOE* may be required for the activation response (Ulrich et al., 2018). *APOE* expression is higher nearer the plaque (Krasemann et al., 2017), but the gradient signal is not known. ApoE suppresses expression of genes related to homeostatic microglia and reinforces proinflammatory gene expression (Krasemann et al., 2017) by activating NF-κB signaling (Ophir et al., 2005; Maezawa et al., 2006). NF-κB is a master regulator of the innate immune system and the inflammatory response (Liu T. et al., 2017). *APOE ε4* exacerbates these effects (Brown et al., 2002; Vitek et al., 2009; Zhu et al., 2012), perhaps by blocking differentiation to the immunosuppressive phase.

TREM2 is part of the microglial surveillance system for monitoring changes in the environment, and regulating microglia responses to those changes, including proliferation, migration and activation. Variants of *TREM2* increase the genetic risk for late-onset AD 2 – 4X, which is second only to the effect size of *APOE ε4* (Jonsson et al., 2013; Guerreiro and Hardy, 2014). *TREM2* is a single-pass receptor that binds damage associated molecular signatures (DAMPS) (Daws et al., 2003), lipoproteins and lipoprotein particles, anionic lipids and sphingomyelins exposed by cellular damage, β-amyloid peptides (Wang et al., 2015; Yeh et al., 2016; Song W. et al., 2017). Ligand binding promotes association between the *TREM2* receptor with the adaptor protein *DAP12* (*TYROBP*), which associate via electrostatic interactions, and activates an intracellular signaling cascade mediating the effects of ligand binding on survival and proliferation, phagocytosis and inflammation (Wang et al., 2015). In mouse AD models, *TREM2* mediated clustering of microglia around β-amyloid plaques and activation of phagocytosis and the 'proinflammatory' response (Jay et al., 2015; Wang et al., 2015; Ulrich et al., 2018; Zhao et al., 2018; Zhong et al., 2018), and was required for the full expression of the response to Aβ pathology across all microglial modules (Friedman et al., 2018). *APOE* and *TREM2* likely operate on the same molecular pathway because *APOE* is a ligand for *TREM2* (Atagi et al., 2015; Bailey et al., 2015; Jendresen et al., 2017), *TREM2* modulates the expression of almost all the genes in the core neurodegeneration bin except *APOE* (Friedman et al., 2018), and $Trem2^{-/-}$ and $Apoe^{-/-}$ mice are phenocopies (Ulland et al., 2017; Ulrich et al., 2018).

A R47H switch in the *TREM2* protein is the most common *TREM2* variant connected with AD. It is associated with increased total tau in the CSF, but does not affect the CSF Aβ peptide levels (Lill et al., 2015). *In vitro*, *APOE* bound to this *TREM2* variant with a lower affinity than to the wild-type receptor (without distinction between the *APOE* isoforms) (Atagi et al., 2015), and the R47H variant decreased the uptake of Aβ-lipoprotein complexes by monocyte-derived macrophages (Yeh et al., 2016).

Chemical dissection has shed additional light on the roles of microglia in AD and related tauopathies. TGF-1β and CSF-1 signaling sustain microglia, and *CSFR1* antagonists or cFMS inhibitors (Dagher et al., 2015; Spangenberg et al., 2016, 2019; Sosna et al., 2018), which deplete resident microglia from the brain, have been used as molecular scalpels. Contrary to expectations, eliminating microglia blocked the development of β-amyloid plaques and accumulation of intraneuronal amyloid, and it prevented the loss of neurons and synapses, and improved memory and learning. These salutary effects occurred whether the inhibitors were added early and maintained for long periods (Sosna et al., 2018; Spangenberg et al., 2019), or were added after plaque formation had reached advanced stages (Spangenberg et al., 2016). Blocking microglial proliferation with an inhibitor of the cFMS kinase, that autophosphorylates and activates the *CSFR*, similarly prevented the formation of amyloid plaques, improved memory and behavior and shifted the brain environment to an immunosuppressive phase (Olmos-Alonso et al., 2016). Hence, and contrary to expectations, microglia evidently are required for formation of amyloid plaques. Moreover, the absence of microglia or impaired microglia function is not detrimental for learning or memory. Shi et al. used the same strategy to learn the role of microglia in tau-mediated neurodegeneration. They demonstrated that microglial-mediated damage, and not tau-mediated toxicities, is responsible for neurodegeneration in a mouse tauopathy model (Shi et al., 2019). Therefore, it appears that microglial proliferation and/or activation is responsible for the neurodegeneration commonly associated with the two major pathologic hallmarks of Alzheimer's disease, neurofibrillary tangles and β-amyloid plaques. It is possible that the microglial-mediated proinflammatory response, or the failure of the microglial immunosuppressive response,

causes the damage leading to cognitive decline and dementia. Microglia are being targeted for neurodegenerative diseases (Dong et al., 2019).

PPARγ and Innate Immunity

PPARγ agonists prime myeloid cells to respond to immunosuppressive stimuli and enhance the differentiation of myeloid cells into an immunosuppressive state (Bouhlel et al., 2007). PPARγ is widely distributed in mouse and human brain (Warden et al., 2016), including in microglia (Bernardo and Minghetti, 2006, 2008), and activation of myeloid cells with a proinflammatory stimulus increases *PPARγ* mRNA and protein expression (Fakhfouri et al., 2012; Song J. et al., 2017). PPARγ activators also increase *PPARγ* mRNA and protein expression. In myeloid cells, the PPARγ binding sites are adjacent to *PU.1* sites on macrophage/microglia-specific targets (Lefterova et al., 2010) and control the expression of *PU.1*-responsive genes (Lefterova et al., 2010). In addition to regulating the cytokines and cytokine receptors directly involved in the inflammation response, *PU.1* regulates expression of factors required for myeloid and lymphoid cell development (Turkistany and DeKoter, 2011), including *M-CSF* (Macrophage-specific CSF) (Zhang et al., 1994). PPARγ blocks *M-CSF* expression (Bonfield et al., 2008), and inhibits the transcription factors *AP-1*, *STAT3* and NF-kB (Ricote et al., 1998). Together with its effects on *PU.1*, the net result is suppression of pro-inflammatory activation and sensitization of microglia for differentiation into the immunosuppressive phenotype. Pioglitazone blocks the synthesis of pro-inflammatory molecules, including IL-1, TNFα, IL-6, iNOS, COX2, MMP9, and Caspase 3 (Kapadia et al., 2008) and promotes the synthesis of immunosuppression-related molecules, including Arg1, IL-4, IL-10, TGF*b,* catalase, SOD, and related genes (Bouhlel et al., 2007).

In vitro studies confirmed that PPARγ controls the cellular response to AD-related pathogenic triggers, including Aβ and LPS (Combs et al., 2000; Heneka et al., 2000; Hunter et al., 2008). These effects go beyond simply regulating expression of pro- and anti-inflammatory molecules. PPARγ overcomes pathogenesis-related developmental blocks that prevent transitioning of microglia to the immunosuppressive phenotype. iPSC-derived microglia that are heterozygous for the pathogenic *TREM2* R47H mutation have a shortfall in glycolytic capacity and cannot execute the metabolic switch that underpins differentiation of microglia to the immunosuppressive phenotype (Piers et al., 2020); consequently, they are deficient in phagocytosis. Pioglitazone corrected the glycolysis deficit, reversed blockade of the metabolic shift, and restored phagocytosis of Aβ42 (Piers et al., 2020). Pioglitazone achieved this by increasing phosphorylation and activation of p38-MAPK, which phosphorylated and activated MAPK2, which, in turn, phosphorylated and activated 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), a key regulatory step in glycolysis.

Pioglitazone's salutary effects on the innate immune system also correlated with a shift toward the immunosuppressive state in pre-clinical models of traumatic brain injury (Deng et al., 2020), depression (Zhao et al., 2016), axonal injury (Wen et al., 2018), neuroinflammation (Kielian and Drew, 2003) and stroke (Tureyen et al., 2007; Cai et al., 2018), and Parkinson's disease (Swanson et al., 2011; Carta and Pisanu, 2013).

CEREBRAL GLUCOSE HOMEOSTASIS

The brain is dependent almost entirely on glucose for its energetics needs and consumes 25% of the body's daily glucose load. In addition to energy production, glucose contributes to the synthesis of neurotransmitters, including acetylcholine, aspartate, glutamate, and GABA. In the fed state, neurons consume glucose directly, and glial cells, mostly astrocytes, store glucose as glycogen. Under oxidative stress, mitochondrial bioenergetics is compromised and neurons divert acetyl-CoA into fatty acids, which astrocytes take up and store as lipid droplets in an *APOE*-dependent process (Liu et al., 2015; Liu T. et al., 2017). These droplets are essential for neuronal health since defective transfer of lipids from neurons to astrocytes causes neurodegeneration (Liu L. et al., 2017). The droplets may represent essential energy reserves. During periods of normal fasting, astrocytes convert stored glycogen via glycogenolysis and glycolysis to lactate, which is consumed by neurons (Calì et al., 2019). Similarly, astrocytes may convert fatty acids stored in the lipid droplets to ketone bodies, for consumption by neurons. Under glucose insufficiency, neurons consume ketone bodies (Ding et al., 2013). Gene expression analysis of human AD subjects and mouse AD models reveal increasing reliance on lipid metabolism with disease progression as glucose consumption decreased (Yao et al., 2011; Demarest et al., 2020). Alternatively, the stored triglycerides may be used to synthesize membranes in support of phagocytosis, or in response to stress (Martínez et al., 2020). Finally, intracellular lipid droplets may be centers for coordinating glial-based responses to infectious agents, by attracting pathogenic microbes and acting as reservoirs for antimicrobial peptides and nucleation sites for other immune proteins, including RSAD2 (Bosch et al., 2020). Nor are these mutually exclusive options. [See Welte and Gould (2017) for a recent review of lipid droplets].

Glucose hypometabolism is a characteristic feature of Alzheimer's disease. It is routinely measured by ^{18}F-deoxyglucose-positron emission tomography (FDG-PET) (Minoshima et al., 1995, 1997; Herholz, 2010), or regional blood flow, measured by ^{15}O-PET (Beason-Held et al., 2013), which are highly correlated. The sodium-insensitive GLUT1 and GLUT3 transporters account for most of the glucose extraction from the blood, and in persons with AD, the levels of these transporters in the brain begin to decline decades before the onset of AD symptoms (Simpson et al., 1994; Patching, 2017). GLUT1 is the predominant glucose transporter in the blood-brain barrier (BBB) and in astrocytes, and is responsible for the uptake from the systemic circulation of all of the glucose consumed by the brain. The high affinity, high capacity GLUT3 transporters are responsible for neuronal glucose uptake. The brain also expresses low levels of the insulin-sensitive GLUT4 transporter, in the cerebellum, cortex, hippocampus and hypothalamus, regions where the insulin receptor is also highly expressed

(McEwen and Reagan, 2004; Alquier et al., 2006). Pioglitazone enhances nerve stimulation-coupled cerebral glucose uptake.

Reduced cerebral glucose utilization in AD is associated with reduced CSF levels of glycolytic intermediates (Bergau et al., 2019)reflecting impaired glycolysis and post-glycolytic pathways (An et al., 2018). It is independent of Aβ42 and Aβ40 levels (Venzi et al., 2017), or brain atrophy (Smith et al., 1992; Ibáñez et al., 1998) or other changes in brain structure (Small et al., 1995, 2000; Minoshima et al., 1997; Reiman et al., 2004; Samuraki et al., 2007; Beason-Held et al., 2013), and emerges decades before the appearance of clinical symptoms (Cutler, 1986; Kennedy et al., 1995; Small et al., 1995; Reiman et al., 1996, 2004; Beason-Held et al., 2013). It is associated with altered expression of energy metabolism genes in brain regions most vulnerable to AD pathology (Xu et al., 2006; Brooks et al., 2007; Wang et al., 2007, 2010; Liang et al., 2008a,b; Bossers et al., 2010), including the emergence of focal temporoparietal hypometabolism, which is distinct from normal aging (Kuhl et al., 1982; de Leon et al., 1983; Duara et al., 1984). Cerebral hypometabolism leads to increased tau phosphorylation (Planel et al., 2004) and amyloid accumulation (Gabuzda et al., 1994). Conversely, re-establishing homeostatic myeloid cell glucose metabolism by inhibition of the EP2 receptor reversed age-associated cognitive decline (Minhas et al., 2021). Thus, defective brain glucose metabolism is an early, consistent, and specific marker for neurodegeneration in AD that is consequential for and precedes AD pathology.

Pioglitazone and Cerebral Glucose Metabolism

In vivo, pioglitazone improves cerebral blood flow and cerebral glucose uptake and disposal (Nicolakakis et al., 2008; Sato et al., 2011; Papadopoulos et al., 2013), in part via enhancing expression of the GLUT4 transporters (Sandouk et al., 1993; Olefsky and Saltiel, 2000), and in part by improving mitochondrial function and biogenesis. Pioglitazone also normalizes glucose metabolism by suppressing PGE2 synthesis and inhibiting PKA signaling that is triggered by EP2 (Subbaramaiah et al., 2012).

CEREBRAL INSULIN RESISTANCE

Insulin resistance (Craft, 2005; Benedict et al., 2012; Willette et al., 2013, 2015a; Ferreira et al., 2018) and type 2 diabetes mellitus (DM2) (Chatterjee and Mudher, 2018; Barbiellini Amidei et al., 2021) are related but independent risk factors for AD. Both DM2 and cognitive impairment share gene expression networks that are enriched in genes involved in inflammation and PI3K-Akt signaling (Potashkin et al., 2019), and direct analysis of post-mortem brain samples revealed impaired insulin- and IGF1-triggered signaling in human and mouse AD brain samples (Bomfim et al., 2012; Talbot et al., 2012).

Insulin, insulin-like growth factor 1 and their respective mRNAs are found throughout the brain (Blázquez et al., 2014). The insulin receptor has been mapped to all cell types throughout the brain, with particularly high concentrations in the hippocampus and hypothalamus, and its roles in processes as diverse as systemic energy homeostasis (Chen et al., 2017), balance and movement (Zhao et al., 2004), and memory formation and consolidation (McNay et al., 2010; McNay and Recknagel, 2011; Kullmann et al., 2016) are well established.

The same gene encodes the brain and the peripheral insulin receptors (IR). However, the brain and peripheral receptors differ in three key ways. The brain receptor arises by alternate splicing of the IR gene and is smaller than the peripheral IR. Unlike the peripheral IR, insulin binding to the brain IR does not promote its internalization. Finally, the brain IR forms hybrid receptors with IGF1 receptors (IGF1R) more readily than the peripheral IR does. The IR and IGF1R receptors belong to the same receptor-tyrosine kinase family. Both are α2β2 heterotetramers, composed of two extracellular ligand-binding α-chains that are disulfide-linked to membrane-spanning β-subunits that possess tyrosine kinase activity. The α-chains are also disulfide-linked to each other. In general, the IR signal elicits metabolic responses, including the translocation of transporters from internal depots to the cell surface, and therefore stimulates glucose and amino acid uptake and metabolism, while the IGF1R predominantly affects protein synthesis and cellular growth. Activation of both receptors trigger changes in gene expression. Hybrid IR-IGF1R receptors are more abundant in the brain than in the periphery. As of this writing, the specific roles of these three receptor types in the CNS have not been resolved.

In the periphery, insulin primarily promotes glucose and lipid homeostasis. A key step is insulin-stimulated translocation of GLUT4 glucose transporters from intracellular pools to surface membranes in adipocyte and muscle, mediated by a cascade of signaling adaptor proteins and kinases that, via a chain of phosphorylations, connect successive kinases with target functional proteins that mediate vesicle translocation, protein synthesis, and activation of metabolic pathways. Ligand binding activates auto-phosphorylation of the receptor on tyrosine residues, creating binding sites for the adaptor proteins, IRS (IR, predominantly) or Shc (IGF1R, predominately), which, themselves, are tyrosine phosphorylated. These form the hubs for signaling cascades. Two cascades stem from the IRS hub: the PI3K-Akt pathway promotes the translocation of transporters from intracellular depots to the cell surface and is responsible for the metabolic effects of insulin. The MAPK pathway, which both IRS and Shc control, mediates insulin's (and IGF1's) effects on gene expression. Both pathways cooperate in regulating cellular growth, differentiation and repair (Boucher et al., 2014).

In the brain, the insulin transduction pathway not only promotes trafficking of GLUT4, but also of the high affinity choline transporter and the AMPA, NMDA and GABA receptors (Zhao et al., 2004; Fishwick and Rylett, 2015; Spinelli et al., 2019). In addition to these post-synaptic effects, insulin promotes dendritic spine and synapse formation (Lee et al., 2011). Insulin does not regulate most of the brain's glucose consumption, because GLUT1, the main glucose transporter of the BBB, and GLUT3, the main glucose transporter within the brain, are not insulin responsive (Simpson et al., 2008). However, insulin does stimulate glucose utilization in the hippocampus, one of the few brain regions that express GLUT4 transporters. To meet the high energy demands associated with memory formation and retrieval, translocation of the GLUT4 transporters

is also mediated by AMPK under the control of the membrane potential (Ashrafi et al., 2017). Both the IR and GLUT4 are concentrated in the synapses, and insulin-stimulated, GLUT4-mediated glucose uptake supports sustained synaptic vesicle recycling (Ashrafi et al., 2017), and is essential for memory formation (Pearson-Leary and McNay, 2016; Pearson-Leary et al., 2018). Post-translational modification of mitochondria by the glucose sensor N-acetylglucosamine O-transferase localize the mitochondria within the same cellular regions as the IR and GLUT4 (Pekkurnaz et al., 2014). O-GlcNAcylation is also required for full activity of the mitochondrial ATP synthase (Cha et al., 2015). The co-localization of insulin receptors with GLUT4 transporters and mitochondria underscore the importance of insulin regulated glucose uptake and metabolism for supporting the energetic requirements of synaptic vesicle trafficking and the action potential. Tau is also O-GlcNAcylated under homeostatic conditions, but insulin resistance perturbs O-GlcNAc cycling and contributes to tau hyperphosphorylation (Liu et al., 2009, 2011; Bourré et al., 2018).

It is clear that, from facilitating the synthesis of acetylcholine at two levels (acetyl-CoA generation via the sequential action of glycolysis and pyruvate dehydrogenase, and choline uptake), to manipulating neurotransmitter release and uptake, to supporting neuritogenesis and repair, and regulating tau phosphorylation, insulin has profound effects on the processes that support cognition.

Given the important role insulin plays in brain physiology, it is not surprising that cerebral hypoinsulinemia, caused by peripheral insulin resistance, or cerebral insulin resistance *per se* are an important risk factors for neurodegenerative diseases, including Alzheimer's disease (Baura et al., 1996; Matsuzaki et al., 2010; Willette et al., 2015b,c; Ekblad et al., 2017; Kong et al., 2018). Insulin resistance in mid-life predicts dementia in late life (Ekblad et al., 2017, 2018; Lutski et al., 2017; Tortelli et al., 2017; Kong et al., 2018). Increasing metabolic control with (Ryan et al., 2006) or without pharmacological intervention (Naor et al., 1997) improves working memory. DM2, which reflects systemic insulin resistance coupled with pancreatic insufficiency, is a risk factor for AD (Ott et al., 1999; Schrijvers et al., 2010; Livingston et al., 2020). The Metabolic Syndrome, reflecting systemic insulin resistance in conjunction with lipid and cardiac co-morbidities, also is associated with AD, independently of the *APOE* genotype (Kuusisto et al., 1997). While these relationships reflect systemic insulin resistance, it is critical to note that brain tissue itself is insulin resistant in AD in subjects who, at the time of death, were without other co-morbidities that feature insulin resistance, such as DM2, obesity or the metabolic syndrome. AD-associated brain insulin resistance is detectable early in life, in high-risk individuals who were not cognitively impaired at death. Reductions in insulin and IGF-1 signaling are detectable by inhibitory phosphorylation of insulin signaling-related proteins *in situ*, by the reduced activities of kinases in the insulin-signaling cascade, by impaired activation of the insulin-signaling cascade *ex vivo* (Steen et al., 2005; Bomfim et al., 2012; Talbot et al., 2012), and by dysregulated expression of genes encoding members of the insulin/IGF1 signaling pathways (Katsel et al., 2018). Deficiencies in insulin signaling are additive and were greater in individuals who suffered from both AD and DM2 (Liu et al., 2011). Mouse EOAD models also exhibit defective insulin signaling (Takeda et al., 2010; Bomfim et al., 2012). For excellent recent reviews of the association of brain insulin resistance with Alzheimer's disease, see Ferreira et al. (2018), de la Monte (2019).

Many of the determinants contributing to AD trigger and sustain brain insulin resistance. Proinflammatory cytokines, including IL-1β and TNFα (Geng et al., 1996; Morrison et al., 2010; Bomfim et al., 2012; Kitanaka et al., 2019) activate 'counter regulatory' kinases such as ERK2, JNK and PKCζ/λ(20), which disrupt insulin/IGF1 – PI3K – Akt signaling. They phosphorylate proteins in the IR/IGFR signaling cascade at sites that interfere with normal docking or kinase activity. β-amyloid fibrils and oxidative stress consequent to mitochondrial dysfunction also activate these kinases (Okazawa and Estus, 2002; Persiyantseva et al., 2013). Inflammation and oxidative stress similarly account for systemic insulin resistance (Czech, 2017).

Pioglitazone Overcomes Cerebral Insulin Resistance

One-significant way pioglitazone reduces the risk for AD is by overcoming cerebral insulin resistance and enhancing blood flow and glucose uptake/utilization. It overcomes each of the drivers behind brain insulin resistance: It restores normal expression of genes of the insulin signaling pathway (Katsel et al., 2018) and promotes glucose uptake in vulnerable neurons, it reduces inflammation and promotes immunosuppression (Zhang et al., 2008; Haraguchi et al., 2008; Swanson et al., 2011; Kaplan et al., 2014), it ameliorates oxidative stress (Gumieniczek, 2003; Wang et al., 2014; Paciello et al., 2018), and blocks the synthesis of Aβ peptides (Liu et al., 2013; Quan et al., 2019b) and promotes their rapid clearance from the brain (Mandrekar-Colucci et al., 2012). By overcoming systemic insulin resistance, including in subjects who are not diabetic, pioglitazone also relieves cerebral hypoinsulinemia (Baura et al., 1996; Miyazaki et al., 2002; Kernan et al., 2003).

MITOCHONDRIAL DYSFUNCTION

Bioenergetics

Mitochondrial dysfunction is a major contributing factor to defective cerebral energy metabolism in AD. Oxidative damage and mitochondrial stress rank among the earliest detectable events in human AD (Hirai et al., 2001; Nunomura et al., 2001; Sultana and Butterfield, 2009), and mouse models (Yao et al., 2009). Altered mitochondrial morphology is evident in dendritic profiles, spines and synaptic terminals, and in astrocytes throughout the brain (Baloyannis, 2011), and belies bioenergetic defects (Parker et al., 1994a; Valla et al., 2001, 2010; Yao et al., 2009), due in part to reduced expression of nuclear-encoded mitochondrial genes and faulty repair of mtDNA defects (Lovell et al., 2000; Weissman et al., 2007; Sykora et al., 2015), and impaired dynamics (Manczak et al., 2011), and proteostasis (Alikhani et al., 2011; Westerlund et al., 2011). Inhibition of mitochondrial energy production elicits amyloidogenic processing of APP (Gabuzda et al., 1994) that,

in turn, worsens mitochondrial function (Manczak et al., 2006; Cenini et al., 2016). Swerdlow (2018); Wang et al. (2020) and others (Lin and Beal, 2006; Reddy and Beal, 2008; Gibson et al., 2010; Moreira et al., 2010; Swerdlow et al., 2010, 2014) have thoroughly reviewed the contribution of mitochondrial damage to the pathogenesis of Alzheimer's, and we note the highlights here.

Although mitochondria possess their own genomes, they encode only 13 of the ∼1500 mitochondrial proteins. The remainder are encoded by the nuclear genome, and the expression of many of these nuclear-encoded mitochondrial genes is dysregulated in early AD. The pattern of disruption roughly parallels the gradient of brain regions that exhibit hypometabolism (Liang et al., 2008b), from the posterior cingulate cortex (PCC), which is severely affected, to the middle temporal gyrus, the hippocampus, the entorhinal cortex, the visual cortex, and the superior frontal gyrus which is relatively spared from metabolic abnormalities (Minoshima et al., 1997; Mosconi et al., 2008b; Herholz, 2010). In addition to mitochondrial genes, glycolytic and TCA pathway genes also are down-regulated in AD (Brooks et al., 2007).

TOMM and TIMM encode components of the outer and inner mitochondrial membrane complexes, respectively, that catalyze import of nuclear-encoded mitochondrial proteins (Wiedemann et al., 2004). In addition to their importance for mitochondrial health (Zeh, 2013), they are critical for controlling cytosolic proteostasis (Liu W. et al., 2018) because blocked importation leads to excessive cytosolic accumulation of misfolded proteins. Mitochondrial protein import is dysregulated in AD (Anandatheerthavarada et al., 2003; Devi and Anandatheerthavarada, 2010; Devi and Ohno, 2012; Cenini et al., 2016; Sorrentino et al., 2017), and this dysregulation follows the same regional pattern as for OXPHOS genes. In AD, 50% of TOM complex proteins and 27% of the TIM proteins were under-expressed in the posterior cingulate cortex, but their expression was reduced by only 17% and 0%, respectively, in the visual cortex (Liang et al., 2008b).

The expression of subunits of each of the five complexes comprising the OXPHOS system is also inhibited in AD, and the extent of under expression in each brain region was consistent with the expression patterns of the respective mRNAs. These studies compliment earlier reports of reduced activity in AD brain of COX (Parker et al., 1994a; Gonzalez-Lima et al., 1997; Bosetti et al., 2002), α-ketoglutaric acid dehydrogenase (Gibson et al., 1988; Bubber et al., 2005) and pyruvate dehydrogenase (Sorbi et al., 1983; Rex Sheu et al., 1985). COX activity also is reduced in blood platelets in AD (Parker et al., 1994b; Bosetti et al., 2002; Valla et al., 2006), suggesting that mitochondrial-related AD pathophysiological determinants are not restricted to the brain.

While these data provide a biochemical rationale for AD-related cerebral hypometabolism, it is possible the observed mitochondrial deficits resulted from AD-related damage. Valla et al. tested this hypothesis in expired young adult *APOE ε4* carriers, who were at risk for developing AD, and in age-matched controls lacking *APOE ε4* (Valla et al., 2010). There were no histologic β-amyloid deposits, neurofibrillary tangles, or soluble Aβ42 in either group, and they were matched for insoluble Aβ42 and soluble Aβ40. None-the-less, the activity and protein levels of COX were lower in the at-risk population, confirming mitochondrial damage occurs prior to detectable pathological signs of AD.

The data summarizing mitochondrial dysfunction in AD and the conceptual picture it has engendered are based on autopsy specimens because it has not been possible to probe mitochondria in living subjects. Rather, mitochondrial function in living subjects has been inferred from FDG-PET analysis, which measures glycolysis directly. Tsukada's group have now introduced a PET ligand that binds to the rotenone-inhibitable site on the mitochondrial OXPHOS complex I, and provides a direct measure of complex I availability in the living brain (Harada et al., 2013; Terada et al., 2020). They discovered the loss of complex I precedes FDG-PET-detectable hypometabolism in the parahippocampus in AD (Terada et al., 2020), confirming that mitochondrial dysfunction is an early event in AD pathogenesis. Mitochondria-related markers in the CSF offer additional tools for directly probing brain mitochondrial health. Podlesniy et al. showed that mtDNA levels in CSF are lower in asymptomatic subjects who are at risk for developing AD and in AD patients relative to cognitively normal, age-matched controls (Podlesniy et al., 2013). This defect was not observed in subjects with frontotemporal lobar degeneration (FTLD), which is nosogenically related to Alzheimer's (Podlesniy et al., 2013). Previously, reductions in mtDNA in AD brain were detected by immunohistochemistry of whole brain (Hirai et al., 2001), and qPCR in single cells isolated by laser capture microdissection (Rice et al., 2014). These *post mortem* data support the use of CSF mtDNA quantification as an *in vivo* measure of mitochondrial health.

Mitochondrial Dynamics

Synaptic loss is evident early in AD pathogenesis and is highly correlated with the severity of AD-related cognitive defects (DeKosky and Scheff, 1990; Terry et al., 1991). Mitochondria are highly dynamic organelles, and are continually redistributing within cells to meet specific regional needs, and undergoing shape changes and continuous and simultaneous rounds of fission and fusion necessary for maintaining functional mitochondria. In healthy neurons, mitochondria are uniformly distributed throughout the neuron, in both the cell body and in synapses, and support synaptogenesis and synapse function via their roles in signal transduction (Werner and Werb, 2002; Yang et al., 2009), ATP production (Rangaraju et al., 2014; Sobieski et al., 2017) and Ca^{2+} -buffering (Contreras et al., 2010; Tarasov et al., 2012). By contrast, in neurons from AD subjects, mitochondria are largely restricted to the cell bodies (Baloyannis et al., 2004; Wang et al., 2009; Pickett et al., 2018). Both APP and tau contribute to disrupted mitochondrial distribution. Axonal trafficking of mitochondria was retarded in *APP/PS1* neurons, and neurons from *PS1* and Tg2576 *APP* mice (Calkins et al., 2011; Trushina et al., 2012). Exposing neurons isolated from *APP* over-expressing mice to Aβ42 in culture inhibited mitochondrial trafficking and reduced axonal mitochondrial density (Du et al., 2010). Exposing hippocampal neurons from control C57Bl/6 mice to the Aβ25-32 peptide produced similar results (Calkins and Reddy, 2011). Disease-associated tau mutations,

including hyperphosphorylated tau and the P301L mutation, disrupt interactions between microtubules and cargo, including mitochondria, and impede normal trafficking (Kopeikina et al., 2011; Schulz K.L. et al., 2012; Shahpasand et al., 2012; Rodríguez-Martín et al., 2016). Depletion of tau protects against Aβ-elicited mitochondrial trafficking deficits (Vossel et al., 2010). This may contribute to the mitigation of neuronal dysfunction observed in tau knock-down APP mice (Roberson Scearce-Levie et al., 2007; Ittner et al., 2010). Insulin resistance and inhibition of O-GlcNAc synthesis, which is necessary for the synaptic tethering of mitochondria (Pekkurnaz et al., 2014), may also disrupt the distribution of mitochondria in neurons.

Impaired mitochondrial dynamics also contributes to mitochondrial functional defects. It is associated with defective expression of the mitochondrial fission genes *DRP1* and *FIS1*, and the mitochondrial fusion genes *MFN1*, *MFN2* and *OPA1* (Wang et al., 2009; Manczak et al., 2011). *FIS1* expression is enhanced, and *MFN1*, M*FN2* and *OPA1* expression is suppressed in brain samples from human AD subjects compared with cognitively normal, age-matched controls. However, while Manczak et al. (2011) reported elevated *DRP1* expression in AD samples, Wang et al. reported decreased Drpt protein expression in AD without a change in its mRNA expression (Wang et al., 2009). These differences could reflect differences in the AD samples (including Braak stage and *APOE* status) used by these investigative teams. The distribution of the fission and fusion proteins matched that of mitochondria in brains from healthy vs AD cases, respectively (Wang et al., 2009). The GTPase activity of Drp1 is enhanced by phosphorylation on S616 (Taguchi et al., 2007). Western blot analysis revealed greater Drp1 S616 phosphorylation in both the mitochondrial and cytosolic fractions from AD subjects than from age-matched, cognitively normal controls (Wang et al., 2009); thus, even though Wang et al. observed lower levels of total Drp1 protein in AD, they detected higher levels of phosphorylated, and presumably activated, Drp1. Amyloid peptides or oligomers may activate Drp1. By co-immunoprecipitation and IHC, Manczak et al. showed monomeric and oligomeric Aβ physically associated with Drp1, which increased with increasing severity of disease. Over-expression of the APPswe mutation in neuroblastoma cells or in primary cultured neurons increased mitochondrial fragmentation and a perinuclear distribution of mitochondria, which was reversed by a BACE inhibitor reversed (Wang et al., 2008). Wang et al. subsequently showed that exposure of cultured neuroblastoma cells to oligomeric Aβ increased Drp1 phosphorylation and accumulation in the mitochondrial fraction, and mitochondrial fragmentation (Wang et al., 2009). Tau also interacts physically with Drp1, in a way that may increase Drp1 GTPase activity. This association has been detected in human AD frontal cortex, but not in controls, and was confirmed in cortical samples from *APP*, *APP/PS1* and 3 x Tg mice, but not in age-matched littermate controls (Manczak and Reddy, 2012).

These results suggest Drp1 activity, or its association with amyloid and/or tau, might be attractive targets for delaying or treating AD. Kuruva et al. used molecular docking simulations to design DDQ, that blocks binding of Drp1 with amyloid (Kuruva et al., 2017). In cultured neuroblastoma cells, DDQ blocked association of Aβ with Drp1, prevented mitochondrial fragmentation and oxidative stress, and enhanced mitochondrial biogenesis and synaptogenesis (Kuruva et al., 2017). We propose that pioglitazone also checks Aβ- and tau-mediated Drp1 activation, by virtue of its effects on Aβ production and tau phosphorylation.

Cells use a series of integrated pathways involving crosstalk among all the major organelles to control the synthesis, folding and trafficking of proteins. Dysfunction of any branch in any compartment has system-wide repercussions. Many cytosolic proteins that are prone to aggregation are imported into mitochondria and degraded (Ruan et al., 2017). Aβ peptides can be imported into mitochondria via the TOM complex (Hansson Petersen et al., 2008), using TOMM20 as the importation 'receptor'(Hu et al., 2018). Overwhelming the mitochondrial proteostasis system with Aβ peptides leads to a number of adverse consequences, including: increased oxidative stress via the generation of ROS that is produced following inhibition of a fatty acid short chain dehydrogenase/reductase (Lustbader et al., 2004) and of OXPHOS complex I (Bobba et al., 2013), inhibition of mitochondrial trafficking and reduced axonal mitochondrial density (Du et al., 2010; Calkins and Reddy, 2011), and increased mitochondrial fragmentation (Baloyannis, 2006). If intramitochondrial degradation of Aβ is impaired (Lautenschläger et al., 2020), or if importation into mitochondria is blocked (Liu Y. et al., 2018), cytosolic proteostasis becomes blocked, leading to protein accumulation and aggregation in the cytosol (reviewed in Lautenschäger and Schierle, 2019). The fact that two proteases involved in mitochondrial protein homeostasis, PreP (Alikhani et al., 2011) and Htr2 (Westerlund et al., 2011), are reduced in AD, and the suggestive evidence that polymorphisms in Htr2 are associated with AD (Westerlund et al., 2011), are consistent with this model. However, the relationship between mitochondrial Aβ accumulation and extramitochondrial protein aggregation is non-linear because Aβ-mediated inhibition of bioenergetics and the processing of APP are related by a feedback cycle, wherein Aβ-mediated inhibition of bioenergetics stalls APP processing and the generation of additional β-amyloid (Wilkins and Swerdlow, 2017). Nonetheless, the intracellular accumulation and mitochondrial localization of both β-amyloid and phospho-tau, contribute to mitochondrial dysfunction and aberrant trafficking and dynamics that characterizes Alzheimer's disease. By inhibiting the generation of Aβ peptides and the phosphorylation of tau, pioglitazone blocks these effects.

Mitochondrial and Cellular Calcium Dysregulation

Calcium homeostasis is perturbed in AD (Khachaturian, 1994), which may contribute to both early and late (Bezprozvanny and Mattson, 2008; Calvo-Rodriguez et al., 2020) phases of the disease. Calcium is essential for multiple neuronal activities in addition to mitochondrial function, including neuritogenesis and synapse formation, synaptic transmission and synaptic plasticity. Altered calcium homeostasis is a cardinal feature of Alzheimer's

and other neurodegenerative diseases (Mattson, 2007; Tong et al., 2018), due, in part, to extrinsic factors, such as β-amyloid accumulation (Bezprozvanny and Mattson, 2008), but also to intrinsic factors. Increased influx through the voltage gated calcium channel and exaggerated calcium release from the ER, coupled with blunted reuptake (Popugaeva and Bezprozvanny, 2013), elevates cytosolic calcium (Thibault et al., 2007). These contribute to mitochondrial calcium overload (Calvo-Rodriguez et al., 2020), contributing to excess ROS production and impaired mitochondrial energy production and apoptosis (Cenini and Voos, 2019). Elevated cytosolic $Ca2^{+}$ also activates CDK5 via calpain, leading to generation of the hyperactive p25 regulatory subunit (Kimura et al., 2014; Seo et al., 2017). The breakdown of neuronal calcium homeostasis extends to expression of genes important in calcium regulation (Emilsson et al., 2006). Oxidative stress and lipid peroxidation (Mattson, 1998), perturbations in the mitochondrial-ER membrane (MAM) (Hedskog et al., 2013; Area-Gomez et al., 2018) and the accumulation of β-amyloid peptides (Mattson et al., 1992) contribute to altered calcium homeostasis in AD.

Taken together, morphological analysis and gene expression, proteomic and functional data all support the conclusion that mitochondrial dysfunction is present early, before the detectible stages of AD pathology, including the accumulation of Aβ plaques or tau tangles, and contributes to AD pathogenesis.

Pioglitazone and Mitochondrial Dysfunction

PPARγ agonists ameliorate AD-related mitochondrial dysfunction by inhibiting Aβ peptide production, discussed above, by eliciting mitobiogenesis (Strum et al., 2007; Miglio et al., 2009), and improving mitochondrial membrane potential (Wang et al., 2002; Pipatpiboon et al., 2012). They also limit oxidative stress damage, by inhibiting ROS generation by complex I (Brunmair et al., 2004; Ghosh et al., 2007) and by increasing expression of glutathione and the antioxidants SOD and catalase (Collino et al., 2006; Aleshin and Reiser, 2013). PPARγ agonists exert additional positive effects on neuronal energy balance by stimulating GLUT3 expression (Garcia-Bueno et al., 2006; Wang et al., 2012), which is decreased in AD (Simpson et al., 1994), by stimulating GLUT4-mediated glucose uptake and by promoting neuronal lactate oxidation (Izawa et al., 2009) and pyruvate flux (Rossi et al., 2020) via enhancing insulin-stimulated Akt activation (Karwi et al., 2020) and inhibiting PDH kinase activity (Way et al., 2001).

PRECLINICAL EFFICACY OF PIOGLITAZONE AND ROSIGLITAZONE

Table 1 summarizes representative pre-clinical studies involving the PPARg agonists rosiglitazone or pioglitazone. This is not meant to be an exhaustive list, but the studies were selected to illustrate several points. First, both drugs exhibit *in vivo* efficacy on at least some AD-related phenotypes, but not all study results could be replicated. Generally, PPARγ agonists protected against oxidative damage, promoted synapse recovery and improved learning and memory, enhanced cerebral blood flow and glucose uptake, reduced corticosterone levels and amyloid deposits, Aβ peptide levels and reactive astrocytes and microglia, and promoted microglial phagocytosis. Two important generalizations can be drawn from these studies, that are important for considering the appropriate design of human trials.

The first is that the timing and length of treatment are critical variables that that are unique for each disease-related phenotype. The time of treatment in the natural history of the disease can be crucial. Some parameters, such as stimulus-coupled cerebral blood flow and glucose uptake, were normalized in both adult (6 months old at initiation of treatment, before visible signs of plaque pathology or significant deficits in learning and memory) and aged (15 – 18 months old at initiation of treatment) mice, while others, such as reversal learning, were improved in the adult mice, but not in aged mice (Papadopoulos et al., 2013). Other traits, such as spatial learning, were only improved when young mice were treated for extended periods (Badhwar et al., 2013). Second, some parameters respond to short-term treatment, while others do not. Cerebral blood flow was normalized after a short (3 day) drug exposure, but improvements in learning did not occur in that time frame (Badhwar et al., 2013). Third, efficacy becomes more pronounced with time of treatment (Escribano et al., 2010; Chen et al., 2015).

The second lesson is that dose matters. These representative studies employed a wide range of drug concentrations. For pioglitazone, they ranged from 80 to 0.04 mg/kg/day, which, after allometric scaling, represent 390 mg/day, down to 0.8 mg/day for a 60 kg human. For comparison, the recommended starting dose of pioglitazone for treating DM2 is 15 – 30 mg/day. The mode is around 20 mg/kg/day/mouse, equivalent to ∼97mg/day for a 60 kg human. The recommended starting dose for rosiglitazone is 4 mg, but the human equivalent doses used for *in vivo* AD-related studies range from 29 to 58 mg/animal/day. PD/PK-type experiments don't appear to have been done that would justify the selection of these doses, and, in addition to the dangers of off-target effects, the use of such high doses is problematic because we don't fully understand the dose-response characteristics of many (if any) of the read-outs. For instance, both human (Knodt et al., 2019) and rat (Crenshaw et al., 2015) studies revealed inverse-U-shaped fMRI BOLD pioglitazone dose response curves. Additionally, Seok et al. found that 2 mg/kg/day pioglitazone was associated with statistically significant improved learning and memory in the Morris water maze test, and reduced IHC-detectable and soluble hippocampal Aβ40 deposits in 9-month-old SAMP8 mice, but the effect on all of these parameters was less at 5 mg/kg/day (Seok et al., 2019). The results of the rat BOLD study also suggest that lower doses were as, if not more, efficacious as higher doses (Crenshaw et al., 2015). These results are reminiscent of cell culture experiments on mitochondrial biogenesis and Aβ clearance (e.g., Miglio et al., 2009; Moon et al., 2012). There is not an agreed-upon explanation for this dose-response pattern, nor can we predict which disease phenotype will respond to PPARγ agonists in this way. Therefore, any evaluation of PPARγ agonists for treating or delaying the onset of AD needs to take into consideration the effect of drug concentration on the parameters being measured.

The lack of PD/PK studies relevant to Alzheimer's disease, in pre-clinical models or humans, including measurements of

TABLE 1 | Summary of PPARγ agonist effects on pre-clinical models of Alzheimer's disease.

Model/references	Dosing	Human Equivalent Dose[a]	Comments	Results
Treatment paradigm				
Tg2576 (Pedersen et al., 2006)	Rosiglitazone, 4 mg/kg, po (chow), vs vehicle control, 4 months.	19.51 mg/d	Male, 9 months old at initiation of treatment; amyloid deposits, hippocampal dendrite spine loss, and defective spatial learning evident when treatment initiated.	Rosiglitazone was statistically associated with improved memory, reduced learning deficits (radial arm maze); reduced insoluble Aβ42 levels; reduced corticosterone levels.
Tg2576, Nenov 2014, 2015 (Nenov et al., 2014, 2015)	Rosiglitazone, 30 mg/kg, po (chow), vs vehicle control, for 30 days.	146.35 mg/d	Equal numbers of males and females, 8 months old at initiation of treatment.	Learning, memory improvements correlated with improved spontaneous synaptic activity and short-term plasticity; engagement of the ERK pathway and expression of synaptic proteins, restoration of mature: immature DG granule cell ratio, normalized Na_v-mediated currents.
APP (V717I), (Heneka et al., 2005)	Pioglitazone, 40 mg/kg/d; po (chow), vs vehicle control, 7 days	195.12 mg/d	Equal numbers of males and females; 10 months old at initiation of treatment; amyloid pathology present.	Decreased BACE1, amyloid plaque deposits, soluble Aβ42 levels, reactive microglia.
APP (Swe/PS1)Δ9, (Mandrekar-Colucci et al., 2012; Skerrett et al., 2015)	Pioglitazone, 80 mg/kd/d, po (gavage) vs vehicle control, 9 days	390.24 mg/d	Equal numbers of males and females; similar results for mice that were 6 or 12 months-old at initiation of treatment and that exhibited differing pathological loads.	Improved memory retention. Decreased amyloid plaque deposits, reduced soluble and insoluble $Aβ_{40}$ and $Aβ_{42}$ in 6-month-old mice; reduced insoluble Aβ42 and Aβ40 and soluble Aβ40 in 12-month-old mice; reduced reactive microglia and astrocytes and enhanced microglial phagocytosis; increased IL-1β, TNFα, Tm1, Fizz1, Arg1 expression;
APP/PSI (Chen et al., 2015)	Pioglitazone, 10 mg/kg/d, ip, vs. vehicle control, 7 and 10 days.	48.78 mg/d	Equal numbers of males and females; 12 months old at initiation of treatment; pathology present.	Improved LTP after 7 days treatment & water maze performance after 10 days. Reduced CDK5 expression and activity as tau phosphorylation surrogate.
APP (Swe/PS1)Δ9 (Toba et al., 2016)	Pioglitazone, 80 mg/kg/d, po (chow), vs vehicle control, 9 days.	390.25 mg/d	Equal numbers of male and female mice, 5 – 6 months old at initiation of treatment, emergent stages of pathology.	Increased motor coordination, LTP; decreased CDK5 regulatory protein (p25 & p35) expression.
J20 (V717F under PDGF promoter) (Escribano et al., 2010)	Rosiglitazone, 5 mg/kg/d, po (gavage) vs vehicle control, treated for 1 month and 4 months.	24.39 mg/d	Equal numbers of males and females; 10 months old at initiation of treatment.	Improved object recognition after one month and progressively improved spatial memory (Morris Water Maze); reduced amyloid plaque and insoluble Aβ42 and Aβ40 levels, and phosphorylated tau, and promoted anti-inflammatory, pro-phagocytic microglial phenotype.
J20 (Nicolakakis et al., 2008)	Pioglitazone, 20 mg/kg/d, po (chow), vs vehicle control, for 1.5 – 2 months.	97.56 mg/d	Equal numbers of males and females; 14 months old at initiation of treatment; amyloidosis, neuronal loss well established.	No effect on water maze performance, amyloid deposits or levels of soluble or insoluble Aβ42 or Aβ40. Improved cerebral blood flow and glucose uptake; restored cerebrovascular function; trend toward improved cortical cholinergic stimulation; reduced astrogliosis; reversed cerebral oxidative stress.
3xTg, Search (Searcy et al., 2012)	Pioglitazone, 18 mg/kg, po (chow), vs vehicle control, for 3.5 months	87.81 mg/d	Female mice, 11 – 12 months of age when treatment initiated; amyloid deposits well-established, tau aggregates present.	Improved learning on the active avoidance task; enhanced LTP; reduced amyloid deposits and hyperphosphorylated tau in CA1.
Prevention paradigm				
J20 (Escribano et al., 2009)	Rosiglitazone, 5 mg/kg/d, po (gavage) vs vehicle control.	24.39 mg/d	Prevention vs. rescue study. Equal numbers of 1.5-month-old males and females were treated for 2.5 months (prevention), and equal numbers of 9-month-old mice were treated for 1 month (rescue).	Improved object recognition in both cohorts. In older mice, reduced corticosterone levels and blocked glucocorticoid receptor down-regulation.
J20 (Badhwar et al., 2013)	Pioglitazone, 20 mg/kg/day, po (chow) vs. control	97.56 mg/d	Treatment initiated with 3-month-old mice for 14 weeks; small cohort for 3 days.	14-week treatment that was initiated in young mice was statistically associated with improved spatial learning, with trend toward improved memory. Three-day treatment rescued cerebral blood flow; effect persisted in the longer-term treated mice.

(Continued)

TABLE 1 | Continued

Model/references	Dosing	Human Equivalent Dose[a]	Comments	Results
Tg2576 (Rodriguez-Rivera et al., 2011)	Rosiglitazone, 30 mg/kg, po(chow), vs vehicle control, for 4, 8 or 12 months	146.16 mg/d	Equal numbers of males and females, 1 month old at initiation of treatment.	Reversed associative learning and memory deficits in 9-month old animals (fed for 8 months), but not 5 (fed for 4 months) or 13 (fed for 12 months) month-old mice.
SCAMP8 (Seok et al., 2019) SCAMP8 is a spontaneous 'Alzheimer's-like' mouse model that exhibits amyloid and tau pathology, neuron and dendrite spine loss, and CNS oxidative stress (Armbrecht et al., 2014; Cheng et al., 2014)	Pioglitazone, 2 or 5 mg/kg/d, po (gavage) versus vehicle control, for 7 weeks.	9.76 or 24.39 mg/d	Equal numbers of male and female mice, 9 months old at initiation of treatment.	Improved water maze performance, reduced amyloid deposits and soluble Aβ40; increased LRP1 expression. All responses were attenuated at 5 mg/kg/d vs 2 mg/kg/d.
Cerebrovascular model				
J20/TGFβ1 (Papadopoulos et al., 2013)	Pioglitazone, 20 mg/kg/d, po (chow), vs vehicle control, 6 months	97.56 mg/d	Also a 'treatment vs prevention study.' Equal numbers of males and females. Two cohorts, 6 and 12 months of age at beginning of treatment. Adult mice treated for 6 months; aged mice treated for 3 months.	No effect on spatial learning or memory; improved reversal learning in adult but not aged mice. In both adult and aged cohorts, improved cerebral blood flow, cerebral glucose uptake; suppressed astrogliosis in cortex but not in hippocampus; suppressed microglial activation in hippocampus. No effect in either cohort on amyloid pathology, or on cerebrovascular reactivity.
TGFβ1 (Lacombe et al., 2004)	Pioglitazone, 18 mg/kg/d, po (chow), vs vehicle control, 2 months.	87.81 mg/d	Equal numbers of male and female mice, 2 months old at initiation of treatment.	Decreased Aβ42 levels and glia activation, and increased hydrocephalus.
TGFβ1 (Galea et al., 2006)	Pioglitazone, 18 mg/kg/d, po (chow), vs vehicle control, 2 months.	87.81 mg/d	Equal numbers of male and female mice, 2 months old at initiation of treatment.	Pioglitazone inhibited cerebral glucose uptake in control (non-transgenic littermate) mice and failed to reverse TGF1b-mediated inhibition in transgenic mice.
Diabetes models				
ICR mice (Jiang et al., 2012) The ICR strain is a general-purpose mouse line. Diabetes was induced by feeding a high fat diet (60% fat, 20% carbohydrate, 20% protein) for 1 month to 10-week-old mice to elicit peripheral insulin resistance and hyperglycemia, followed by injection with streptozotocin (100 mg/kg), to cause insulin deficiency and cerebral hypoinsulinemia.	Pioglitazone, 18 mg/kg/d, po (chow) or 9 mg/kg/d vs vehicle control, for 6 weeks.	87.81 mg/d or 43.9 mg/d	Equal numbers of male and female mice, 16 – 18 weeks old at the initiation of treatment, with similar body weights and degree of hyperglycemia were randomly assigned to equally sized treatment groups. Non-diabetic controls were similarly divided into treatment groups.	HFD/strep-diabetes was associated with memory impairments; pioglitazone treatment improved learning and memory, and reduced soluble Aβ42 and Aβ40, BACE1, NF-κB and RAGE.

(Continued)

TABLE 1 | Continued

Model/references	Dosing	Human Equivalent Dose[a]	Comments	Results
Sprague-Dawley rats (Gao et al., 2017) 12-week-old rats were fed 60% fat diet for 20 weeks, then injected with streptozotocin (27 mg/kg).	Pioglitazone, 10 mg/kg/d, po (chow) vs vehicle control, for 10 weeks.	48.78 mg/d	Equal numbers of 20-week-old male and female mice were divided into treatment groups, as described for the ICR mice.	HFD/strep rats exhibited memory impairments versus the control and diabetes+pioglitazone groups, which did not differ from each other. Pioglitazone corrected impaired ERK1/2 mRNA and protein expression caused by hyperglycemia.
APOE Model				
APOE TR mice (To et al., 2011) 3-month-old male mice fed 60% fat diet for 32 weeks.	Pioglitazone, 20 mg/k/d, po (gavage) vs vehicle control, for 3 weeks.	97.56 mg/d	40-week-old male HF or LF mice were divided into control and pioglitazone treatment groups and dosed for 3 weeks.	HF diet elicited insulin resistance and impaired glucose tolerance; reduced all phospho-tau epitopes in all mice. Neither diet nor pioglitazone affected APP metabolism. In HF mice, pioglitazone was associated with reduced AT8 p-tau in *APOE ε3* mice and increased AT8 p-tau in A*POE ε4* mice.
Aging-associated neuropathology				
Reversal of aging effects in Wistar rats (Cowley et al., 2012)	Rosiglitazone, 3 mg/k/g, po (chow), vs vehicle control, 56 days.	14.63 mg/d	Equal numbers of male and female rats, 22 months old vs 3 months old (control) at the initiation of treatment.	Rosiglitazone improved T1 relaxation times, improved post-synaptic component of LTP, decreased astrogliosis and RANTES expression, mediated by rosiglitazone-enhanced endothelial cell-astrocyte interactions. No effect on microglial activation.
Prevention of aging effects in Wistar rats (Wang et al., 2012)	Rosiglitazone, 6 mg/kg/d, po (chow), vs vehicle control, 40 days.	29.27 mg/d	Equal numbers of male and female rats, 12 – 14 months old (middle aged) vs 1-month-old controls at the initiation of treatment.	Rosiglitazone improved water maze learning, enhanced synaptic plasticity, place cell activity, improved post-synaptic component of LTP, and restored hippocampal GLUT3 expression.
BOLD imaging Pharmacodynamics				
Young adult Wistar Rats (Crenshaw et al., 2015)	Pioglitazone, 0.04, 0.08, 0.16, 0.32 mg/kg/d, po (gavage) versus vehicle control, for 2 and 7 days.	0.195, 0.39, 0.78 or 1.56 mg/d	Doses were chosen to bracket the dose used in NCT02284906 (TOMMORROW) (0.8 mg/day), after allometric scaling. Study underpowered for the large number of comparisons.	Resting state functional connectivity increased between two regions after two days of 0.08 mg/kg/day; after seven days 17 connections were changed vs. baseline across all 5 dose groups. On day 7, connectivity between CA1 and ventral thalamus was increased in all pioglitazone doses but was weakest at 0.32 mg/kg/day.

[a] *https://www.fda.gov/regulatory-information/search-fda-guidance-documents/estimating-maximum-safe-starting-dose-initial-clinical-trials-therapeutics-adult-healthy-volunteers.*

target engagement in the brain, contributes to difficulties in planning reliable human studies, and hinders the development of testable theories of drug mechanisms of action on the observed responses. In fact, none of the studies summarized in **Table 1** confirmed that, under these treatment regimens, the drugs entered the rodent brain. Rosiglitazone has essentially no BBB penetrance (GSK, unpublished), and pioglitazone has low BBB penetrance (Maeshiba et al., 1997). Yet, pioglitazone ranging from 0.04 to 0.32 mg/kg/d increased functional connectivity between the CA1 region and the ventral thalamus in young adult Wistar rats, but the connectivity fell off at the highest dose (Crenshaw et al., 2015). It appears that either (Longhe, 2020) high pioglitazone concentrations are unnecessary, at least for some responses; or (Patterson, 2018) that some processes are responsive to small amounts that penetrate the BBB and large doses are necessary to overcome the transport barrier (by mass action); or (Zissimopoulos et al., 2015) that some processes respond to actions of the drug outside the BBB. One investigation concluded that rosiglitazone modulates astrocyte behavior *in vivo* indirectly, via regulating interactions between BBB endothelial cells and astrocytes (Cowley et al., 2012). To the best of our knowledge, these observations have not been followed-up. Given the potential for cross-talk between astrocytes and microglia (Clark et al., 2021), such an indirect pathway might also contribute to PPARγ agonist-mediated *in vivo* regulation of microglial function.

In designing *in vivo* efficacy and MOA studies, and to provide guidance for human clinical trials, doses and treatment times should be optimized through implementation of detailed PD/PK studies that include quantification of drug substance and target engagement in the brain and the BBB, as well as in relevant peripheral cells.

HUMAN STUDIES

Observational Cohort Studies

Several longitudinal observational cohort studies have shown that pioglitazone reduces the risk and delays the onset of dementia in the context of type 2 diabetes mellitus. These studies were performed using data extracted from national health insurance records, on subjects who were diagnosed with type 2 diabetes mellitus (DM2) and without dementia (as coded by the International Classification of Diseases, Ninth (ICD-9) or Tenth (ICD-10) editions) on the index date. The index date was the date of first prescription of the drug. In most cases, observations ended after 5 years or when subjects were diagnosed with dementia. Rosiglitazone had a neutral effect (Tseng, 2019). Meta-analysis of these observational studies have been published (Ye et al., 2016; Zhou et al., 2020).

Miller et al. used Department of Veterans Affairs (VA) records to conduct an analysis of US veterans with diabetes but without a recorded diagnosis of AD for two years prior (Miller et al., 2011). Their analysis included subjects prescribed with either rosiglitazone or pioglitazone. The study population was heavily white (79%) and male (98%) type 2 diabetics, who were followed from the time of drug initiation until the first AD diagnosis, which was made using the ICD-9 codes. In this population, the hazard ratio (HR) of thiazolidinedione (TZD) only vs. insulin only was 0.81 (95% CI, 0.73 – 0.89). When insulin and TZD use were combined to improve glycemia control, the HR for insulin followed by TZD was 0.63 (95% CI, 0.53 – 0.74), and for TZD followed by insulin was 0.72 (95% CI, 0.61. – 0.84).

Using German public health insurance company records, Heneka et al. considered only subjects who did not receive insulin and who were dementia-free for two years prior to the index date, which was the date of first pioglitazone use, and they followed subjects for 5 years (Heneka et al., 2015b). The populations they followed were: $\geq$ 60 years old, free of dementia at the beginning of the study, diabetics not taking pioglitazone, diabetics taking pioglitazone (broken down by length of time on drug), and non-diabetics. Long-term pioglitazone use (receiving pioglitazone prescriptions $>$ 8 quarters) was associated with lower risk for dementia relative to non-diabetics [Relative Risk (RR), 0.53 (95% CI, 0.301 – 0.936, P = 0.029)], while short-term users ($<$8 quarters) had an RR $\sim$ nondiabetics (RR, 1.16, P = 0.317). For diabetics without a pioglitazone prescription, the relative risk was 1.23 ($P < 0.0001$). Neither rosiglitazone nor metformin use altered the risk in this dataset. The RR for insulin use was 1.608 (95% CI, 1.459 – 1.773, $P < 0.001$).

Chou et al. confirmed that pioglitazone reduced the risk of dementia in patients with DM2, among Taiwanese (Chou et al., 2017). They extracted data from the Longitudinal Health Insurance Database subset of Taiwan's National Health Insurance Research Database (NHI), for 'ever pioglitazone' vs 'never users'. The cohort were dementia free at the index date, and they incorporated a 5-year follow-up period. The pioglitazone cohort had a higher prevalence of stroke and hypertension than the comparison cohort, which was matched to the pioglitazone cohort by age, sex and index date. They used a 'defined daily dose' (DDD, 30 mg/day), as recommended by the World Health Organization, to quantify the daily pioglitazone use. Overall, the risk for dementia was 23% lower in the pioglitazone use group versus in the comparison group; HR = 0.77 (95% CI, 0.62 – 0.95, P = 0.015). The pioglitazone effect was time- and dose-dependent. The hazard ratios were 0.50 (95% CI, 0.34 – 0.75, P = 0.001) in the high cumulative user group ($>$444 defined daily dose), 0.53 (95% CI, 0.36 – 0.77, $P < 0.001$) in the long-term user group ($>$536 days), and 0.66 (95% CI, 0.49 – 0.90, P = 0.009) in the high-mean daily dose user group ($>$mean daily dose).

Tseng also followed subjects in Taiwan's NHI, but restricted his analysis to a 2 year follow-up period (Tseng, 2018). Unlike Chou et al., Tseng matched the pioglitazone- and comparison-cohorts for comorbidities, including hypertension, dyslipidemia, ischemic heart disease, peripheral arterial disease, Parkinson's disease, statin use and other glycemic control agents. Analysis of the unmatched and the matched cohorts confirmed that pioglitazone use was associated with significantly lower risk for dementia. Metformin was also found to be protective in this study, but pioglitazone's protective effects were independent of metformin. The effect of pioglitazone was largest in patients who had never taken metformin and among those who had taken pioglitazone for $>$ 20 months. In the matched cohort, among patients who had never used metformin, the hazard ratios for ever vs never pioglitazone were: $<$ 11 months: 0.588 (95% CI, 0.272 – 1.273, P = 0.1778), 11 – 19.6 months: 0.690 (95% CI, 0.338 – 1.409, P = 0.3084), $>$ 19.6 months: 0.265 (95% CI, 0.102 – 0.688, P = 0.0064). By contrast, for all patients, the hazard ratio for ever pioglitazone vs never users was 0.716 (95% CI, 0.545 – 0.940, P = 0.163), and the hazard ratios for $<$ 110 months use, 11 – 19.6 months and $>$ 19.6 months use were 0.806 (95% CI, 0.544 – 1.193, P = 0.2809), 0.654 (95% CI, 0.430 – 0.994, P = 0.0467), and 0.694 (95% CI, 0.469 – 1.026, P = 0.067).

Tseng's findings that metformin was protective are at odds with Heneka et al., who observed no benefits for metformin users. Interestingly, Bohlken et al. also reported that metformin use did not reduce incidence of dementia in a German cohort (Bohlken et al., 2018). Their study relied on the German Disease Analyzer database (IQVIA), representative of General Practices (as opposed to all sources of health case, including Neurology), and involved a disease cohort that was dementia-free for at least one year prior to the index date. The 'ever' and 'never' drug cohorts were matched for age, sex and comorbidities. The odds ratio of developing dementia for those taking glitazones (pioglitazone or rosiglitazone) was 0.80 (95% CI, 0.68 – 0.95, P = 0.011) and the odds ratio for metformin was 0.96 (95% CI, 0.88 – 1.04, P = 0.153). The discordance between the German and Taiwanese metformin results could reflect differences in dementia subtypes (Neff et al., 2021), other genetic background differences of the ethnic groups represented in the respective databases, comorbidities of the 'ever' and 'never' groups in the respective studies, the severity of diabetes, or methodological differences in data collection and analysis.

Numerous observational studies of other hypoglycemic agents, including insulin, glycosidase inhibitors, metformin,

sulfonylureas and DPP-4 inhibitors have been conducted, and the results have been examined and summarized in two meta-analysis (Ye et al., 2016; Zhou et al., 2020). Generally, the DPP-4 inhibitors were associated with the lowest risk of dementia, followed by metformin and the thiazolidinediones, which lumped pioglitazone and rosiglitazone together (Zhou et al., 2020). Another study has shown that no hypoglycemic agents, including thiazolidinediones or metformin, delayed the onset of AD (Imfeld et al., 2012). Because the effects of rosiglitazone are neutral (Tseng, 2019), lumping it together with pioglitazone produces confounding results in these meta-analyses. DPP-4 inhibitors block the degradation of the incretin hormones (GIP, GLP-1), which regulate microglial function (Spielman et al., 2017). Because incretins trigger PPARγ expression (Svegliati-Baroni et al., 2011; Onuma et al., 2014), it is not possible to disentangle the PPARγ and incretin contributions to the DPP-4 effects evident in these data.

Overall, the longitudinal cohort studies demonstrate pioglitazone use is associated with a reduced risk for dementia in populations of adult-onset diabetics, and the effect is time and dose dependent. These results are in line with the pilot clinical studies conducted using DM2 cases (Hanyu et al., 2009, 2010; Sato et al., 2011). The strength of the 'never vs ever' longitudinal cohort studies is they approximated placebo controlled, clinical trials that matched subjects for age, sex and co-morbidities and had realistic follow-up periods (2 – 5 years). However, they all relied on ICD diagnostic codes to define dementia and often there was a lag between onset of dementia and diagnosis. Heneka et al. attempted to overcome that shortcoming by adopting a multi-layer approach to diagnosis. Additionally, none of these studies accounted for *APOE* or other genetic risk factors, life-style factors, or the severity of diabetes and the degree of insulin resistance and glycemic control. To overcome the difficulties of these observational studies and of the small-scale pilot clinical studies with DM2 (section 10.2), a large-scale blinded, placebo controlled clinical trial in populations with adult-onset diabetes, that are at increased risk of developing Alzheimer's disease, seems warranted.

Clinical Studies

Table 2 summarizes twelve clinical trials that reported data evaluating thiazolidinedione PPARγ agonists as therapeutics for Alzheimer's disease. We will focus on those studies that highlight important points. We include two pharmacodynamics studies using measures of brain energetics as readouts, two small studies that included measures of drug effects on Aβ peptide levels, two that correlated drug effects on insulin-lowering, and three small studies evaluating the efficacy of pioglitazone in volunteers with pre-existing DM2; we did not include in the table an additional study reporting similar outcomes by this group, but we do discuss it below. Aside from the studies in DM2 patients, all of the studies excluded individuals with a history of diabetes or who were taking medications to control glucose. Two of the studies were prevention trials with participants who were free of dementia at the time of enrollment, that reflected one of the important lessons from the pre-clinical studies, that beginning treatment before the onset of AD-related pathology preserved learning and memory (Badhwar et al., 2013). The rest were treatment studies that involved subjects with mild-to-moderate Alzheimer's disease or mild cognitive impairment at the outset. With the exception of NCT00348309, NCT00348140 and NCT01931566, all of the studies may have been underpowered or conducted for too short a duration relative to the conversion rate in the controls from normal cognition to MCI or mild-AD.

Several small pilot studies evaluated the efficacy of rosiglitazone and pioglitazone in the context of the metabolic risk factors insulin resistance and type 2 diabetes mellitus (Luchsinger et al., 2004; Biessels et al., 2006; Muller et al., 2007; Xu et al., 2009; Willette et al., 2015a,b; Wu et al., 2016). Watson et al. enrolled volunteers with early AD and amnestic MCI for a 24-week trial with rosiglitazone (Watson et al., 2005). These volunteers were also mildly insulin resistant, based on the HOMA-IR scores calculated from the reported average baseline insulin and glucose values (calculated 2.0; scores > 1.9 are mildly insulin resistant) (Matthews et al., 1985). In addition to changes from baseline cognitive scores, these authors they quantified plasma insulin and, unlike most of the other studies that we located, the plasma biomarkers Aβ42 and Aβ40. Over the course of the 6-month trial, rosiglitazone preserved delayed memory scores and selective attention, while memory deteriorated in the control group (Watson et al., 2005). The Aβ42/Aβ40 ratio fell in the placebo group but was stabilized by rosiglitazone. Rosiglitazone also elicited a small but statistically significant drop in peripheral insulin, and the degree of memory preservation and error rates on the interference test were inversely related to changes in plasma insulin levels. Risner et al. also found suggestive evidence of an interaction between changes in insulin levels and cognition (Risner et al., 2006). This effect may be related to the correction of central hypoinsulinemia, which peripheral hyperinsulinemia causes (Baura et al., 1996). Glucose levels rose, but the change did not attain statistical significance.

APOE ε4 is the most significant genetic risk factor for developing late-onset Alzheimer's disease, and several studies investigated whether there was an interaction of *APOE ε4* carriage with rosiglitazone or pioglitazone effects on cognition. Risner et al. conduced a small, 24-week, dose-response study (average N = 128) (Risner et al., 2006). Overall, rosiglitazone did not have a significant effect on cognition, but tests for interaction between ADAS-Cog score and APOE status were significant. Cognition improved at the highest rosiglitazone dose (8 mg) in *APOE ε4* negative subjects. Notably, *APOE ε4* negative subjects also experienced a greater drop in plasma insulin elicited by 8 mg rosiglitazone than subjects who carried at least one *APOE* ε4 allele. The relationship between insulin lowering and cognitive improvement is reminiscent of Watson et al. (2005), but unlike in the latter study, the interaction was not formally analyzed here.

GlaxoSmithKline studied the interaction between *APOE* status and rosiglitazone efficacy further in two larger-scale, dose-response trials, which compared the efficacy of low (2 mg) versus high (8 mg) rosiglitazone in test populations that were stratified by APOE status, as adjunctive therapy to AChEIs (Gold et al., 2010; Harrington et al., 2011). NCT00428090 ran for 24 weeks, with average N = 124 subjects (Gold et al., 2010), and the NCT00348309 and NCT00348140 studies ran for 48

TABLE 2 | Summary of PPARγ agonist clinical trial results for Alzheimer's disease.

Study	Treatment	Study Design	Population	Results
A. Rosiglitazone				
A.1. Phase 2 Intervention				
Preserved cognition in patients with early Alzheimer disease and amnestic mild cognitive impairment during treatment with rosiglitazone (Watson et al., 2005)	Rosiglitazone 4 mg daily, vs placebo.	24-week, placebo-controlled, double blind, parallel-group study in subjects with early AD and Amnestic MCI. Outcome measures: cognition, plasma insulin, plasma Aβ.	Placebo, N = 10, rosiglitazone, N = 20. Average age, 73 years, 70% F, 100% White. Baseline insulin, 8.1 μU/mL; MMSE mean baseline, 23. Subjects taking medications to control glucose were excluded.	Rosiglitazone was statistically associated with better delayed recall at 4 and 6 months, selective attention at 6 months, stable plasma Aβ42, Aβ40, and Aβ42/Aβ40 ratio.
Efficacy of rosiglitazone in a genetically defined population with mild-to-moderate Alzheimer's disease (Risner et al., 2006)	Rosiglitazone 2, 4 or 8 mg daily vs placebo.	24-week, placebo-controlled, double blind, parallel-group pilot study in subjects with mild-to-moderate AD (phase 2). Outcome measures: ADAS-Cog, CIBIC+	Average N = 128, average age, 70.7 years, 60% F, 100% White. Balanced for *APOE* ε4. Baseline insulin, 14.2 μU/mL. MMSE mean baseline, 21.3. Subjects with history of T1DM or T2DM, or with fasting glucose ≥ 7mM or HbA1c ≥ 8.5% were excluded.	*Overall*: no statistically significant effect of rosiglitazone on outcome measures. *In APOE ε4 non-carriers*, treatment with 8 mg rosiglitazone was statistically associated with improved ADAS-Cog.
NCT00265148 Effects of rosiglitazone on cognition and cerebral glucose utilization in subjects with mild to moderate Alzheimer's disease (Tzimopoulou et al., 2010)	Rosiglitazone 4 mg daily for one month, increasing to 8 mg daily for the remainder of the study, vs placebo.	52-week parallel group, double blind, phase 2 study. Outcome measures: 12-month cerebral glucose metabolic rate change, brain volume, ADAS-Cog, CIBIC+	Average N = 31 completed the study, average age, 71.25 years, 46.2% F, 94.9% White. Balanced for *APOE* ε4. Subjects with history of T1DM or T2DM or taking medications* to control glucose were excluded.	No sustained treatment effect on total or regional glucose metabolic rate or brain volume; no effect on ADAS-Cog or CIBIC+.
A.2. Phase 2 – Diabetes				
Rosiglitazone and cognitive stability in older individuals with type 2 diabetes and MCI (Abbatecola et al., 2010)	Rosiglitazone 4 mg daily, vs metformin, 500 mg daily, vs. rosiglitazone + metformin vs. diet.	36-week, prospective, randomized, open-controlled study in subjects with mild-to-moderate AD in association with T2DM. Outcome measures: changes in neuropsychological test scores and metabolic control parameters (FIRI, FPG, HbA1C).	Average N = 32.2, average age, 76 years, 45% F; FPG mean baseline, 8.44 mmol/L; FIRI, 148 pmol/L; mean baseline HbA1C, 7.5%; MMSE mean baseline, 24; TMT-A mean baseline, 67.6; TMT-B mean baseline, 161.1; DIFFBA mean base line, 101.2; RAVLT mean baseline, 24.5.	Metformin/rosiglitazone combination stabilized all neuropsychological tests. Metformin stabilized MMSE, TMT-A, TMT-B; diet stabilized MMSE, TMT-A. In linear-fixed effects model, FIRI x time correlated with metformin/rosiglitazone RAVLT.
A.3. Phase 3 Intervention				
NCT00428090 Rosiglitazone (Extended Release Tablets) as monotherapy in subjects with mild to moderate Alzheimer's disease (Gold et al., 2010)	Rosiglitazone 2 or 8 mg extended release, daily vs placebo (REFLECT-1).	24-week, double blind, double dummy, randomized, parallel group phase 3 study, stratified for APOE ε4 status in subjects with mild-to-moderate AD. Outcome measures: ADAS-Cog, CIBIC+	Average N = 159, average age, 72.3 years, 37% F, 72% White, balanced for *APOE* ε4. ADAS-Cog mean baseline, 19.1. Subjects with history of T1DM or T2DM or taking medications* to control glucose were excluded.	No statistically or clinically significant effect of either rosiglitazone dose in full population; no significant evidence of interaction between treatment with rosiglitazone and APOE genotype.
NCT00348309/ NCT00348140 Rosiglitazone (Extended Release Tablets) As Adjunctive Therapy For Subjects With Mild To Moderate Alzheimer's Disease (Harrington et al., 2011)	Rosiglitazone 2 or 8 mg extended release, as adjunctive to donepezil (REFLECT-2), or adjunctive to any AChEIs (REFLECT-3).	48-week, double blind, randomized, placebo-controlled, parallel group, phase 3 studies, stratified for APOE ε4 status in subjects with mild-to-moderate probable AD. Outcome measures: ADAS-Cog, CDR-SB.	*NCT00348309* (REFLECT-2): Average N = 464, average age, 74.1 years, 60% F, 90.67% White, balanced for *APOE* ε4. ADAS-Cog mean baseline, 25.3; MMSE mean baseline, 19.46. *NCT00348140* (REFLECT-3): Average N = 476.3, average, 73.9 years old, 55.6% F, 91.76% White, balanced for *APOE* ε4. ADAS-Cog mean baseline, 24.1; MMSE mean baseline, 19.7. Subjects with history of T1DM or subjects with T2DM taking medications* to control glucose were excluded.	No statistically or clinically significant effect of either rosiglitazone dose in full population; no significant evidence of interaction between treatment with rosiglitazone and APOE genotype in either REFLECT-2 or REFLECT-3.

(Continued)

TABLE 2 | Continued

Study	Treatment	Study Design	Population	Results
B. Pioglitazone **B.1. Phase 1 - Dose-ranging**				
NCT01456117 Study to assess the effects of daily administration of pioglitazone on brain hemodynamics in cognitively healthy elderly subjects (Knodt et al., 2019)	Pioglitazone, 0.6 mg, 2.1 mg, 3.9 mg, 6.0 mg daily, vs. placebo	A 2-week, multiple-dose, single-blind, randomized, parallel design, placebo-controlled, phase 1 dose-ranging study. Outcome measure: episodic memory-related hippocampal activity, measured via blood oxygen level-dependent (BOLD) functional magnetic resonance imaging.	Average N = 11, average age = 66.08 years, 71% F, 85.45% White. CERAD-WLM mean baseline, 8.02, TMT-B mean baseline, 97.58. Diabetic subjects taking medications to control blood glucose*, or with HbA1C > 6% were excluded.	Statistical association of 0.6 mg/day pioglitazone with increased right hippocampal activation during encoding of novel face-name pairs at day 7 and day 14, relative to baseline. No statistically significant improvement at 2.1, 3.9 or 6.0 mg/day.
B.2. Pilot - Diabetes				
Role of tumor necrosis factor-alpha in cognitive improvement after peroxisome proliferator activator receptor gamma agonist pioglitazone treatment in Alzheimer's disease (Hanyu et al., 2010)	Pioglitazone, 15 mg daily vs. none.	24-week, prospective, randomized, open-controlled study, in subjects with mild-to-moderate Alzheimer's disease in association with T2DM. Outcome measures: ADAS-JCog, MMSE, TNFα, IL-6, C-reactive protein.	N = 17 for both groups, average age 78.7 years, 50% F, 100% White, balanced for APOE ε4 and donepezil use; MMSE mean baseline, 21.85; ADAS-JCog mean baseline, 15.65; TNFα mean baseline, 1.38 pg/mL; IL-6 mean baseline, 2.62 pg/mL; C-reactive protein mean baseline, 0.08 mg/dL.	Pioglitazone was statistically associated with improved ADAS-JCog and TNFα, and changes in ADAS-JCog were correlated with changes in TNFα.
Efficacy of PPARγ agonist pioglitazone in mild Alzheimer disease (Sato et al., 2011)	Pioglitazone, 15 or 30 mg daily vs. none.	24-week prospective randomized, open-controlled study in subjects with mild-to-moderate Alzheimer disease in association with T2DM. Outcome measures: ADAS-JCog, MMSE, WMS-R, rCBF, plasma Aβ40 and Aβ42, HOMA-R, HbA1c, FIRI.	N = 21 for both groups, average age, 77.5 years, 52% F, 100% White, balanced for *APOE* ε4; balanced for other hypoglycemic agents, donepezil.	Pioglitazone was statistically associated with improved MMSE, ADAS-JCog and WMS-R, with improved blood flow in the parietal lobe, and with improved metabolic factors. The plasma Aβ40/Aβ42 ratio did not change in the pioglitazone group and increased in the control group. ADAS-JCog significantly worsened in the control group.
B.3. Phase 2 Intervention				
NCT00982202 Pioglitazone in Alzheimer's disease safety trial (Geldmacher et al., 2011)	Pioglitazone, 15 mg daily, escalating weekly to 45 mg daily, vs. placebo.	72-week, double-blind, randomized, placebo-controlled, group comparison study of mild-to-moderate probable Alzheimer's disease Outcome measures (collected at 3-month intervals): (Longhe, 2020). Measures of cognition, including ADAS-Cog, CDR-SB. (Patterson, 2018). Estimate for effect size calculations.	Average N = 14.5, average age, 70.95 years, 62% F; MMSE mean baseline, 21; ADAS-Cog mean baseline, 21; CDR-SB mean baseline, 5.8.	Pioglitazone was not statistically associated with any improved measure of cognition; the adjusted mean for ADAS-Cog per month was lower in the pioglitazone group, but not statistically significant. For α = 0.05 and power = 0.80, sample sizes of 340 (170 pio, 170 placebo) and 155 (78 pio, 77 placebo) subjects would be required for their estimated regression coefficients of the pioglitazone effect on ADAS-Cog (-0.746) and CDR-SB (-0.354), respectively, to be significant.
NCT00736996 Pioglitazone and exercise effects on older adults with MCI and metabolic syndrome (POEM) (Hildreth et al., 2015)	Pioglitazone, 15 mg daily escalating to 45 mg daily after one month, versus placebo; or 45-75 minutes exercise training 3X/week, vs. *status quo* exercise. Exercise regimen initiated at 50-60% HR max, escalated to 80-85% HR max over the course of the study.	24-week double-blind, randomized, placebo-controlled pilot study in sedentary adults with MCI and central obesity. Outcome measures: Change in baseline for cognition, insulin clamp, body composition, metabolic and inflammatory markers.	Average N = 22, average age 65.6 years, 51.8% F, 87.8% White, balanced for *APOE* ε4; average compliance, pioglitazone, 76%, placebo, 89%; glucose mean baseline, 101 mg/dL; insulin mean baseline, 16.1 μU/mL; C-reactive protein mean baseline, 3 mg/L; IL-6 mean baseline, 1.7 pg/mL; TNFα mean baseline, 1.56 pg/mL; MMSE mean baseline, 28.6; ADAS-Cog mean baseline, 6.	Pioglitazone was not statistically associated with any improved measure of cognition; performance on the Visual Reproduction Test; scores worsened in the pioglitazone group vs. placebo; ADAS-Cog improved with exercise (-1.3 EX vs. -0.3 CON; P = 0.05). No statistically significant correlations between glucose disposal rates and cognitive performance.

(Continued)

TABLE 2 | Continued

Study	Treatment	Study Design	Population	Results
B.4. Phase 3 Prevention				
NCT01931566 A study to simultaneously qualify a biomarker algorithm for prognosis of risk of developing MCI Due to AD and to test the safety and efficacy of pioglitazone to delay the onset of MCI due to AD in cognitively normal subjects (Alexander et al., 2019; Burns et al., 2019) *NOTE: The full publication describing this study was under review when the current paper was submitted.*	Pioglitazone, 0.8 mg extended release daily, vs placebo.	Event-driven (anticipated 5 yr.), double-blind, randomized, parallel group placebo-controlled Phase 3 prevention study in cognitively normal adults susceptible for AD (*APOE, TOMM40* genotypes and age). Outcome measures: Delay onset of MCI in normal participants who are at increased risk due to age and genetic risk factors.	N = 433 low-risk placebo, 1516 high-risk placebo, 1545 high risk pioglitazone. Average age, 73.1 years, 56.16% F, 96.6% White; average *APOE ε4* carriage in the high-risk groups, 92.45%; MMSE mean baseline, 28.56. Outcome measure: Time to diagnosis of MCI due to AD for pioglitazone-treated subjects vs placebo in high-risk stratum. Pre-specified futility threshold, 30% conditional probability that a 40% treatment difference would be detected.	Study terminated due to futility analysis. After 1278 days, total events in placebo, 46; total events with pioglitazone, 39. Pioglitazone risk ratio vs placebo was 0.8 (95% CI, 0.45 – 1.4), P = 0.307; post-hoc subgroup analysis suggests possible benefit of pioglitazone for males.

ADAS-Cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; ADAS-JCog, Japanese version of the ADAS-Cog; ATP III, Adult treatment panel III criteria for central obesity; rCBF, regional cerebral blood flow; CDR-SB, Clinical Dementia Rating scale-Sum of Boxes; CIBIC+, Clinician's interview-based impression of change with caregiver input; DIFFBA, TMT-A minus TMT-B – a measure of cognitive efficiency; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-R, homeostatic model assessment for insulin resistance; MCI, mild cognitive impairment; MMSE, Mini-mental state examination; RAVLT, Rey Auditory-verbal learning test; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TNFα, tumor necrosis factor-alpha; TMT-A, TMT-B, Trail marking test A and Trail marking test-B, respectively.
**Insulin, sulfonylureas, PPARγ agonists or glitinides.*

weeks with average N = 464 subjects (Harrington et al., 2011). Rosiglitazone did not have a statistically significant effect on cognition in either *APOE ε4+* or *APOE ε4-* subjects at either dose, in either trial. There was essentially no difference in HbA1c values between the two rosiglitazone concentrations in Gold et al. (2010) and increased with increasing dose of rosiglitazone in Harrington et al. (2011). The authors did not report any statistical interaction between these changes and scores for cognition. Fasting glucose and fasting insulin values were not measured in any of these studies.

As demonstrated in volunteers with DM2 (Sato et al., 2011) as well as in mouse models of AD (Nicolakakis et al., 2008; Papadopoulos et al., 2013), PPARγ agonists promote central glucose metabolism. Tzimopoulou et al. measured cerebral glucose metabolic rates (CMRglu) and brain atrophy as pharmacodynamics markers, of central rosiglitazone action. The volunteers for this study had mild-to-moderate Alzheimer's disease, and were age- and sex-matched with cognitively normal controls for a 52-week trial (Tzimopoulou et al., 2010). Rosiglitazone (8 mg extended release/day) was statistically associated with a modest (1.5%) increase in glucose utilization, compared with a 4.7% decrease for the placebo over the first month of treatment. However, this immediate increase was not robust and the mean CMRglu rates decreased in both groups over the remaining 11 months of the trial. Although the rate of decline of CMRglu was lower in the rosiglitazone group than in the placebo, the trend was only suggestive, and there was no evidence that rosiglitazone affected changes in brain volume or cognition. *APOE ε4* carriage did not affect any outcome. As above for the REFLECT studies, no fasting insulin or glucose values were recorded, so it was not possible to assess interaction between changes in insulin level or insulin resistance and cognition or these pharmacodynamics markers.

In a series of small pilot studies with DM2 cases, Hanyu's group reported that pioglitazone (15 – 30 mg/day) improved cognitive measures in type 2 diabetics after 6 months; ADAS-JCog scores improved in DM2 cases taking the drug, but worsened in the control diabetics who did not (Hanyu et al., 2009, 2010; Sato et al., 2011). Regional cerebral blood flow also improved with pioglitazone (Sato et al., 2011), as did peripheral TNFα levels (Hanyu et al., 2010). Pioglitazone stabilized the Ab42/Ab40 ratio, which decreased in the controls (Sato et al., 2011).

While Hanyu's group monitored cases with pre-existing DM2 to evaluate pioglitazone's effectiveness in delaying cognitive decline, Abbatecola et al. took a different tack, to learn if a PPARγ agonist could ameliorate pre-existing MCI. Another difference was they studied the combination therapy of rosiglitazone added to metformin, vs monotherapy of metformin alone or diet alone to control glycemia. Combining rosiglitazone with metformin was superior to metformin alone and diet alone in slowing cognitive decline (Abbatecola et al., 2010). These data are consistent with the longitudinal cohort study, showing a trend toward increased protection in metformin users in the first 12 months following the addition of rosiglitazone (Tseng, 2019).

Of the remaining three studies, one was a pharmacodynamics study and two were prevention studies. Hildreth et al. directly investigated the role of a metabolic risk factor, insulin resistance, on cognitive impairment in genetically low-risk populations, using a high pharmacological dose of pioglitazone. The TOMMORROW study measured (NCT01931566) the efficacy of pioglitazone to delay the onset of cognitive impairment in a population that was metabolically robust but genetically at risk for developing late-onset Alzheimer's disease; it involved a very low pioglitazone dose established with the help of the PD study.

Knodt et al. used BOLD fMRI as a pharmacodynamics tool, to determine pioglitazone's effect on the hippocampal function and as a tool to inform dose selection for the TOMMORROW study (NCT01931566) (Knodt et al., 2019). Healthy, cognitively normal volunteers received daily for 14 days vehicle (placebo), or 0.6 mg, 2.1 mg, 3.9 mg or 6.0 mg pioglitazone. For perspective, the starting dose, and smallest tablet size, for treating DM2 is 15 mg, and these doses ranged from 0.4 to 40% of that dose. Overall, reaction times for correctly recalled face-name pairs were negatively correlated with activity in both the right and left hippocampus during encoding. 0.6 mg pioglitazone was associated with increased right hippocampal activation from baseline to day 7 and from baseline to day 14. The placebo group exhibited decreased right hippocampal activation from day 7 to day 14. These data support that pioglitazone has an effect in conscious humans on brain function, and moreover suggest a hormetic dose-response effect on hippocampal function, similar to what was observed in the rat BOLD study for interactions between the CA1 region and hypothalamus and ventral thalamus (Crenshaw et al., 2015). Together with the findings from Hanyu's group summarized above and the observational cohort studies, that involved clinical levels of pioglitazone (Heneka et al., 2015b; Chou et al., 2017; Tseng, 2018), these results suggest the overall salutary effect of pioglitazone on risk of dementia may be mediated through multiple targets, each responsive to a unique pioglitazone concentration range.

The POEM study evaluated and compared the efficacies of exercise and pioglitazone on changes in cognition scores and on metabolic parameters (Hildreth et al., 2015). Additionally, they measured circulating markers of inflammation (CRP, IL-6 and TNFα). As in Watson et al. (2005), the participants in this study were insulin resistant at baseline (HOMA-IR score, 4.0), but unlike Watson et al. they were cognitively normal (mean baseline MMSE, 28.6). Neither pioglitazone nor exercise affected circulating inflammatory markers. Fasting insulin and insulin resistance, as measured by euglycemic-hyperinsulinemic clamp, improved in the pioglitazone group, but neither exercise nor pioglitazone affected cognitive performance, and there was no interaction between improved glucose disposal rate and any domain of cognitive performance. Cognitive performance did improve in *APOE* ε*4*-negative participants, but the change was not statistically significant. As Hildreth et al. pointed out, if there was any cognitive impairment among the participants at baseline, it was very mild. With only an average of 22 subjects, followed for only 6 months, likely there would have been too few conversions from normal cognition to MCI in the placebo group to detect possible effects of pioglitazone.

The TOMMOROW study (NCT01931566) was designed to determine if low dose (0.8 mg/day, extended release) pioglitazone would delay-of-onset of mild clinical impairment due to Alzheimer's disease, in cognitively and metabolically normal subjects who, due to genetic risk factors, are at high risk of developing MDI due to AD within 5 years. It involved 3500 participants; aged 65 – 83. Subjects were assigned as either low or high risk to develop MCI due to AD in the subsequent 5 years, stratifying risk by age at entry and genotypes at *APOE* and *TOMM40 '523* loci. High-risk subjects, most of whom carried at least one *APOE* ε*4* allele, were assigned to either the placebo or treatment arm (average N = 1530). The baseline average MMSE score was 28.56, and volunteers with a history of diabetes or who were taking drugs that affected glycemia were excluded. The study outcome was the delay of onset of MCI, and it was sufficiently powered to detect a 30% difference in change from base line over the anticipated running time of 5 years had there been one. However, after the study was initiated the futility criteria was changed to 40% difference, and futility analysis led to an early termination of the trial, when the majority of subject had less than 3 years' drug exposure. Pioglitazone in high-risk non-Hispanic/Latino Caucasian subjects did not have a statistically significant effect different from placebo (39/1430 [2·7%] *vs* 46/1406 [3·3%]; HR 0·80; 99% CI, 0·45 – 1·40; p = 0·307). Although not statistically significant, a pre-specified sex subgroup analysis revealed potential differences in male subjects (pioglitazone HR 0·56; 95% CI, 0·30–1·06; p = 0·074) (Alexander et al., 2019). No pharmacodynamic measures were collected, including plasma Aβ peptide levels. No metabolic parameters aside from HbA1c were collected, and analysis of possible interactions between changes in performance on the cognitive battery and changes in fasting insulin or insulin resistance is not possible.

In addition to the clinical studies summarized in **Table 2** for PPARγ agonists, a dual PPARδ/PPARγ agonist currently is being evaluated for its effects on the risk of developing dementia in subjects with mild to moderate AD. T3D-959 is 15X more potent against PPARδ than PPARγ. PPARδ agonists are hypothesized to reduce risk for AD because they regulate glucose and fatty acid utilization and enhance anti-oxidant and anti-inflammatory signaling (Liu Y. et al., 2018). In an exploratory phase IIb study, T3D-959 increased cerebral glucose utilization, and provided suggestive improvement in cognitive assessments (Chamberlain et al., 2020). A double-blind placebo-controlled phase 2 dose-ranging study is currently underway. Its primary outcomes are effects on cognition and global function, and exploratory measures include plasma Aβ 42/40 ratio, Nfl and tau, and cerebral glucose utilization (Clinicaltrials.gov NCT04251182) (Didsbury et al., 2020).

Overall, pioglitazone and rosiglitazone were ineffective in restoring cognitive function or in delaying the onset of MCI due to Alzheimer's disease in non-DM2 volunteers. However, several caveats should be considered before we can consider this a settled issue. First, most of these studies were insufficiently powered or were not conducted long enough to detect changes with statistical confidence (Watson et al., 2005; Hanyu et al., 2009, 2010; Abbatecola et al., 2010; Tzimopoulou et al., 2010; Geldmacher et al., 2011; Sato et al., 2011; Hildreth et al., 2015; Knodt et al., 2019). For instance, Geldmacher et al. conducted an 18-month, Phase 2 pioglitazone (15 mg/day initially, escalating to 45 mg/day after one month) safety study in volunteers with Alzheimer's disease, that also permitted effect-size calculations (Geldmacher et al., 2011). They enrolled an average of 14.5 volunteers in each cohort, with average MMSE baseline scores of 21. They administered five separate cognitive tests every 6 months, and calculated the regression coefficients for a multilevel model for each test. The ADAS-Cog parameter they obtained (−0.746) was not significant, nor were the ones for any other of the tests they ran. From their data, Geldmacher et al. estimated the average cohort size would have to be ∼170 for the observed ADAS-Cog parameter to have been significant, with $\alpha = 0.05$ and power = 0.8. We cannot directly compare this study with any of the others, it begs the question of what the results would be if similar calculations were applied to the other studies.

Second, both Watson et al. and Risner et al. showed that rosiglitazone had a positive effect on cognitive decline after taking changes in fasting insulin into account, and Risner et al. also found that changes in fasting insulin were more extensive in individuals without an *APOE ε4* allele. Peripheral hyperinsulinemia causes central hypoinsulinemia (Baura et al., 1996), which underlies this effect. Several groups (Watson et al., 2005; Gold et al., 2010; Tzimopoulou et al., 2010; Harrington et al., 2011) measured fasting glucose and/or HbA1c, but these measurements were not useful. Insulin itself is a better covariate than glucose or its surrogates for use in clinical trials of PPARγ action. Yet most of the studies subsequent to Risner et al. (2006) failed to take drug effects on fasting insulin into account.

Third, the concordance of rosiglitazone's effects on cognition and the Aβ42/Aβ40 ratio in Watson et al. (2005) and Sato et al. (2011) suggests that Aβ peptides are useful biomarkers for monitoring PPARγ - mediated effects in clinical trials of this sort, especially since the mechanism underlying the PPARγ effect on APP processing and Aβ release is well-understood, and standardized blood tests are available for clinical use. PPARγ agonists also inhibit tau phosphorylation, and because plasma p-tau 181 is associated with the metabolic and cognitive deficits associated with AD (Lussier et al., 2021), incorporation of a standardized, sensitive test for p-tau 181 (Karikari et al., 2020) would also be useful. The attempt to use cerebral glucose metabolism as a pharmacodynamics marker for PPARγ agonist efficacy in AD (Tzimopoulou et al., 2010) was unsuccessful due to study limitations. While Tzimopoulou et al. revealed a sustained protective effect of rosiglitazone on glucose metabolism (Tzimopoulou et al., 2010), the study did not run long enough for statistically or clinically meaningful conclusions to be drawn. BOLD fMRI as a PD marker also has theoretical justifications summarized in Knodt et al. (2019), but only one pre-clinical study has been published related to pioglitazone's effects on BOLD signaling (Crenshaw et al., 2015), and its suitability vis a vis more established biomarkers has not been established. In light of the difficulties with these PD biomarkers, and the informativeness of alternations in Aβ levels, it is disappointing that plasma Aβ42 and Aβ40 measurements were not incorporated more widely in clinical trials testing the efficacy of PPARγ agonists to delay the onset of dementia due to AD.

It would be informative to both our understanding of the underlying pathophysiology of Alzheimer's disease and for drug development purposes to learn if the relationships between changes in cognitive scores and changed fasting plasma insulin, and changed Aβ42 and Aβ40 peptide levels recorded in the Pilot and Phase 2 trials, were simply type I errors. As we've shown in this review, the relationship between each of these parameters and PPARγ MOA is empirically justified, and we recommend that future large-scale AD drug trials of PPARγ agonists or of the PPARγ/δ dual agonist T3D-959, incorporate measurements of fasting insulin, Aβ peptides and p-tau 181 as covariates.

CONCLUSION

Pioglitazone represents 'polypharmacy in a pill', and improves multiple etiopathologic determinants of Alzheimer's disease, including inflammation and oxidative stress, microglial defects, the development of amyloid plaques and neurofibrillary tangles, impaired cerebral glucose consumption and mitochondrial dysfunction, involving suppressed bioenergetics and disrupted dynamics. Pre-clinical studies have shown pioglitazone improves learning and memory, which correlate with improved synaptic activity and reduced amyloid and tau pathology, and better effects are seen when treatment is initiated before the onset of AD pathology. Longitudinal cohort studies have shown that pioglitazone is a time- and dose-dependent protective factor in individuals with DM2. These results are consistent with small scale, pilot studies in DM2 cases that showed pioglitazone increased cerebral blood flow as well as delayed the onset of dementia. Most of the clinical studies that have been conducted to date have been small and underpowered, or have not run long enough to be decisive. However, they are suggestive that pioglitazone's effects on cognition interacts with its effects on insulin lowering, even in cases without DM2.

AUTHOR CONTRIBUTIONS

WG wrote the manuscript. All authors discussed and edited the manuscript, and read and approved the final manuscript.

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Conflict of Interest: AS is President and CEO of Zinfandel Pharmaceuticals, Inc. DB is Senior Vice President and COO of Zinfandel Pharmaceuticals, Inc. WG has received consulting fees from Zinfandel Pharmaceuticals, Inc.

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