RESEARCH ARTICLE

Proteomic analysis of brain proteins in APP/PS-1 human double mutant knock-in mice with increasing amyloid b-peptide deposition: Insights into the effects of in vivo treatment with N-acetylcysteine as a potential therapeutic intervention in mild cognitive impairment and Alzheimer's disease

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Proteomics analyses were performed on the brains of wild-type (WT) controls and an Alzheimer's disease (AD) mouse model, APP/PS-1 human double mutant knock-in mice. Mice were given

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hypothesis associated with AD has brought to the forefront the possible therapeutic utility of antioxidant compounds. This is based on the observed increase in reactive oxygen species (ROS) in AD brain that leads to downstream increases in protein oxidation [2], DNA and RNA oxidation [3–7], and lipid peroxidation [8–9]. The oxidative stress present in AD brain is also a result of decreased endogenous antioxidant defenses [10–11] and ultimately leads to neuronal death. Because of the profound consequences of oxidative stress in AD brain and reports that high doses of compounds such as a-tocopherol (vitamin E) may be effective in delaying disease progression [12], the potential of antioxidant compounds as AD therapeutics seems promising.

Our laboratory has provided substantial evidence that amyloid-beta (Ab) (1–42), known to be heavily implicated in AD, mediates oxidative stress in AD brain [13]. Ab (1–42) is derived from the amyloid precursor protein (APP) through cleavage of APP byb- and g- secretases, in which presenilin 1 (PS-1) is a component of the g-secretase complex. Genetic mutations in APP, PS-1, and PS-2 are associated with familial AD (FAD) [14]. PS-1 mutations lead to altered processing of APP and thus an increase in Ab peptides which are present as toxic oligomers or in senile plaques, pathological hallmarks of AD.

A mammalian model of FAD was developed by Borchelt et al. (licensed to Cephalon, Frazer, PA, USA), in which mice were backcrossed to carry the APP^{NLh}/APP^{NLh} $PS\text{-}1^{P264L}/PS\text{-}1^{264L}$ mutations in order to humanize the mouse Ab sequence and to include the PS-1 mutation identi ed in human AD (APP/PS-1 human double mutant knock-in mice) [15]. APP/PS-1 mice have increasedbA production and accelerated amyloid deposition [15]. We have shown that neurons from APP/PS-1 mice (generated using the Cre-loc& knock-in technology) compared to wild type (WT) exhibit increased protein oxidation, lipid peroxidation, and susceptibility to oxidation by exogenous oxidants [16]. Furthermore, cerebral amyloid deposition increases in an age-dependent manner in APP/PS-1 mice [17–19]. Oxidative stress levels (as measured by protein carbonyls (PCO), 3-nitrotyrosine (3NT)-modi ed proteins, markers of protein oxidation, and 4-hydroxynonenal (HNE)-bound proteins, a marker of lipid peroxidation) also increase in brain in an age-dependent manner in APP/PS-1 knock-in mice when compared to WT controls [20].

We recently explored the antioxidantN-acetylcysteine (NAC), as an AD treatment at different disease stages in an APP/PS-1 human double mutant knock-in mouse model [21]. NAC is currently an FDA-approved drug for acetaminophen-based liver toxicity [22] and heavy metal poisoning. NAC serves as an antioxidant by indirectly increasing intracellular glutathione (GSHr5565F9 1 1e-464.9(g0d)-571.445ndaminopc46([2the)]tly (3NT-345.3(3-4[3TJ 1.2203 -166772 TD [(A)-315s.1(tha9-no330.6(76)-3ab-593.9(i0A)668.6(7ownsded)-98s.6(whi1(ins,))-1002.36ssoci3

2 Materials and methods

2.1 Chemicals

Unless otherwise indicated, chemicals were purchased from

fold-change and a p-value < 0.05 (using a Student's t-test). It should be noted that herein we only assessed changes that occur between treatment groups at each time point independently (i.e. only 4–9 months or only 7–12 months). For example, in the 4–9-month-aged group, we made two way

investigated the expression levels of Pin1 using Western blot analysis and determined that Pin1 was signi cantly decreased in the brains of APP/PS-1 mice relative to WT controls [21] at 12 months of age and Pin1 levels slightly increased after in vivo NAC treatment. These results are consistent with these observations that Pin1 is signi cantly decreased in the brains of 12-month-old APP/PS-1 H_2O mice relative to WT H_2O mice (see Table 4).

3.4 Western blotting analysis

Western blotting analysis was performed to validate changes in protein expression for a-enolase in the 7–12-month treatment group. Figure 3A shows a Western image of three samples from each treatment group after being probed with primary enolase antibody (actin was used as a loading control). Figure 3B is a histogram plot representation of the results in which no signi cant changes are detected in normalized enolase expression levels across the treatment groups. However, there is a trend towards increased enolase expression in APP/PS-1 mice given drinking water treated with NAC relative to APP/PS-1 mice given drinking water. This result is generally consistent with the proteomics results r si

Table 2. Differentially expressed proteins in 4–9-month treatment groups

Protein identi ed	WT H $_2$O versus WT NAC[a)]		WT H_2O versus APP/PS-1 H_2O[b)]		APP/PS-1 H_2O versus APP/PS-1 NAC[c)]	
	Ratio	p-Value	Ratio	p-Value	Ratio	p-Value

Based on the proteomics results obtained, we observe several proteins whose brain levels are altered in APP/PS-1 mice relative to WT controls in both the 4–9-month and 7–12-month groups given drinking water. Below we discuss biological processes of proteins that are substantially altered in APP/PS-1 mice and where applicable, the potential bene ts of NAC in in uencing protein levels in WT and APP/PS-1 mice. Some of the changes reported in Tables 2 and 4 are consistent with results obtained from studies in our laboratory that have previously investigated changes to the proteomes of oxidized proteins in AD and MCI [39–43].

4.1 Energy-related enzymes

Alterations to proteins involved in various aspects of energy production through changes in expression levels or modi- cations could contribute to the overall dysregulation in glucose metabolism evidenced in AD [39–44]. Prior to A (1–42) deposition (e.g. 4–9 months) the subunits of ATP synthase protein, a and d, have decreased expression in APP/PS-1 H_2O mice relative to WT H_2O mice. On the other hand, the V-type proton ATPase subunit B, which is the brain speci c isoform, is increased in

APP/PS-1 H_2O mice relative to WT H_2O mice. In the 7–12-month-old group, ATP synthase subunita was observed to be increased in APP/PS-1 H_2O mice relative to WT H_2O mice. ATP synthase is involved in the synthesis of ATP and work by utilizing a proton gradient that is established through the electron transport chain (ETC) located in the inner mitochondrial membrane coupled to complex physical rotations of components of this complex [45].

Cox5A levels were increased in the brains of APP/PS-1 H_2O mice relative to WT H_2O in the 4–9-month treatment group and decreased in the 7–12-month treatment group. Cox5A is a mitochondrial enzyme involved in the final aspects of the ETC by providing reducing equivalents from cytochrome C to oxygen [46]. Elevated expression of Cox5A prior to substantial Abeta deposition could increase the amount of ROS present by leading to increased numbers of electrons coming out of the ETC. Alternatively, increased Cox5A expression could lead to better mitochondrial function to provide ATP. Decreased Cox5A expression however, may be an indirect or direct result of increased levels of Abeta deposition and correlate with increased levels of ROS and oxidative stress. It is possible that while we measure decreased Cox5A expression in the 7–12-month animals, there may be a concurrent increase in Cox5A oxidation due to increased oxidative stress. Additional experiments, such as redox proteomics, would be necessary however to support this hypothesis.

a-Enolase, fructose-bisphosphate aldolase C, GAPDH, and pyruvate kinase are enzymes important in the glycolytic and tricarboxylic acid (TCA) cycles and ATP production.

brain. These mice have levels of bA deposition and plaque formation that are lower than that observed in 12-month-old animals whom better mimic the pathology observed in later stages of AD. Additionally, in the 7–12-month treatment group, NAC signi cantly increases a enolase and PK expression, suggesting that NAC through downstream mechanisms may in uence certain aspects of glucose metabolism, entry into the TCA cycle, and glutamate synthesis.

4.2 Excitotoxicity

GS was reported as oxidatively modi ed in AD brain, [47]; thus, increased GS levels observed in 12-month-old APP/PS-1 H_2O mice could represent a cellular response to elevated glutamate as a result of bA induced oxidative stress. GDH is a mitochondrial enzyme that catalyzes the oxidative deamination of glutamate to form a-ketoglutarate, thereby replenishing the TCA cycle. The levels of GDH do not change in APP/PS-1 H_2O mice; however, NAC indirectly or directly causes an increase in GDH in APP/PS-1 NAC mice in the 9-month-old mice. Altered glutamate regulation is observed in AD [48], and we reported oxidative modi cation of the glutamate transporter in AD [8]. Calbindin, a calcium-binding protein that buffers cytosolic Ca^{21} levels, was signi cantly reduced in 9-month-old APP/PS-1 H_2O mice. Reduced calbindin may contribute to Ca^{21} dyshomeostasis through increasing the levels of intracellular Ca^{21}. Other Ca^{21}-binding proteins such as calcineurin have been linked to Ca^{21} dyshomeostatis in aging [49] and AD [50]. These data are consistent with the reports of altered Ca^{21} levels in APP and other model systems of AD [51–53].

4.3 Cell cycle, tau phosphorylation, and A b production

Increased oxidation, decreased levels, and decreased activity of Pin1 in AD and MCI brain has been reported [54, 55]. Similarly, decreased levels and activity of Pin1 exist in the brains of APP/PS-1 H_2O mice relative to WT H_2O mice in 9- and 12-month-old mice [21]. Pin1, by binding to p-Ser/p-Thr-Pro motifs and conversion of the Pro residue of the target protein from cis-to-trans conformation and vice versa, regulates the activity of target proteins [56]. In particular, Pin1 regulates target Tau-relevant kinases and protein phosphatase 2A, important for the phosphorylation/dephosphorylation of tau, and is necessary for cell growth and proper cell cycle functions [57, 58]. Pin1 has also been implicated in bA production, in which Pin1 inhibition promotes APP processing in the amyloidogenic pathway and therefore causes elevated levels of Ab [48, 59] and oxidative stress [13, 60]. This result is consistent with and may contribute to elevated numbers of senile plaques in AD and in neuritic plaques in this APP/PS-1 model [38].

4.4 Synaptic abnormalities

One pathological hallmark of AD is synapse loss [61]. SNAP-25 is a synaptosomal protein that is a part of the Q-SNARE complex that functions in neurotransmitter release at

signi cantly decreased in the 4–9-month treatment group in APP/PS-1 H_2O mice.

4.8 Effects of NAC on the proteome of WT mice

Fructose bisphosphate aldolase A and SOD2 were signi - cantly increased in 9 month-old WT mice treated with NAC relative to WT H_2O. Fructose bisphosphate aldolase A is an enzyme involved in the glycolytic pathway. SOD2, also known as MnSOD, is an antioxidant enzyme localized in the mitochondrion that protects cells from toxic superoxide anion. Elevated SOD2 upon in vivo NAC treatment in WT mice may be an early protective mechanism against augmented oxidative stress which is known to increase with aging.

In the 12-month-old age group, NAC treatment of WT mice led to prohibitin being signi cantly increased and co lin 1 signi cantly decreased in brain. Prohibitin is localized to the inner mitochondrial membrane and is known to inhibit DNA synthesis, regulate the cell cycle, and be involved in apopotosis and aging [69]. Prohibitin has shown cellular defense against oxidative stress in epithelial cells [70, 71] and decreases in expression as a function of cellular senescence in human and chicken broblasts [69]. Because prohibitin also has roles as a chaperone and participates in the assembly of subunits in the mitochondrial respiratory chain complex, this protein regulates mitochondrial respiratory activity and aging [72, 73]. Elevated prohibitin expression following NAC may be helpful in delaying detrimental changes associated with mitochondrial respiration in aging by increasing the availability of components involved in the ETC. Co lin 1 is the non-muscle isoform of co lins that constitute a major portion of actin rods and plays roles in actin depolymerization and polymerization. Taken together, the results suggest that NAC, which many persons use as an over-the-counter dietary supplement, can affect levels of brain proteins involved in metabolism, prevention of oxidative stress, mitochondrial function, and cytoskeletal integrity. Each of these processes could be important in slowing mitochondrial alterations associated with aging.

Examining the proteomes of WT and APP/PS-1 mice given drinking water prior to (i.e. 9 months) and after (i.e. 12 months) periods of signi cant Ab (1–42) deposition revealed several altered brain proteins involved in energy production, cell signaling, defense systems, excitotoxicity, synapse-related, cellular structure, and mitochondria in APP/PS-1 mice. In vivo NAC treatment for 5 months has been reported to provide protection against oxidative stress in brain of APP/PS-1 human double mutant knock-in mice at 9 and 12 months of age, consistent with the notion that NAC has potential to be a therapeutic approach for both MCI and AD

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