Alzheimer's Disease associations of ferritin and glutathione with oxidative damage and neuronal loss

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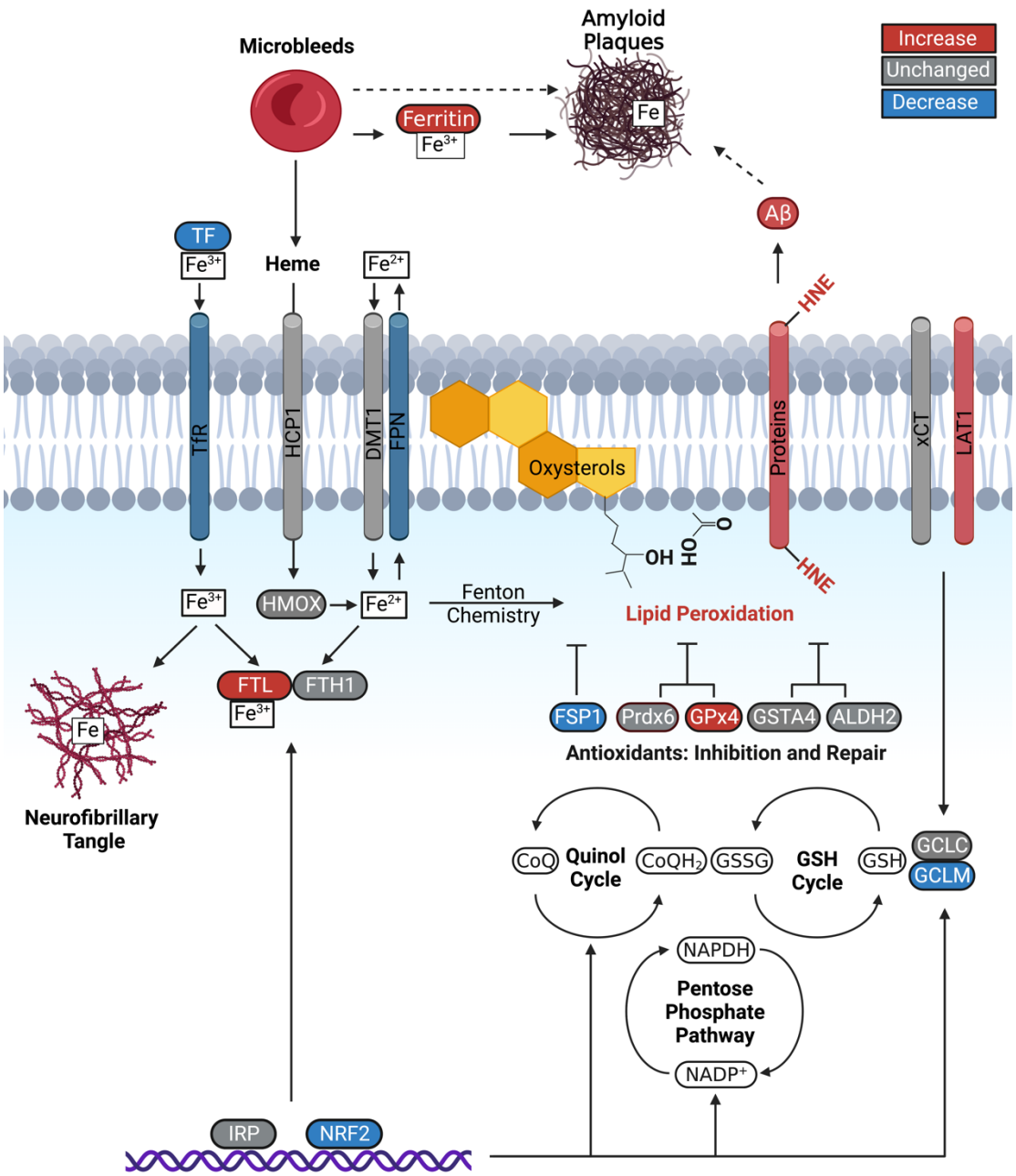
Abstract: The role of reactive iron in Alzheimer's Disease (AD) has major gaps. Little is known of AD changes in iron transport, glutathione-mediated oxidative repair, and associations with ApoE alleles. Intravascular blood was minimized by washing minced postmortem brains. HNE from iron-associated lipid peroxidation was increased in AD prefrontal cortex by 50% for whole tissue and subcellular lipid rafts, where Aβ-peptides are produced. Proteins mediating iron deposition and oxidative repair were extensively altered by AD. Protein oxidation was proportionate to the iron storage protein ferritin light chain (FTL); both higher in ApoE4. Loss of neurons and synapses in AD was proportionate to FTL. Iron transport was impaired with lower transferrin, transferrin receptor, and ferroportin. AD decreased Ferroptosis suppressor protein 1 and glutamate cysteine ligase modulator subunit (GCLM), which regulates glutathione synthesis. These findings provide a mechanistic framework for iron-associated neurodegeneration during AD through impairment of lipid peroxidation repair mechanisms involving glutathione.



Graphical Abstract: Hypothesis: lipid peroxidation is driven by increased iron stores and decreased antioxidant defenses during AD. Schema shows proteins that mediate iron metabolism in relation to lipid peroxidation (HNE) and antioxidant defenses in prefrontal cortex. AD-associated increase (red), decrease (blue), or no change (grey) relative to cognitively normal elderly controls. Aβ; amyloid beta, ALDH2; alcohol dehydrogenase, APP; amyloid precursor protein, DMT1; divalent metal transporter 1; FPN, ferroportin; FSP1, ferroptosis suppressor protein 1; FTH1, ferritin heavy chain; FTL; ferritin light chain; GCLC, glutathione cysteine ligase catalytic subunit; GCLM, glutathione cysteine ligase modulator; GPx4, glutathione peroxidase 4; GSH, glutathione; GSSG, glutathione disulfide; GSTA4, glutathione S-transferase A4; HMOX; heme oxygenase; IRP, iron regulatory protein; LAT1, large neutral amino acid transporter 1; LOOH, Lipid hydroperoxides; Nrf2, Nuclear factor erythroid 2-related factor 2; Prdx6, peroxiredoxin 6; TF, transferrin, TfR; Transferrin receptor; xCT, cysteine-glutamate antiporter.

Introduction:

Postmortem AD neuropathology is weakly linked to cognitive status in two large longitudinal studies[1,2]. Less than 35% of variation in terminal cognitive decline is attributable to multiple neuropathological markers including the AD traditional indicators of amyloid plaques and neurofibrillary tangles (NFT). What is missing? We hypothesize that iron-associated membrane damage contributes to pathway-specific neurodegeneration in AD.

Iron has long been linked to AD. "*The pathogenesis of Alzheimer's disease…may be a primary disturbance in the cerebral metabolism of iron"*, was proposed in 1953 by Louis Goodman based upon examination of AD cases that showed iron around senile plaques and neurofibrils[3] analyzed by Prussian blue histochemistry. Recent measurements by ICP-MS show correlations of total iron in prefrontal cortex with NFT density and rates of cognitive decline. Extending the largely cancer-based ferroptosis hypothesis[4] to AD, Ayton *et al.* hypothesized that increased iron found in AD contributes to increased lipid peroxidation products. The present study examined molecular details of lipid peroxidation by iron by examining twenty proteins that mediate brain iron metabolism and repair of lipid peroxidation.

In 1964, Hochstein hypothesized that iron oxidation caused membrane lipid peroxidation[5], followed by Ursini's 1982 discovery that the chemical reduction of phospholipid hydroperoxides by glutathione was mediated by glutathione peroxidase 4 (GPx4)[6]. The hydroxyl radical (·OH) produced from hydrogen peroxide by iron (Fenton chemistry) is widely recognized in lipid peroxidation[7].

$$\mathbf{H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}}$$

Knockout of the other seven glutathione peroxidases showed that GPx4 elimination of phospholipid hydroperoxides rather than decreased H_2O_2, is essential for preventing lethal oxidative membrane damage *in vivo*[8]. Subsequently, iron was shown to directly enhance aggregation of amyloid β-peptides[9], consistent with abundant iron in senile plaques and NFT detected by x-ray spectroscopy[9]. Specifically, plaque cores contain iron in multiple oxidation states[10]. The chemical presence of ferric iron in plaques[10,11] and in NFTs[12] confirms Roger's initial Prussian blue histochemistry; now known to react with ferric iron[13]. Next, a specific oxidized lipid component was identified as HNE (α,β-unsaturated hydroxyalkenal 4-hydroxynonenal)[14], which is elevated in AD cerebrospinal fluid[15] and tissue[16]. HNE can form conjugates with cysteine (most reactive), histidine, and lysine[17]. In cultured neurons, iron-induced HNE and toxicity was blocked by conjugation to the antioxidant glutathione (GSH)[18]. In 2012, the term ferroptosis was introduced to cancer research for cell death mediated by iron-dependent lipid peroxidation. Ferroptosis was soon considered for AD and other neurodegenerative diseases[4,19,20].

Seven decades after Rogers' seminal study, brain iron levels were observed to be specifically associated with cognitive decline and AD neuropathology in the longitudinal Memory and Aging Project[21]. A mechanistic link to iron was experimentally shown for GPx4 depletion in cultured neurons exposed to hemin from degraded hemoglobin, which was blocked by chelation of iron with deferoxamine[22]. However, AD changes in CSF ferritin, and tissue iron were inconclusive[21,23–28]. Postmortem studies in the AD brain by Ashraf et al in 2020 reported increases in prefrontal cortex of HNE and ferritin protein, and decreased ferroportin, while total brain iron did not differ in AD[24]. Conclusions were limited by the small sample (7 brains, AD and age-matched controls) that did not allow for analysis by sex and ApoE allele, and by long postmortem intervals before freezing. Major unknowns include the impact of AD on glutathione-dependent repair of lipid peroxidation[29] and how AD stages may alter the twenty proteins mediating brain iron transport and storage within ferroptotic processes in brain and other tissues[4,30].

To address these gaps, we assayed ferritin light chain (FTL) and other iron related proteins and GSH metabolic enzymes in 24 AD and 24 age-matched control brains. Prefrontal cortex was compared with cerebellum, which has minimal AD neurodegeneration[31]. Whole tissue was compared with lipid rafts, a subcellular fraction containing the aspartyl proteases (ADAM10, BACE1, PSEN1/2)

that cleave amyloid β-peptides from the amyloid precursor protein (APP). We recently reported that lipid rafts from AD brains have less APP than controls[32]; the present studies used these same brains.

We first considered how to minimize residual erythrocytes in brain tissues that were not perfused before freezing - brain fragments are often reddish with surface blood. Residual blood will confound assays for brain iron and GSH-utilizing enzymes, because erythrocytes contain iron, ferritin, and GSH enzymes[33,34]. We introduce a washing procedure to minimize adherent and intravascular hemoglobin in unperfused brain specimens.

Results

Minimization of residual blood in postmortem brain

Cardiac perfusion of mice with heparinized saline decreased heme iron in cerebral cortex by 35% (**Table 1A**). Because perfusion is not feasible for pieces of thawed human cerebral cortex, we developed a washing procedure to minimize entrapped blood in the vasculature where prefrontal cortex was minced into 2 mm cubes, followed by gentle washing with isotonic buffer (PBS). The resulting 'washed' brain had 68% less heme than the non-perfused, a greater decrease than by perfusion.

When applied to human cortex (**Table 1B)**, washing decreased heme levels by 50%. For verification and comparison with Ayton et al. 2020[21] and Ashraft et al 2021[26]: total iron in human brain was assayed by ICP-MS. Washing decreased iron by -45%, similar to the decrease by -50% in heme levels. Total iron levels for unwashed human cerebral cortex approximated those of Ayton et al. 2021[21]. Concentrations of total aluminum, copper, and zinc in human brain were not altered by washing (**Supplemental Fig. 1**), consistent with their low levels in blood. The removal of blood was further documented with α-hemoglobin, which was decreased 50% with washing (**Supplemental Fig. 2C,D).** Thus, entrapped intra-vascular blood may contribute 50% of the total iron in unperfused or unwashed postmortem human brain, which was reported to have 2- to 5-fold higher levels of total iron (**Table 1B)**. Thus, residual vascular blood can inflate estimates of total iron and extravascular heme.

Table 1 Heme and Iron content of prefrontal cortex

A. Mouse, wildtype

	Non-perfused	**Perfused PBS**	**Perfused PBS + Heparin**	**Non-Perfused + Washed**
Heme/mg wet brain	3.1 ± 0.2	2.1 ± 0.2* (-32%)	2.0 ± 2.3** (-35%)	1.3 ± 0.2*** (-68%)

B. Human

	Unwashed	**Washed**
Heme/mg wet brain	7.1 ± 0.4	3.6 ± 0.2, ****, -50%
Total iron, ug/g wet brain	45 ± 4.9	26 ± 1.4 ****, -45%
Ayton et al. 2021[21]	~55	N/A
Ashraf et al. 2020[24]	~290	N/A

C. Sources of Iron and Ferritin

Compartment	**Total iron**	**Ferritin**
Brain, unwashed	50 ug/g wet brain	0.135 ug/g
Blood	3500 ug/mL	3.8-14 ug/mL
Serum	1.3 ± 0.14 ug/mL	0.011-3.4 ug/mL
CSF	0.061 ± 0.006 ug/mL	0.012 ug/mL

Table 1: Heme and total iron in prefrontal cortex were assayed for effects of washing to minimize residual intravascular blood. **A**) mouse (C57BL/6J, 6 mo; n=5-6); **B**) human (AD; n=24) and controls (65+ yr; n=24) prefrontal cortex (Brodmann regions 8-10); **C)** Iron[21,35,36] and ferritin[23,36,37] in brain, blood, serum, and CSF. The ferritin complex in erythrocytes and tissue include FTL and FTH1. Isotonic phosphate buffered saline (PBS; pH 7.4) was used for aortic perfusion of mouse and for washing of minced mouse and human brain. Prefrontal cortex 30 mg was minced into 2 mm cubes and 'washed' by gentle vortexing in PBS. Heme and α-hemoglobin per mg tissue were assayed spectrophometrically relative to unwashed brain (**Supplemental Fig. 3**). For total iron, brain cubes were gently sheared in PBS by polyethylene pestle (PES-15-B-SI, Axygen) for measurement by ICP-MS. Iron from Ashraf was estimated as Fe/g protein, assuming 1 gram protein per 12 gram wet weight brain[39]. Brain iron and ferritin by compartment for human are >95% below blood levels, assuming 1 ml volume is equivalent to 1 gram wet brain. Significance by one way ANOVA (mouse) with Tukey's posthoc, or 2- tailed t-test (human): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Lipid peroxidation and heme are increased in AD

Washed brain samples were assayed for oxidative damage by measuring two protein adducts, HNE and 3-nitrotyrosine (NT) (**Fig. 1**). For the following data, cognitive status was defined by clinical criteria for AD and age-matched controls; subsequent studies (**Fig. 6**) include postmortem Braak scores for NFT[40].

Prefrontal cortex AD total iron did not differ (**Fig. 1B**), while heme increased +25% (**Fig. 1C**). AD had 50% more HNE and NT than controls (**Fig. 1D,E**). HNE correlated with heme (r=0.32, p=0.025), unlike NT (r=0.11, p=0.47; not shown). The absence of heme-NT correlation suggests a different pathway than for heme-iron production of HNE. For example, aggregated human Aβ42 stimulates production of neurotoxic peroxynitrite, shown by us *in vitro*[41].

Cerebellum (**Extended Figs.**) was compared with prefrontal cortex as an adjacent brain region with less AD neurodegeneration[31]. AD cerebellar heme was 40% higher, paralleling cortex (**Extended Fig. 1C**). Cerebellum HNE increased 30% in AD (**Ext. Fig. 1E**), while NT did not differ (**Ext. Fig. 1D**).

ApoE alleles and sex influenced HNE levels, but not NT. The ApoE4 prefrontal cortex had 35% more HNE than ApoE3 (**Fig. 1F**); cerebellar HNE did not differ by ApoE allele (**Ext. Fig. 1F**). Sex differences did not parallel apoE alleles. For female AD, prefrontal cortex had 60% more HNE than cognitively normal, whereas males did not differ in HNE by cognitive status (**Fig. 1G**). Sex-ApoE interactions were not statistically evaluated because of the small sample size; data are shown in **Supplemental Tables 1 and 3**.

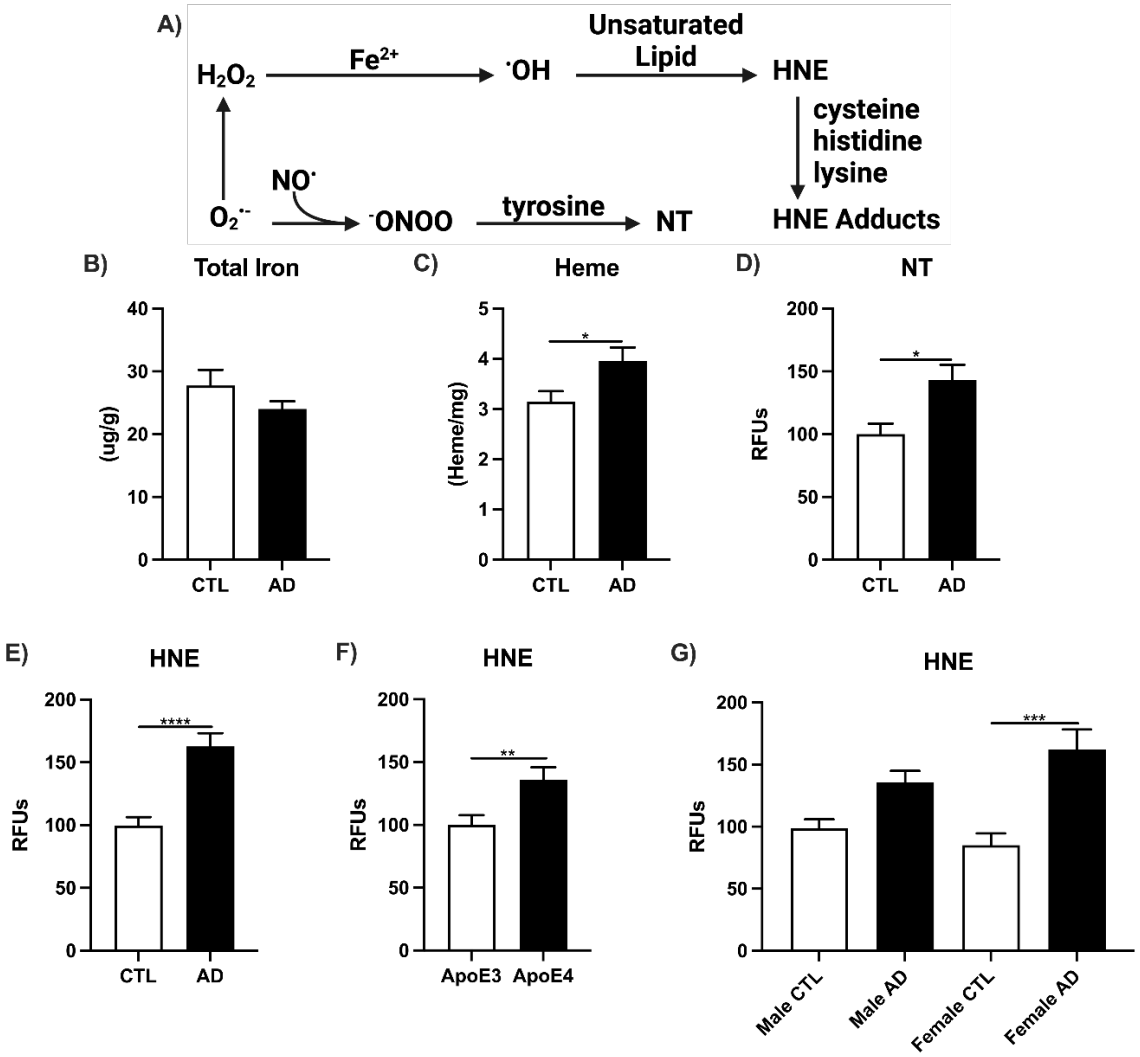


Figure 1: Oxidative damage to prefrontal cortex proteins (HNE, NT) in age matched AD and cognitively normal control (CTL). **A)** Schematic representing oxidative damage to lipids and proteins. Hydroxyl radicals (·OH) are produced during oxidation of Fe^{2+} or Cu^{2+} ('Fenton chemistry') and may oxidize adjacent proteins, lipids or nucleic acids. HNE can alter proteins via Michael addition products through its double-bonded carbons, or by forming Schiff bases through its aldehyde moiety. The bottom reaction shows the iron-independent oxidative pathway to form peroxynitrite -ONOO from $O_2^{\cdot-}$ and ·NO, which can then oxidize tyrosine to NT. Aggregated human Aβ42 stimulated formation of peroxynitrite which was neurotoxic in vitro[41]. Non-heme iron also mediates lipoxygenase activity[42]. Prefrontal cortex from Brodmann area 8, 9, or 10 and cerebellum was washed measurements for **B)** Total iron, measured by ICP-MS **(Table 1B)**, **C)** heme, and dot blots for **D)** NT, and HNE presented as **E)** cognitively normal vs AD, **F)** ApoE allele, or **G)** sex as relative fluorescent units (RFUs). Significance by 2-tailed t-test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Next, we analyzed AD associations with oxidative damage and heme iron in specific pathways that mediate brain iron transport and storage. GSH is the main oxidative repair mechanism, biochemically reduced by the NADPH produced by the pentose phosphate pathway (**Graphical abstract**). Iron deposits and membrane oxidative repair were analyzed for levels of twenty proteins that had statistically significant AD changes.

Iron protein metabolism is decreased in AD

Brain cellular iron is imported by the divalent metal transporter 1 (DMT1) together with transferrin (TF) and its receptor TfR; in AD, levels of TF and TfR differ by cell type [28]. Heme iron is imported by heme carrier protein 1 (HCP1) and degraded by hemeoxygenases (HMOX1 or HMOX 2). Intracellular ferric iron is stored in complexes with ferritin light chain (FTL) after oxidation of ferrous iron (Fe^{2+}) by ferritin heavy chain (FTH1). Export of ferrous iron is mediated by ferroportin (FPN).

Iron transport proteins were decreased in AD (**Fig. 2A-E**): transferrin and its receptor TfR (-25%); iron export protein FPN (-30%), with a trend for DMT1 (-30%; p=0.13). Correspondingly, AD increased ferritin light chain (FTL) (+75%) (**Fig. 2H**), while ferritin heavy chain (FTH1) was not changed by AD (**Fig. 2I**). The increased FTL in AD confirms Ashraf et al 2020[24] who also reported increased FTH1, not found in our larger sample. Increased brain FTL is consistent with reports in studies of cerebrospinal fluid, microglia, and neuritic plaques during AD[23,25,27], which could arise from multiple cellular sites (**Table 1C**). Relationships for iron import/export included negative correlations between iron storage protein FTL with iron import by TfR (r=-0.30, p=0.04) and export by FPN (r=-0.36, p=0.01; **Fig. 2J**). ApoE4,4 had 2-fold higher FTL than ApoE3,3 and ApoE3,4 in prefrontal cortex.

The cerebellum differed from prefrontal cortex in the absence of higher FTL in AD (**Ext. Fig. 2H**) or ApoE4 carriers **(Supplemental Table 3)**. AD cerebellum had higher transferrin (TF), while TfR and DMT1 were decreased, paralleling the AD cortex (**Ext. Fig. 2A-C**). HMOX1 decreased 40% in AD cerebellum, while HMOX2 was unchanged **(Ext. Fig. 2F-G**). These brain region differences in oxidative damage are consistent with the relative resistance of cerebellum to AD damage.

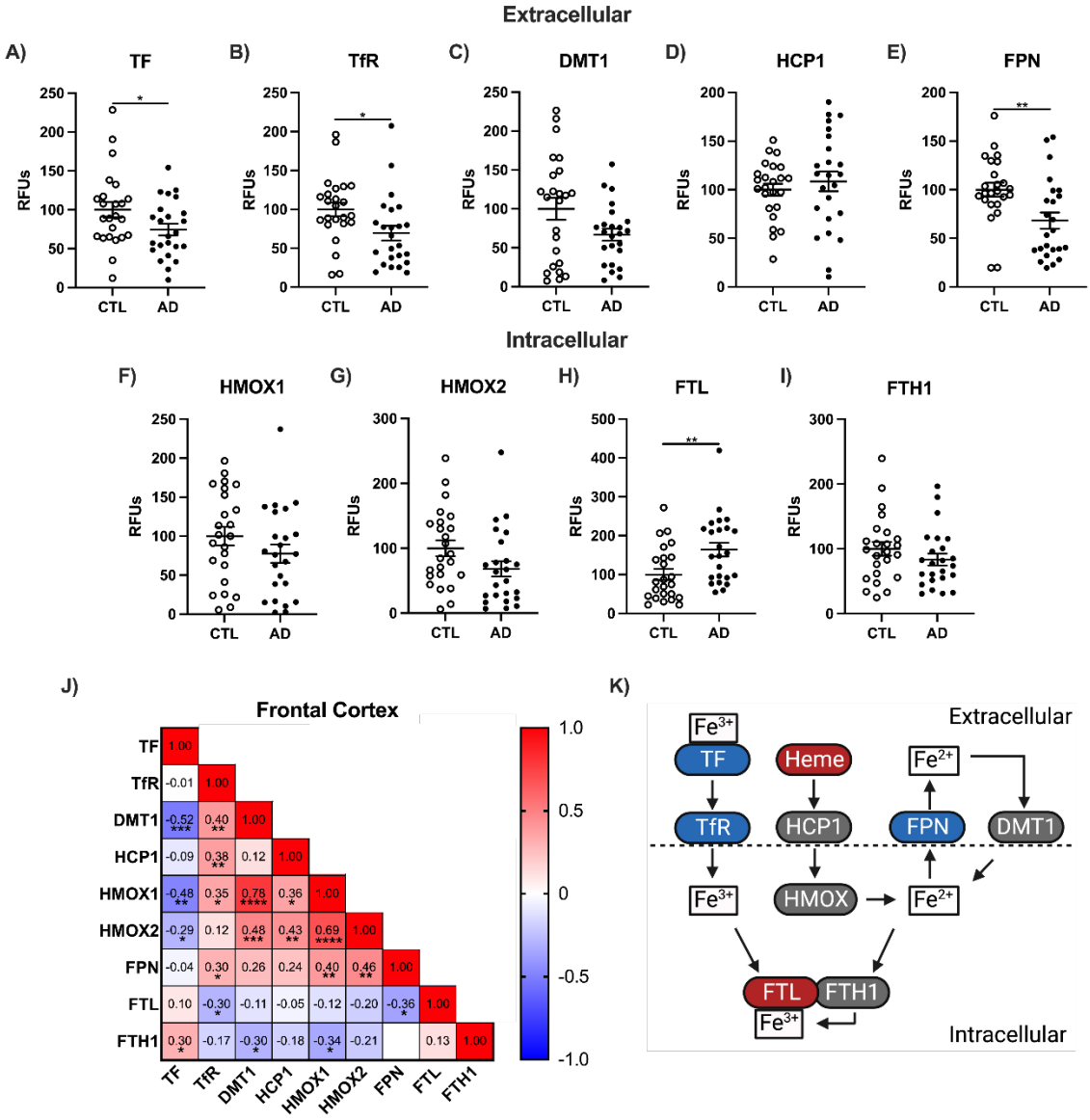


Figure 2: Iron transport and metabolism in AD prefrontal cortex. Relative fluorescent units (RFUs) from Western blots. **A)** transferrin (TF), **B)** transferrin receptor (TfR/CD71), **C**) divalent metal transporter 1 (DMT1), **D)** heme carrier protein 1 (HCP1), **E)** ferroportin (FPN), **F)** hemeoxygenase 1 (HMOX1), **G)** heme oxygenase 2 (HMOX2), **H)** ferritin light chain (FTL), **I)** ferritin heavy chain 1 (FTH1), **J)** Correlation matrix for iron metabolism in clinical AD, **K).** Summary matrix of AD changes: blue, decrease; red, increase. Significance, 2-tailed t-test (A-I) or Spearman correlation (J): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

GSH synthesis is decreased with AD

The tripeptide GSH mitigates the oxidation of lipids, protein amino acids, and conjugation of HNE to proteins by several enzymes (**Graphical Abstract)**. GSH is utilized to reduce hydroperoxides by the glutathione peroxidase (GPx) family and peroxiredoxin 6 (Prdx6), while the glutathione S-transferases (GST) conjugate GSH to diverse metabolites and xenobiotics. Biosynthesis of GSH depends on cystine import via the proteins xCT (SLC7A11) and LAT1 (SLC3A2/CD98). Imported cystine is then rapidly reduced to cysteine because of the intracellular reducing environment. The rate limiting step in GSH synthesis[43] is formation of γ-glutamylcysteine catalyzed by glutamate cysteine ligase, which is composed of GCLC (catalytic) and GCLM (modulatory) subunits; binding of GCLM to

GCLC accelerates GSH synthesis by four-fold. Next, GSH is formed from addition of glycine to γ-glutamylcysteine by glutathione synthetase (GSS). The electrophile-activated transcription factor Nrf2 regulates gene expression of GCLC, GCLM, GPx4, and Prdx6.

GSH enzymes showed AD selective changes in relation to iron transport proteins in whole brain tissue, and in subcellular lipid rafts wherein neurons generate amyloid β-peptides from the amyloid precursor protein (APP)[32]. Lipid antioxidant mechanisms independent of GSH include ferroptosis suppressor 1 (FSP1), which utilizes $CoQH_2$ to reduce lipid hydroperoxides through the quinol cycle. In cortex, GPx4 protein level was doubled in AD relative to controls, while FSP1 was decreased 40% (**Fig 3A,D**). FSP1 was decreased in AD cerebellum; additional data in **Ext Fig. 3**.

Because GSH has a critical role in prevention of lipid peroxidation, we examined enzymes of the GSH synthesis cycle (**Graphical Abstract)**. In AD prefrontal cortex, the glutamate-cysteine ligase subunits were differentially altered for AD: only the modulatory subunit GCLM was decreased by -60%; the catalytic subunit GCLC was unaltered (**Fig. 3H,I**). LAT1 (L-type amino acid importer transporter) increased 33% in AD, while xCT did not differ, contrary to the 20% increase observed by Ashraf *et al.* 2020[24] (**Fig. 3J,K**). Two enzymes of GSH synthesis were not altered in AD cortex: glutathione synthase (GSS), the final step in GSH synthesis (**Fig. 3L**) and glutathione reductase (GSR) (**Fig. 3M**), which utilizes NADPH to reduce glutathione disulfide back to GSH. Cerebellum GCLM protein decreased by 35%, consistent with cortex (**Extended Fig. 3H**).

Related metabolic cycles also showed selective AD changes. For the pentose phosphate pathway, glucose 6-phosphate dehydrogenase (G6PD) was decreased by 45% in AD cases relative to controls, while phosphogluconate dehydrogenase (PGD) was unchanged (**Fig. 3N,O**). These enzymes oxidize glucose to generate NAPDH required by GSR for reduction of GSSG. The NADPH-producing enzymes G6PD and PGD were positively correlated with GSR (both $r=0.31$, $p=0.03$). The amino acid importer LAT1 and cystine importer xCT were positively correlated ($r=0.49$, $p<0.001$; **Fig. 3P**). Cerebellum GSR was also positively correlated with G6PD ($r=0.42$, $p=0.003$; **Ext. Fig. 3Q**).

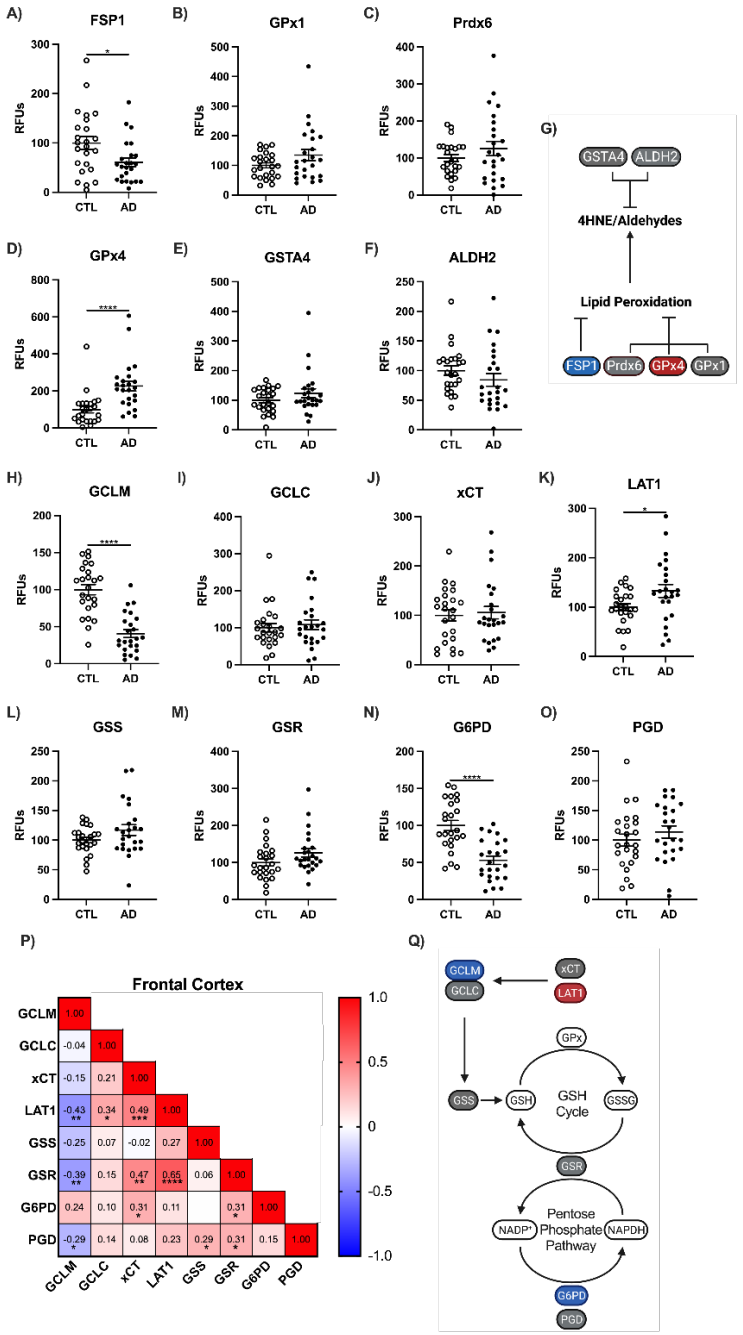


Figure 3: Enzymes that repair or mitigate lipid peroxidation and facilitate GSH production. Western blots shown as relative fluorescent units (RFUs) for frontal cortex from whole tissue lysate for antioxidants **A)** GPx4, **B)** GPx1, **C)** Prdx6, **D)** FSP1, **E)** GSTA4, and **F)** ALDH2. **G)** Enzymatic repair of lipid peroxidation. GSH cycle enzyme levels by Western blots, as relative fluorescent units (RFU). **H)** GCLM, **I)** GCLC, **J)** xCT/SLC7A11, **K)** LAT1, **L)** GSS, **M)** GSR, **N)** G6PD, and **O)** PGD. **P)** Correlation matrix for GSH cycle enzymes and **Q)** Schema of GSH metabolic enzymes and pentose phosphate pathway. AD increase (red), decrease (blue), no change (grey), relative to cognitively normal controls; not measured (white). Significance by 2-tailed t-test (A-H) or Spearman correlation (I): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Iron transcription factors did not differ by AD

Transcription factors were examined for DNA binding proteins that are directly influenced by intracellular iron (IRP1/2, NCOA4), or indirectly (Nrf2) (**Ext. Fig. 8A**). IRP1 and IRP2, did not differ in AD cases compared to controls in cortex (**Ext. Fig. 8B,C**). However, nuclear receptor coactivator A4 (NCOA4; lysosomal degradation of ferritin) was 30% higher in AD (**Ext. Fig. 8F**).

Transcription factors Nrf2 and NF-kB that regulate multiple genes of detoxification and inflammation were differentially altered: Nrf2 was -75% lower in AD, while NF-κB p65 transcription factor subunit was 2-fold higher (**Ext. Fig. 8D,E**). Other transcription factors were unchanged by AD (**Ext. Fig. 8**).

Lipid peroxidation is increased and antioxidant defense is diminished in AD lipid rafts

Subcellular lipid rafts (LR) were examined in these same samples for oxidative changes relevant to amyloid β-peptide production from APP (**Fig. 4**). Before evaluating AD, we defined which GPx isoforms are present in lipid rafts. As expected, GPx4 was present, as well as GPx1 (**Supplemental Fig. 3**). Prdx6, another GSH utilizing lipid hydroperoxide reducing enzyme, was absent in lipid rafts (not shown), despite its presence in whole tissue cortex (**Fig. 3D**).

AD cortex had increased lipid raft oxidation: HNE, +165%; NT, +65% (**Fig. 4G,H**), paralleling increases observed in total tissue (**Fig 1C,D)**. ApoE4,4 had 2-fold higher HNE than ApoE3,3 and ApoE3,4 in cortex, but not cerebellum (**Ext. Fig. 4**). Similarly, lipid raft HNE had a smaller AD increase in cerebellum (+45%) than cortex (+160%; **Ext. Fig. 4H**).

AD decreased the membrane protective enzymes GPx4 (30%), GPx1 (50%), and ALDH2 (40%; **Fig. 4B-D**), consistent with membrane damage in AD. The Discussion considers how these changes may influence the production of amyloid β-peptides.

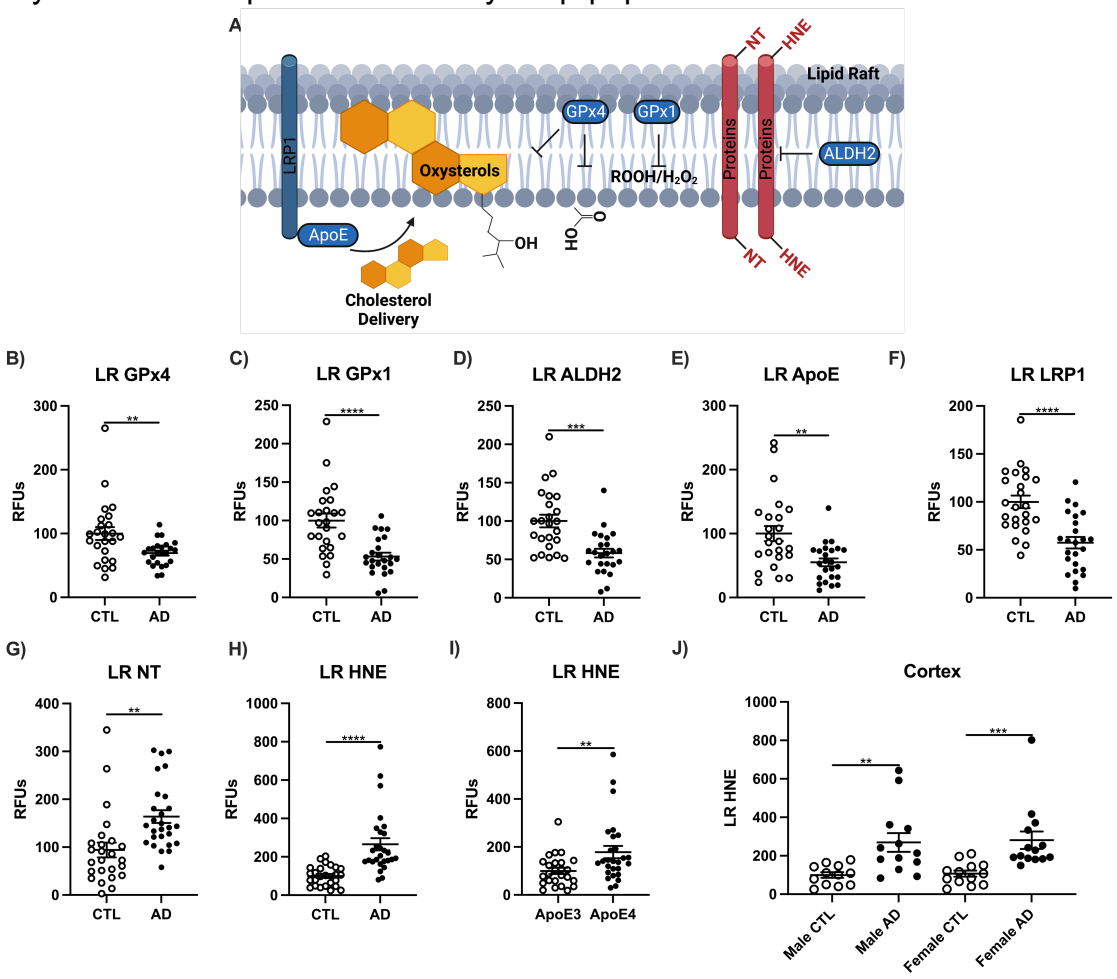


Figure 4: A) Schema of lipid raft damage, protective mechanisms, and cholesterol shuttling in AD prefrontal cortex. Western blots as relative fluorescent units (RFUs) of lipid raft **B)** GPx4, **C)** GPx1, **D)** ALDH2, **E)** ApoE, **F)** LRP1, **G)** NT, and HNE presented as **H)** CTL vs AD, **I)** ApoE allele, and **J)** sex. B-I) Significance by 2-tailed t-test or J) one-way ANOVA with Tukey's posthoc: **p<0.01, ***p<0.001, ****p<0.0001.

Ferroptosis markers in AD

Proteins and oxidative modifications involved in ferroptosis differed by clinical AD status in whole tissue prefrontal cortex and lipid rafts (**Fig. 5A**). Both whole tissues and rafts showed AD increases of iron storage and reduced iron export (**Fig. 5B,C**); increased lipid peroxidation particularly in membranes (**Fig. 5D,E**); increased ferritin degradation (ferritinophagy) (**Fig. 5F**), lower Nrf2 (**Fig. 5G**) and its target antioxidant genes that mitigate lipid peroxidation (**Fig. 5H-M**). Cerebellum had fewer AD alterations of ferroptosis-related proteins (5/10) than cortex (**Ext. Fig. 5**). Notable changes in cerebellum are decreased GCLM (-35%), lipid raft GPx4 (-40%), and FSP1 (-40%) that paralleling cortex (**Ext. Fig 5I,L,M**).

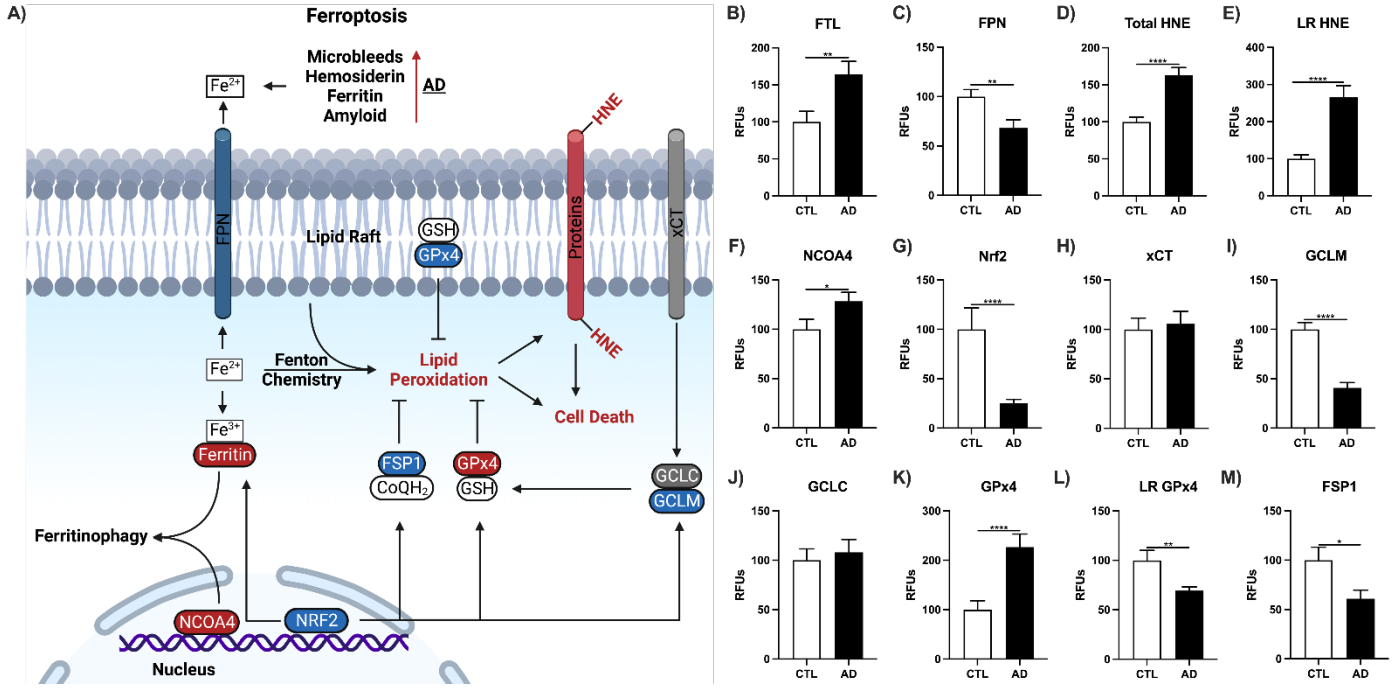


Figure 5: Ferroptosis schema with consolidated data from prior figures. **A)** Schema of ferroptosis markers related to iron transport, lipid rafts, and oxidative damage mitigation. **B)** FTL, **C)** FPN, **D)** HNE, **E)** lipid raft HNE, **F)** NCOA4, **G)** Nrf2, **H)**, xCT, **I)**, GCLM, **J)** GCLC, **K)** GPx4, **J)** Lipid raft GPx4, and **M)** FSP1. Significance by 2-tailed t-test: *p<0.05, **p<0.01, ****p<0.0001.

Neuronal loss in AD is associated with increased ferritin, lipid peroxidation, and diminished antioxidant defense

While neuronal loss dramatically increases in AD, the relation of neuronal loss is undefined for iron stores and membrane damage in relation to clinical AD and Braak stage. Neurons and synapses of prefrontal cortex were analyzed for organelle-specific proteins of NeuN (neuronal nuclear antigen), presynaptic SYP (synaptophysin1), and postsynaptic PSD95 (postsynaptic density protein 95). Statistical significance was analyzed in two ways: as above, by comparing pre-mortem clinical status of dementia versus controls; additionally, we examined neuron loss markers in relation to postmortem Braak scores 0-6 for density and distribution of NFTs. By both clinical and Braak measures, AD cortex had the expected major loss of neuron cell bodies (NeuN, -50%) and synapses (PSD95, -40%;

Fig. 6A,C). Neuronal markers varied inversely with cortex tissue levels of heme by Braak score (**Fig. 6D**), while NeuN varied inversely with ferritin light chain, FTL (r=-0.56, p<0.0001). The relationship for NeuN and heme was significant for Braak stage (r=-0.29, p=0.04) but not for clinical status. These inverse associations are the first direct associations of brain iron markers with brain region-specific neuron loss during AD. The inverse variation of NeuN with HNE (r =-0.46, p<0.001) strengthens the relationship of lipid peroxidation to neuron loss. Many of these relationships were weakened or absent from cerebellum, consistent with its reduced neuronal loss (**Ext. Fig. 6**).

Because cognitive status had shown limited correlation for postmortem AD-related indices in two large longitudinal studies[1,2], we examined the present data for disjunction and overlap by both premortem clinical AD and by postmortem Braak. The Venn diagram for neuronal markers shows 41% overlap of AD vs controls by clinical status and Braak score (**Fig. 6E**) for all metrics in **Fig. 6D**. The positive correlation between NeuN and FPN protein levels was the only relationship shared by both criteria and in cortex and cerebellum: (**Ext. Fig. 6D-F**). This singularity suggests the importance of iron export by FPN for neuronal survival.

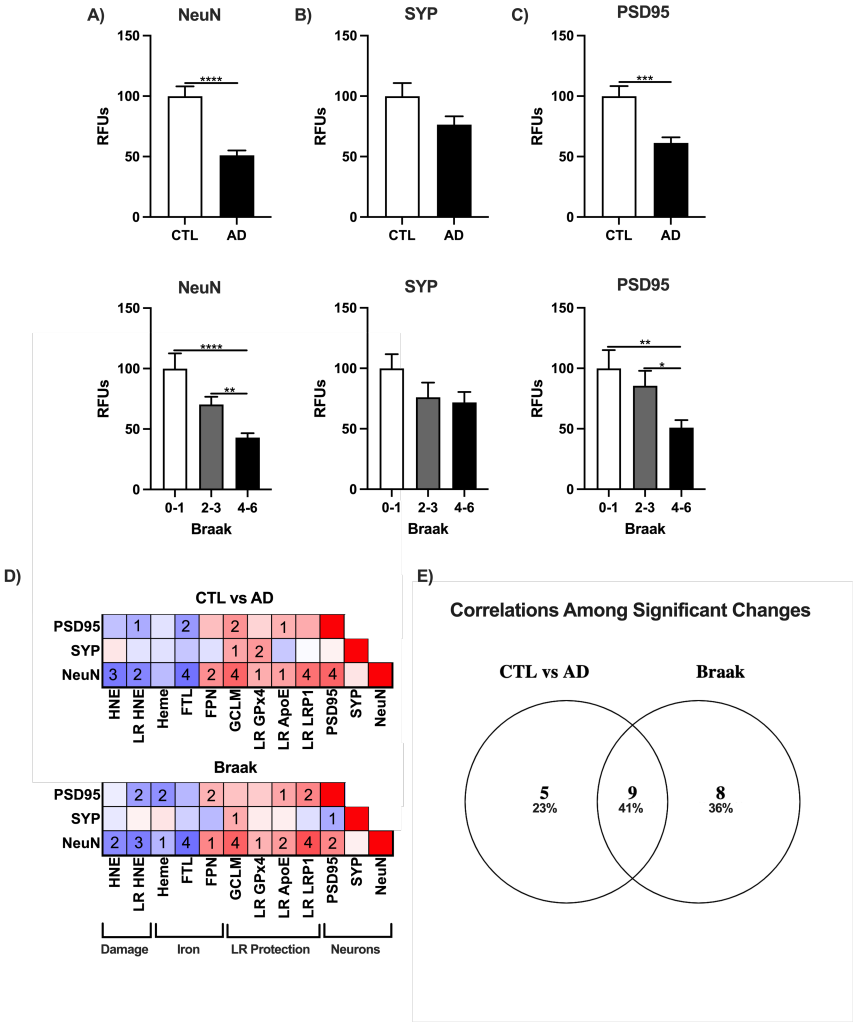


Figure 6: Neuronal loss in AD prefrontal cortex. Western blots as relative fluorescent units (RFU) of neuronal markers presented as CTL vs AD or Braak stage[32]. **A)** neuronal nuclei antigen (NeuN), **B)** synaptophysin 1 (SYP), **C)** postsynaptic density protein 95 (PSD95). **D)** Correlation matrix for variables with significant relationships to NeuN and **E)** Venn diagram comparison. Significance by 2-tailed t-test (CTL vs AD), one way ANOVA (Braak) with Tukey's posthoc, or by Spearman correlation: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Correlation matrix: 1, p<0.05; 2, p<0.01; 3, p<0.001; 4, p<0.0001.

ApoE allele associations with lipid rafts, lipid peroxidation, and antioxidant defense

NeuN was 50% lower in ApoE4,4 carriers than E3,3 and 3,4 (**Fig. 7A**). LR total cholesterol (-25%) and protein (-50%) had the largest deficits in ApoE4,4 carriers (**Fig. 7C,D**). Total Aβ fibrils were higher by 140% in ApoE4,4 carriers than E3,3 (**Fig. 7F**). The amyloid clearance proteins ApoE and LRP1 were 50% lower in ApoE4,4 (**Fig. 7G,H**). Iron storage protein FTL (+110%) was highest in ApoE4,4 carriers with matched increases in LR HNE (+185%) adducts (**Fig. 7I,J**). Antioxidant GPx4 (-50%) and GSH cycle enzyme GCLM (-60%) were both decreased in ApoE4,4 cortex (**Fig. 7K,L**). All LR markers were negatively correlated with Aβ fibrils (**Fig. 7M**). Aβ fibrils also correlated with LR HNE levels (r=0.56, p<0.0001).

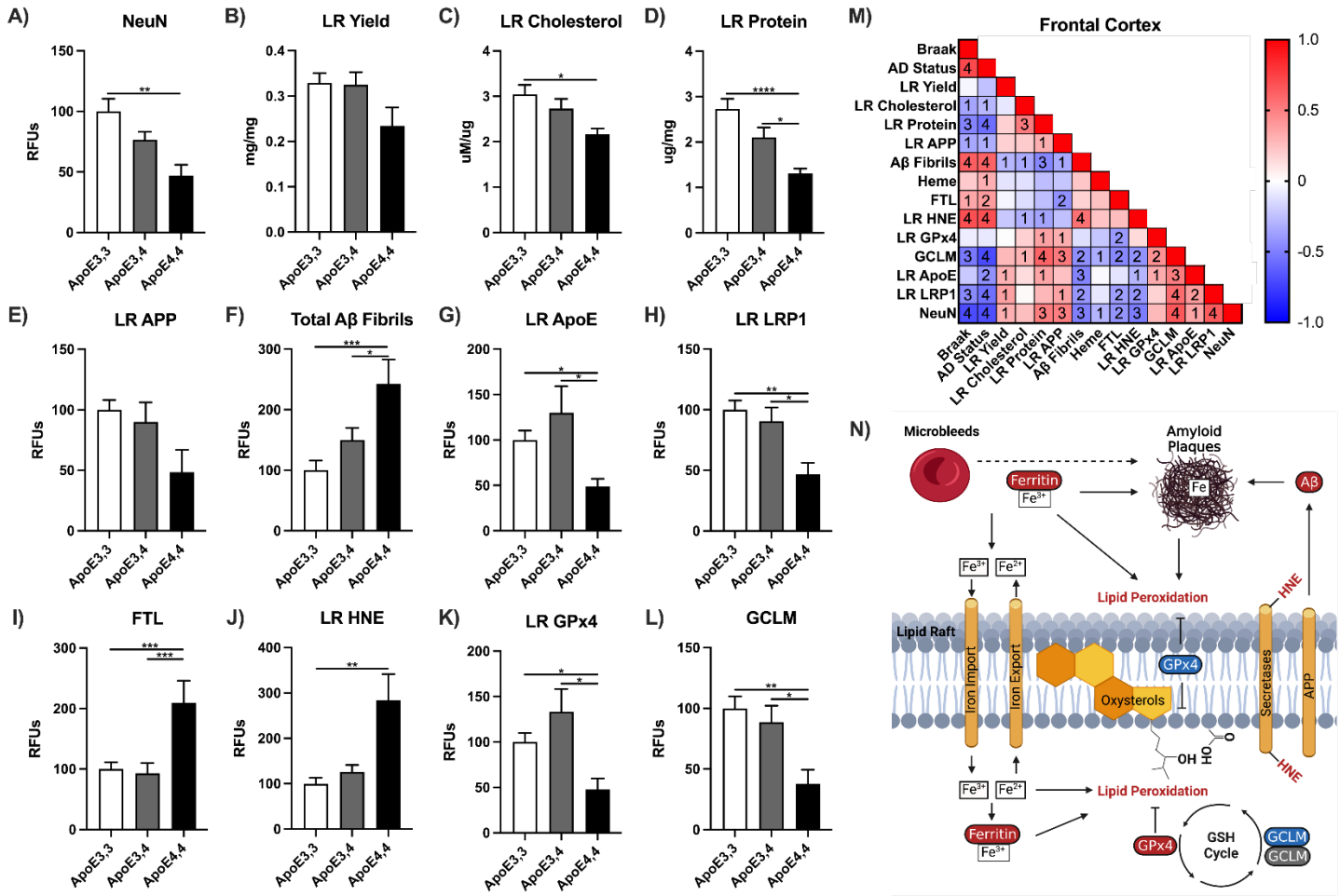
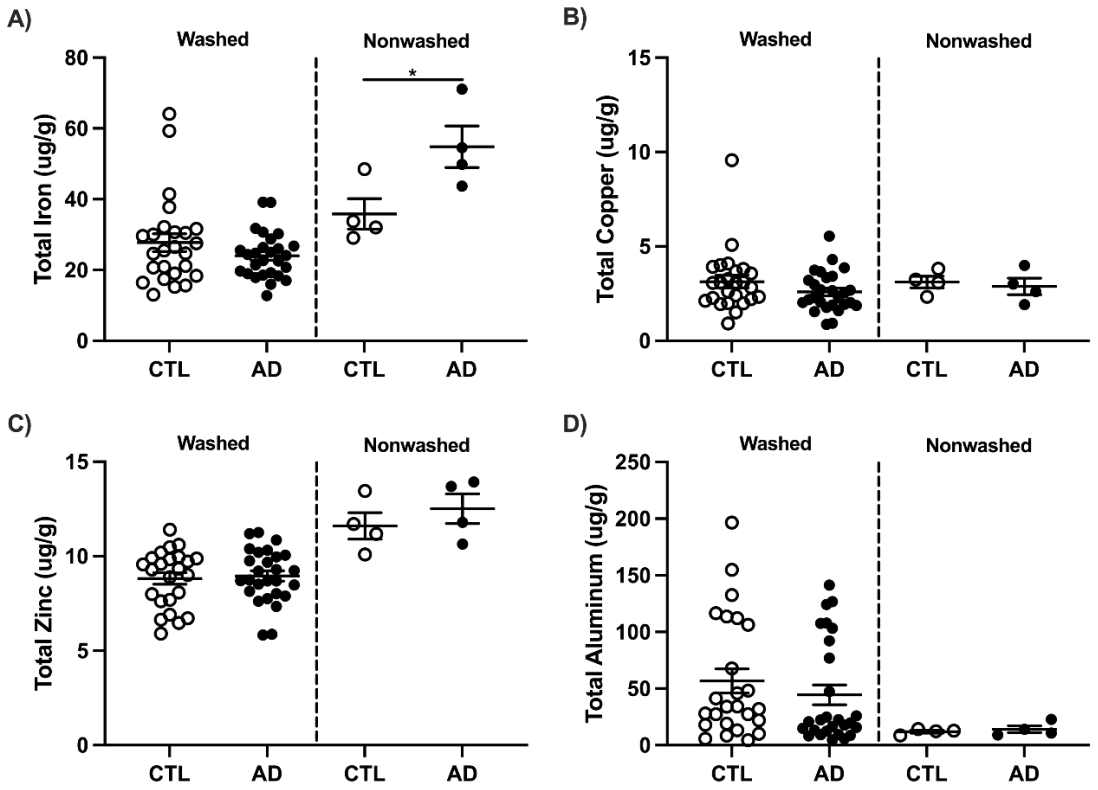
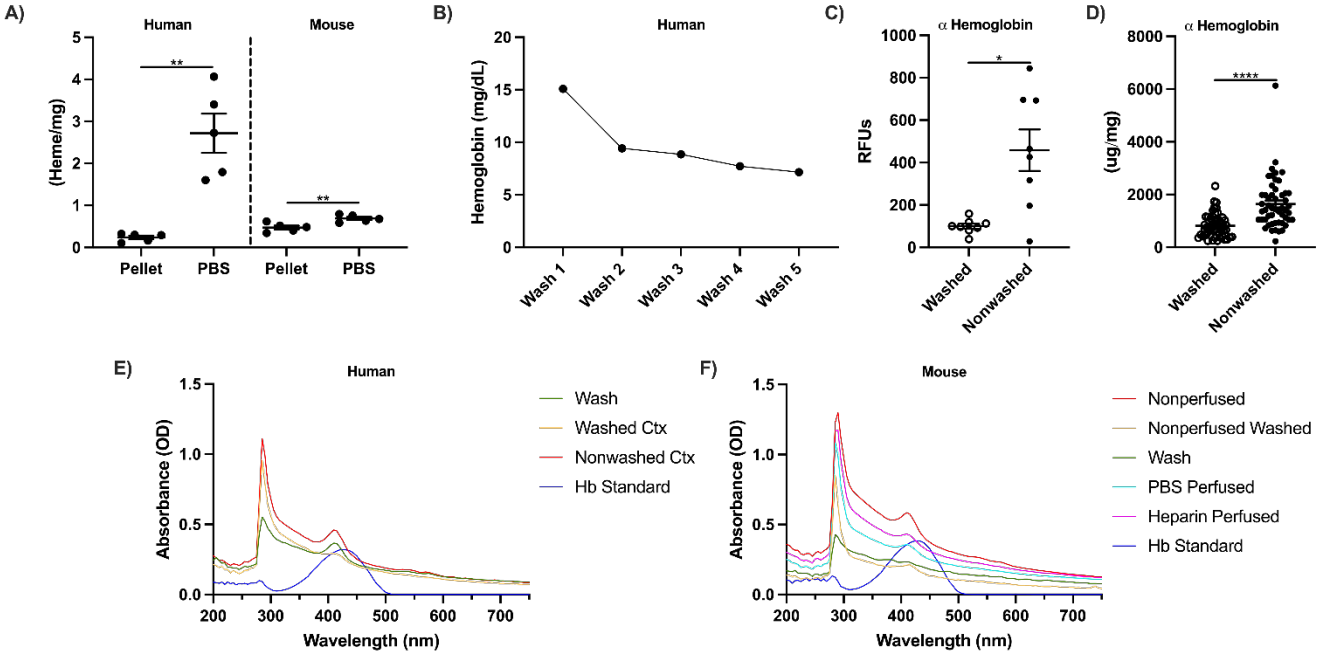


Figure 7: ApoE allele differences are associated with lipid rafts, antioxidant defense, and amyloid. Levels of **A)** NeuN, **B)** LR yield, **C)** LR cholesterol, **D)** total LR protein, **E)** LR APP, **F)** total Aβ fibrils, **G)** LR ApoE, **H)** LR LRP1, **I)** FTL, **J)** LR HNE, **K)** LR GPx4, and **L)** GCLM in prefrontal cortex of cognitively normal and AD. **M)** Correlation matrix of proteins analyzed by ApoE allele. **N)** Hypothesis linking oxidized lipid rafts to amyloid processing. One way ANOVA with Tukey's posthoc, or by Spearman correlation: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Correlation matrix: 1, p<0.05; 2, p<0.01; 3, p<0.001; 4, p<0.0001.

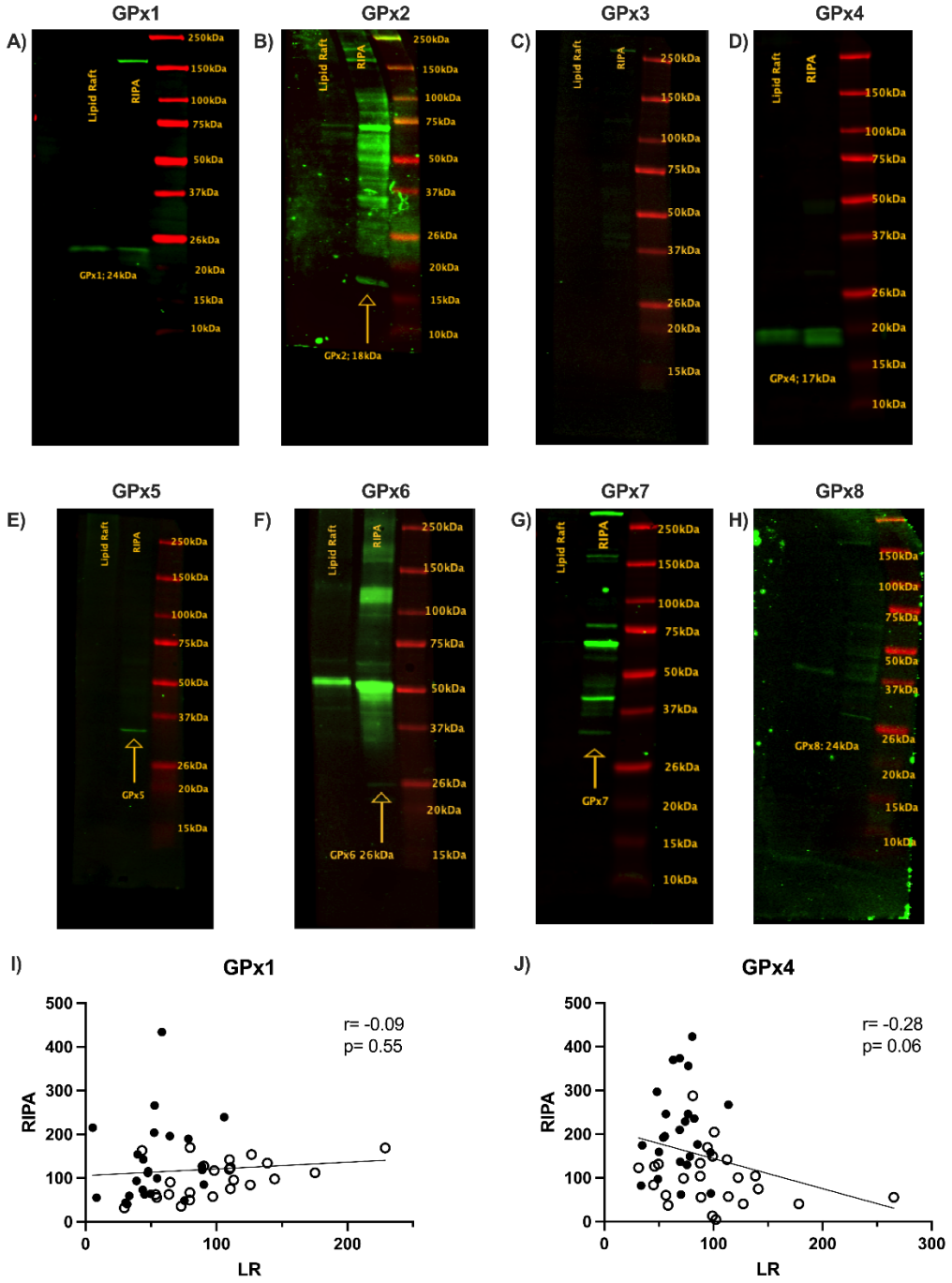


Supplemental Figure 1: Total **A)** Iron, **B)** Copper, **C)** Zinc, and **D)** Aluminum concentrations measured by ICP-MS (Table 1). **A-D,** Human prefrontal cortex was washed with PBS or non-washed. Significance by 2- tailed t-test (CTL vs AD): *p<0.05.



Supplemental Figure 2: A) Nonperfused mouse and human brain tissues were washed to minimize residual blood with PBS and pelleted by centrifugation. **B)** Collected wash was assayed for heme in 5 consecutive washes. Washes 3-5 yielded <10% further decreases. The reduction in α-hemoglobin in human frontal cortex was

confirmed by **C)** Western blot and **D)** immunoassay. Corresponding spectra for hemoglobin at 410 nm confirmed removal for **E)** human and **F)** mouse cortex. Significance by 2- tailed t-test: **p<0.01.



Supplemental Figure 3: Glutathione Peroxidases (GPx) 1 and 4 in lipid rafts from prefrontal cortex. The GPx isoforms of lipid rafts from human prefrontal cortex were immunologically identified by Western blots of lipid raft and whole cell lysates (RIPA buffer extract). **A)** GPx1, **B)** GPx2, **C)** GPx3, **D)** GPx4, **E)** GPx5, **F)** GPx6, **G)** GPx7, **H)** GPx8. Coplots of **I)** GPx1 and **J)** GPx4 for RIPA extract and lipid raft fractions.

Age	Sex	AD Status	Braak	ApoE Allele	PMI (hours)	Frontal Cortex	Cerebellum	MMSE	MMSE Interval (Months)	ADRC
99	Female	CTL	0	3,3	9	x	x	28	23.4	USC
91	Female	CTL	0	3,3	8.75	x	x	20	84	USC
85	Female	CTL	0	3,3	7	x	x	N/A	N/A	USC
95	Female	CTL	1	3,3	3.25	x	x	N/A	N/A	USC
87	Female	CTL	2	3,3	10.75	x	x	29	1	USC
95	Female	CTL	2	3,3	2.92	x	x	N/A	N/A	UCI
86	Female	CTL	3	3,3	6.17	x	x	30	35.5	UCI
90	Female	CTL	3	3,3	2.2	x	x	N/A	N/A	UCI
89	Female	CTL	3	3,3	3.58	x	N/A	24	5.9	UCI
94	Female	AD	4	3,3	10.5	x	x	N/A	N/A	USC
81	Female	AD	5	3,3	7.5	x	x	N/A	N/A	USC
89	Female	AD	5	3,3	1.5	x	x	N/A	N/A	USC
66	Female	AD	6	3,3	17	x	x	N/A	N/A	USC
70	Female	CTL	1	3,4	4.17	N/A	x	28	20.1	UW
86	Female	CTL	3	3,4	9.33	N/A	x	29	12.3	UW
97	Female	CTL	3	3,4	0	N/A	x	26	6.6	UW
91	Female	CTL	3	3,4	11.2	x	x	23	10.1	UW
85	Female	CTL	3	3,4	4.2	N/A	x	29	37.9	UW
89	Female	CTL	3	3,4	3.5	N/A	x	27	28.7	UW
74	Female	CTL	3	3,4	4.5	N/A	x	30	44.3	UW
91	Female	CTL	3	3,4	3.33	x	x	27	42.5	UCI
87	Female	CTL	3	3,4	4.57	x	x	29	85.6	UCI
93	Female	CTL	5	3,4	4.15	x	x	30	81.6	UCI
81	Female	AD	5	3,4	5.5	x	x	N/A	N/A	USC
84	Female	AD	5	3,4	6	x	x	N/A	N/A	USC
80	Female	AD	5	3,4	19	x	x	N/A	N/A	USC
96	Female	AD	6	3,4	9	N/A	x	N/A	N/A	USC
77	Female	AD	6	3,4	8.25	x	x	N/A	N/A	USC
87	Female	AD	6	3,4	4.5	N/A	x	N/A	N/A	USC
78	Female	AD	4	4,4	15.5	x	x	N/A	N/A	USC
81	Female	AD	5	4,4	4	x	x	N/A	N/A	USC
80	Female	AD	5	4,4	3.75	x	x	N/A	N/A	USC
76	Female	AD	5	4,4	5.25	x	x	N/A	N/A	USC
82	Male	CTL	0	3,3	9	x	x	28	N/A	USC
93	Male	CTL	0	3,3	12	x	x	30	1229.2	USC
93	Male	CTL	0	3,3	3.75	x	x	N/A	N/A	USC
76	Male	CTL	0	3,3	11.25	x	x	N/A	N/A	USC
86	Male	CTL	2	3,3	4.42	x	x	25	48.4	UCI
83	Male	CTL	2	3,3	3.18	x	x	N/A	N/A	UCI
86	Male	CTL	3	3,3	2.92	x	x	20	37	UCI
80	Male	CTL	3	3,3	4.05	x	x	26	95.1	UCI
97	Male	AD	3	3,3	5.25	x	x	N/A	N/A	USC
87	Male	AD	4	3,3	4.75	x	x	N/A	N/A	USC
76	Male	AD	5	3,3	9.75	x	x	N/A	N/A	USC
88	Male	AD	5	3,3	6.75	x	x	N/A	N/A	USC
92	Male	CTL	0	3,4	5.25	x	x	N/A	N/A	USC
87	Male	CTL	1	3,4	4	N/A	x	25	8.9	UW
90	Male	CTL	3	3,4	5.8	x	x	N/A	N/A	UCI
97	Male	CTL	4	3,4	5.83	x	x	26	7	UCI
87	Male	CTL	4	3,4	6.28	x	x	22	101.5	UCI
77	Male	AD	2	3,4	6	x	x	N/A	N/A	USC
81	Male	AD	3	3,4	7.25	x	x	N/A	N/A	USC
85	Male	AD	5	3,4	7.25	x	x	N/A	N/A	USC
72	Male	AD	6	3,4	9	x	x	N/A	N/A	USC
75	Male	AD	0	4,4	9.5	x	x	N/A	N/A	USC
88	Male	AD	5	4,4	5	x	x	N/A	N/A	USC
92	Male	AD	5	4,4	8.25	x	x	N/A	N/A	USC
70	Male	AD	5	4,4	3.5	x	x	N/A	N/A	USC
81	Male	AD	5	4,4	6.54	N/A	x	N/A	N/A	USC

Supplemental Table 1: Data provided for brain specimens by each ADRC source: USC, 37; UCI, 14; UW, 8. White, 76%; Latino, 14%; African American, 3%; Asian, 2%; N/A, 5%. ApoE3,3, 42%, ApoE3,4, 42%, ApoE4,4, 16%.

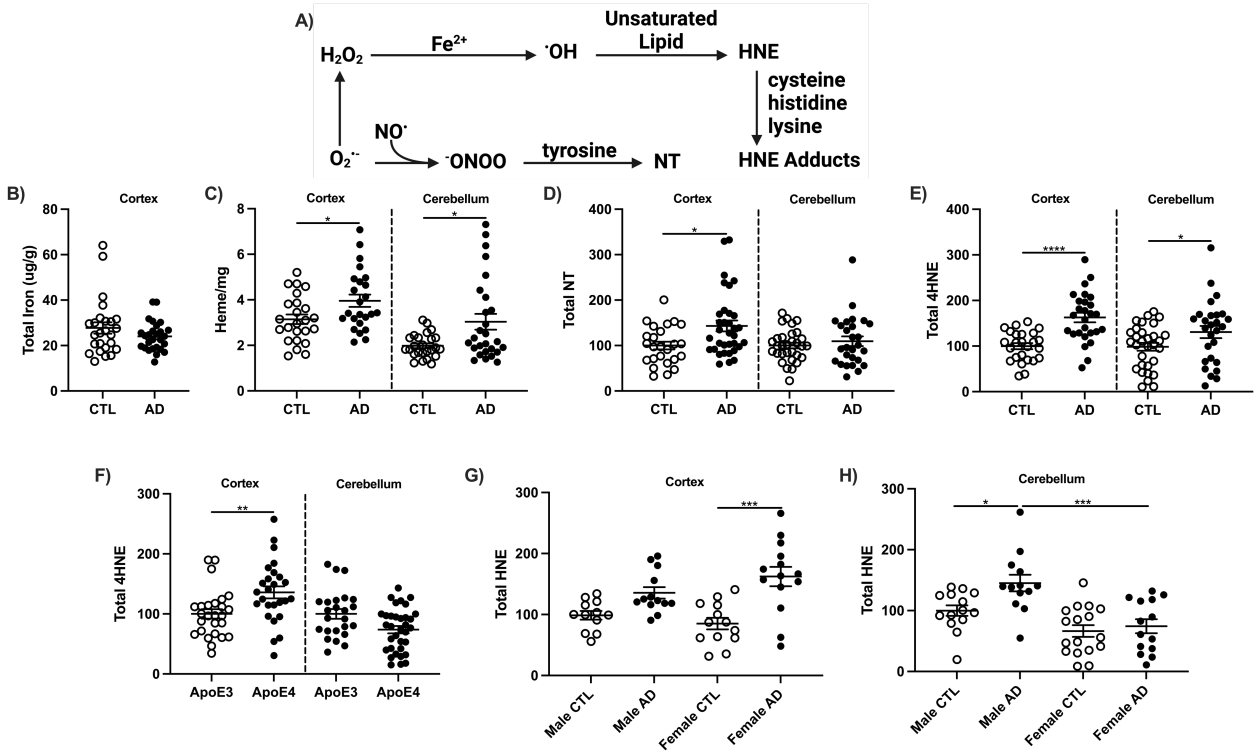
	NFT-Bearing Neurons				**NFT-Free Neurons**		
	NFT	Cytoplasm	Nucleus	Neuropil	Cytoplasm	Nucleus	Neuropil
AD	65 ± 9	20 ± 3	42 ± 5	14 ± 3	12 ± 2*	44 ± 7*	11 ± 1*
CTL	N/A	N/A	N/A	N/A	4 ± 0.5	17 ± 2	4 ± 0.5

Supplemental Table 2: Iron in NFT free and bearing neurons presented as mass charge ratio (m/z) by Good et al. 1992[12]. Significance by 2-tailed t-test: *$p<0.05$.

Measurement	Cortex (AD vs CTL)	Cerebellum (AD vs CTL)	Cortex (ApoE3,3 vs 3,4 & 4,4)	Cerebellum (ApoE3,3 vs 3,4 & 4,4)	Cortex Male (AD vs CTL)	Cortex Female (AD vs CTL)
FTL	+65% **	+10%, n.s.	+30%, n.s.	-30%, n.s.	+55%, n.s.	+75% *
FSP1	-40% ****	-30% ****	-40% *	-20% **	-50%, n.s.	-30% *
GCLM	-60% ****	-35% *	-30%, n.s.	-15%, n.s.	-65% ****	-55% ***
GPx4	+110% ****	-15% *	+45% *	-30, n.s.	+45%, n.s.	+210% ***
GPx4, LR	-30% **	-40% ****	-5%, n.s.	-5%, n.s.	-25% **	-35%, n.s.
Heme	+25% *	+30% **	+5%, n.s.	+10%, n.s.	+20%, n.s.	+25%, n.s.
HNE, Total	+60% ****	-20%, n.s.	+35% **	-25% *	+35% **	+90% ***
HNE, LR	+160% ****	+45% **	+80% **	+35% *	+170% **	+180% ***
NF-κB, nuclear	+175% **	+5%, n.s.	+70%, n.s.	+5%, n.s.	+45%, n.s.	+290% **
Nrf2, nuclear	-75% ****	-15%, n.s,	-65% ***	-15%, n.s.	-85% **	-60% **

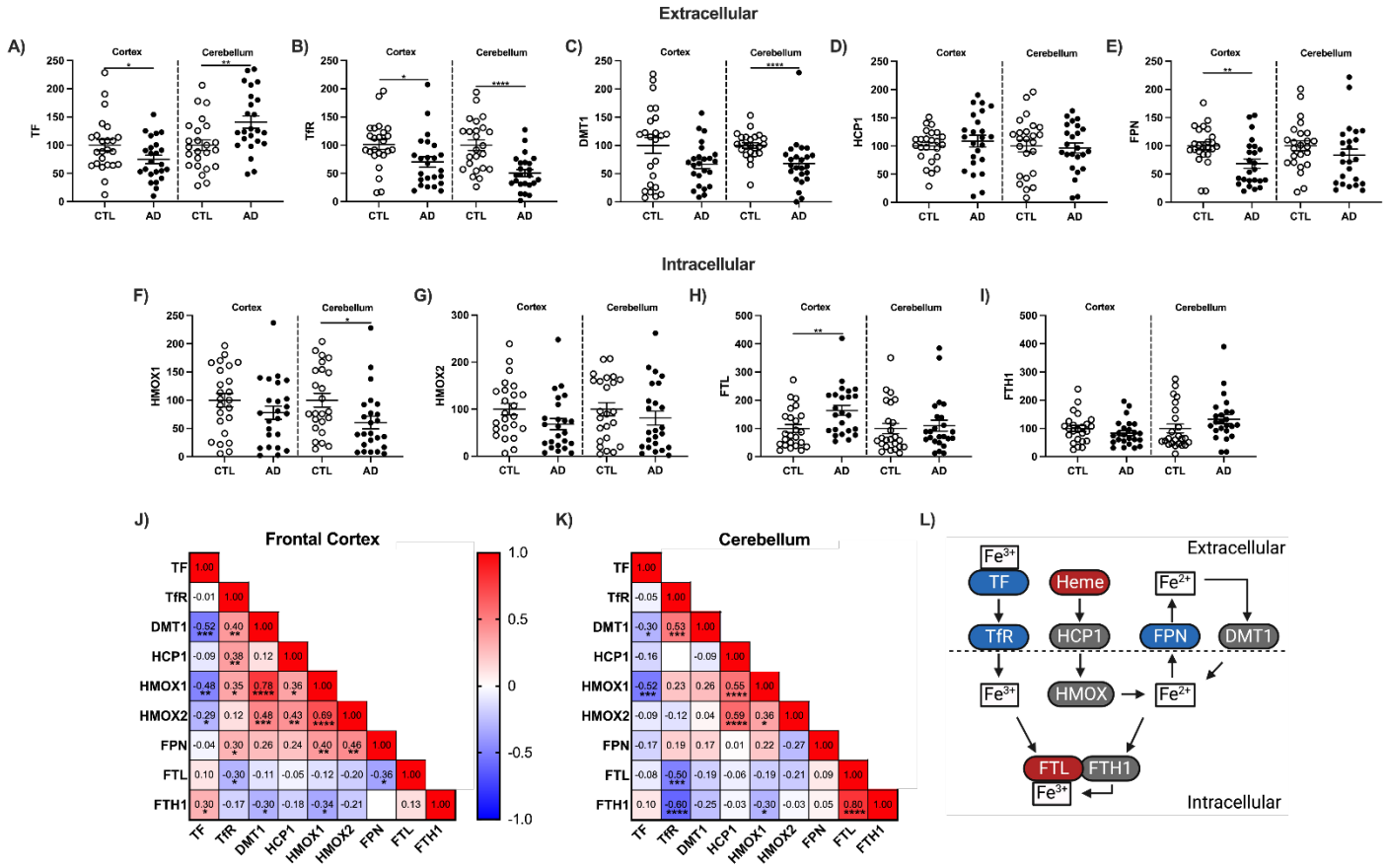
Supplemental Table 3: AD change in prefrontal cortex (Brodmann 8-10) and cerebellum by ApoE3 and 4 alleles. FTL, ferritin light chain; FSP1, ferroptosis suppressor protein 1; GPx4, glutathione peroxidase 4. t-test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; n.s, not significant, p>0.05.

Extended Figures

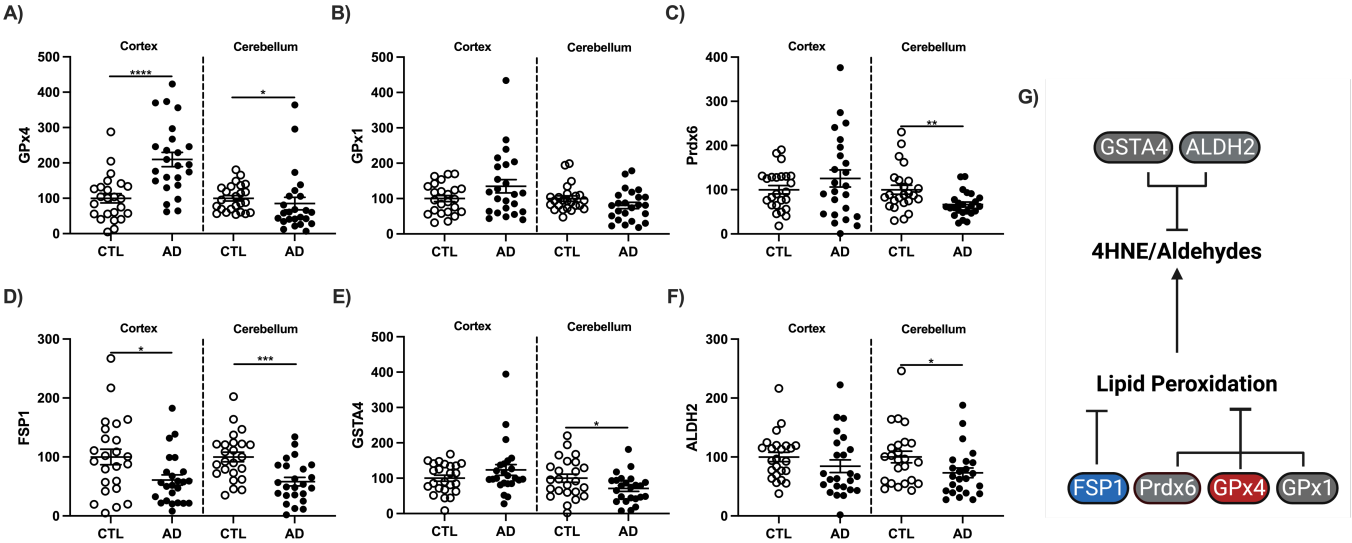


Extended Figure 1: Oxidative damage to prefrontal cortex and cerebellar proteins (HNE, NT) in age matched AD and cognitively normal control (CTL). **A)** Schematic representing oxidative

damage to lipids and proteins. Hydroxyl radicals (·OH) are produced during oxidation of Fe^{2+} or Cu^{+} ('Fenton chemistry') and may oxidize adjacent proteins, lipids or nucleic acids. HNE can alter proteins via Michael addition products through its double-bonded carbons, or by forming Schiff bases through its aldehyde moiety. The bottom reaction shows the iron-independent oxidative pathway to form peroxynitrite $^{-}$ONOO from $O_2^{\bullet-}$ and ·NO, which can then oxidize tyrosine to NT. Aggregated human Aβ42 stimulated formation of peroxynitrite which was neurotoxic in vitro[41]. Non-heme iron also mediates lipoxygenase activity[42]. Prefrontal cortex from Brodmann area 8, 9, or 10 and cerebellum was washed measurements for **B)** Total iron, measured by ICP-MS **(Table 1B)**, **C)** heme, and dot blots for **D)** NT, and HNE presented as **E)** cognitively normal vs AD, **F)** ApoE allele, or **G)** sex as relative fluorescent units (RFUs). Significance by 2-tailed t-test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Extended Figure 2: Iron transport and metabolism in AD prefrontal cortex and cerebellum. Relative fluorescent units from Western blots. **A)** transferrin (TF), **B)** transferrin receptor (TfR/CD71), **C**) divalent metal transporter 1 (DMT1), **D)** heme carrier protein 1 (HCP1), **E)** ferroportin (FPN), **F)**] hemeoxygenase 1 (HMOX1), **G)** heme oxygenase 2 (HMOX2), **H)** ferritin light chain (FTL), **I)** ferritin heavy chain 1 (FTH1), Correlation matrix for iron metabolism in clinical AD for **J)** prefrontal cortex and **K)** cerebellum. **L)** Summary matrix of AD changes in prefrontal cortex: blue, decrease; red, increase. Significance, 2-tailed t-test (A-H) or Spearman correlation (I,J): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.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B)
C)
D)
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G)
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Cerebellum
GPx4
GPx1
Prdx6
FSP1
GSTA4
ALDH2
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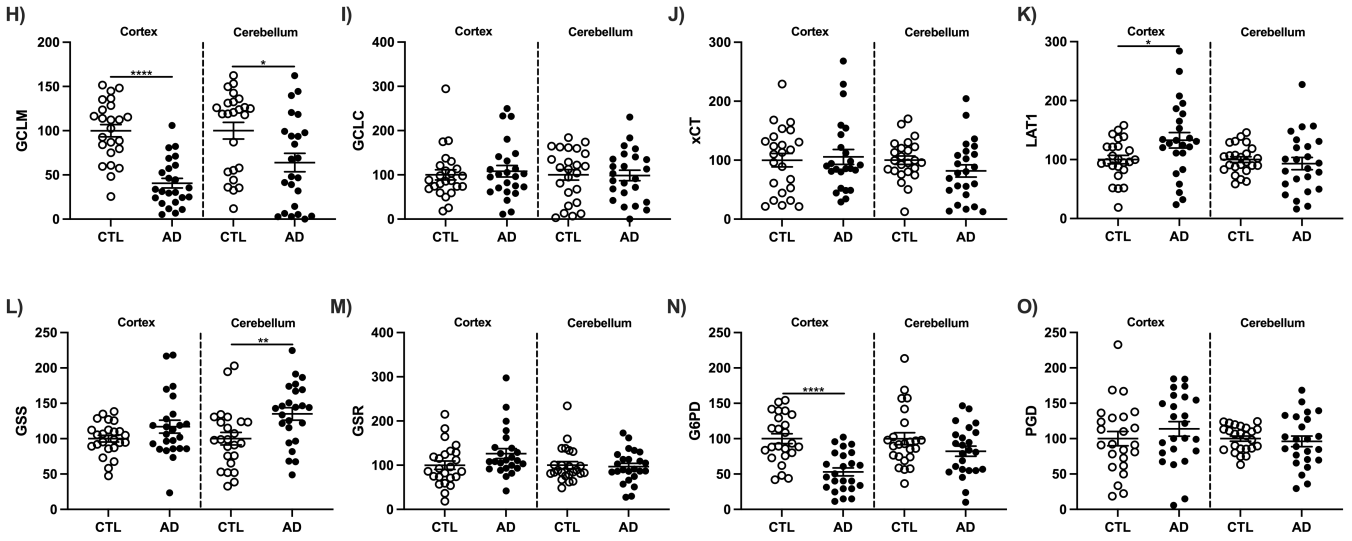
4HNE/Aldehydes
Lipid Peroxidation
FSP1
Prdx6
GPx4
GPx1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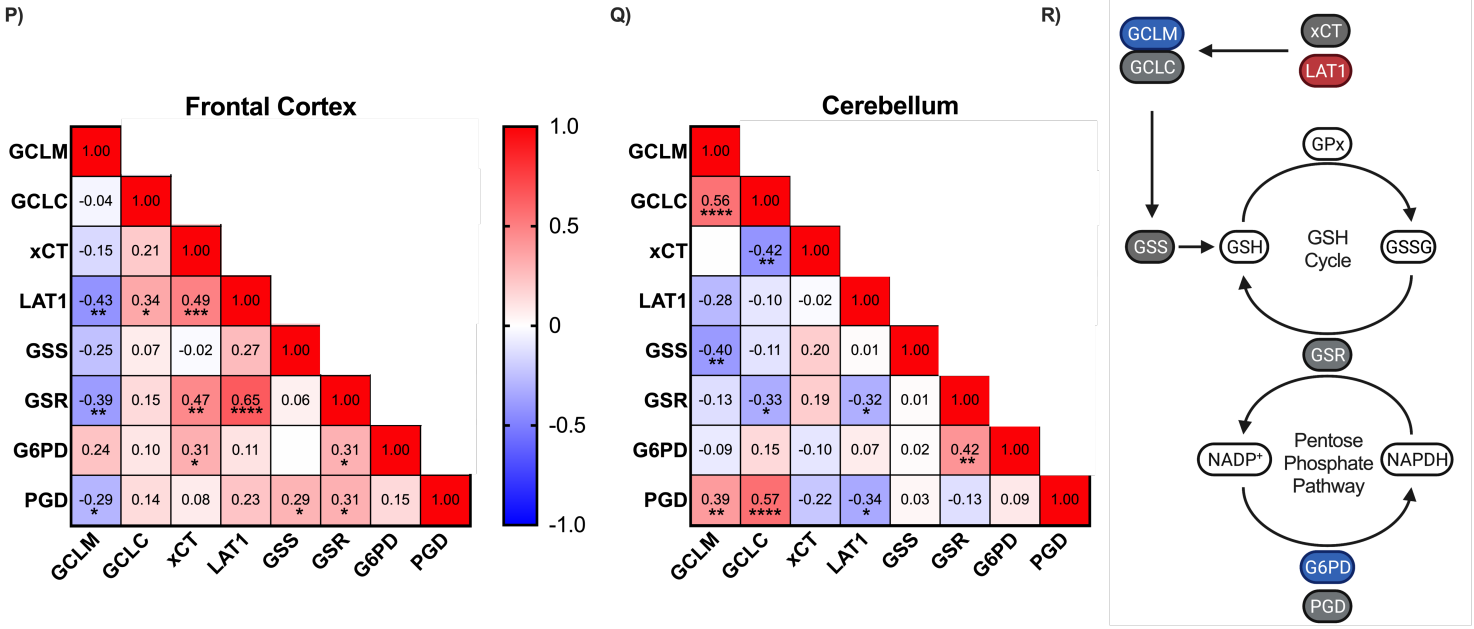
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Frontal Cortex
Cerebellum
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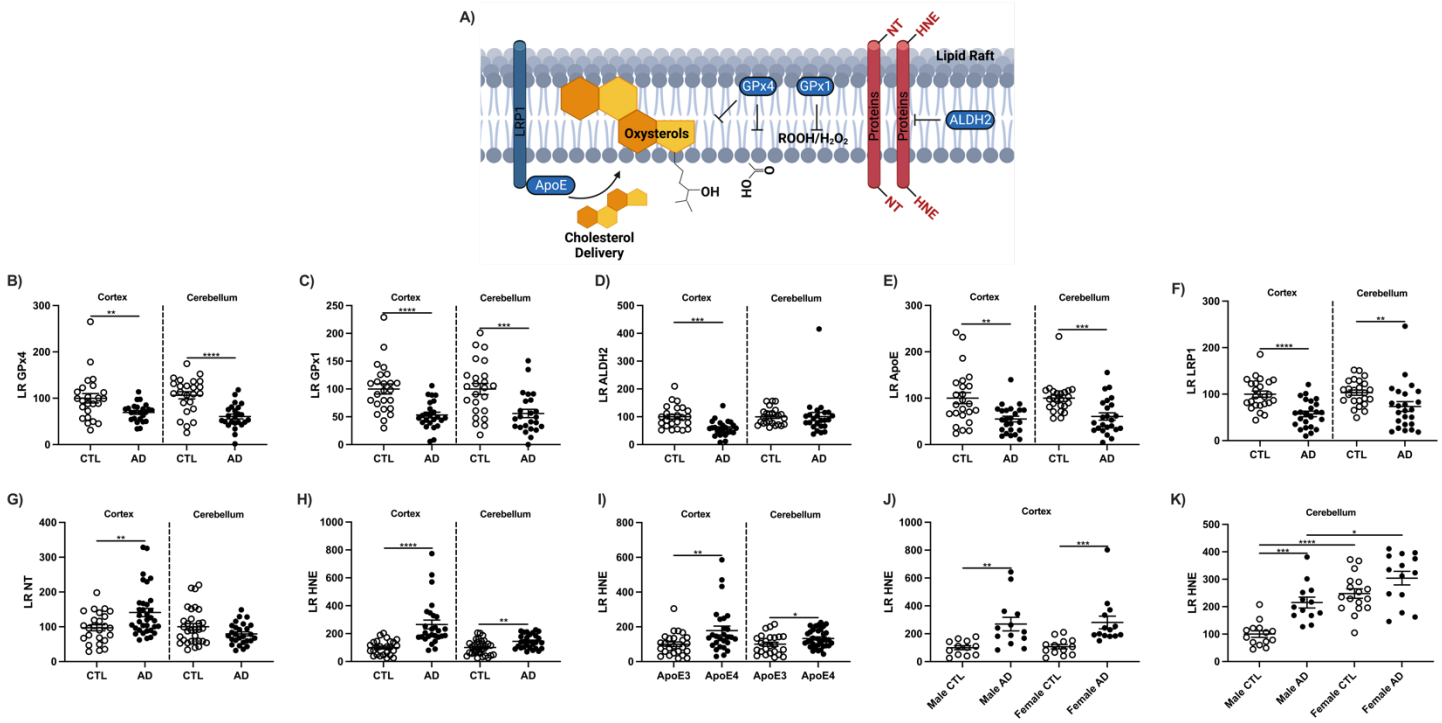
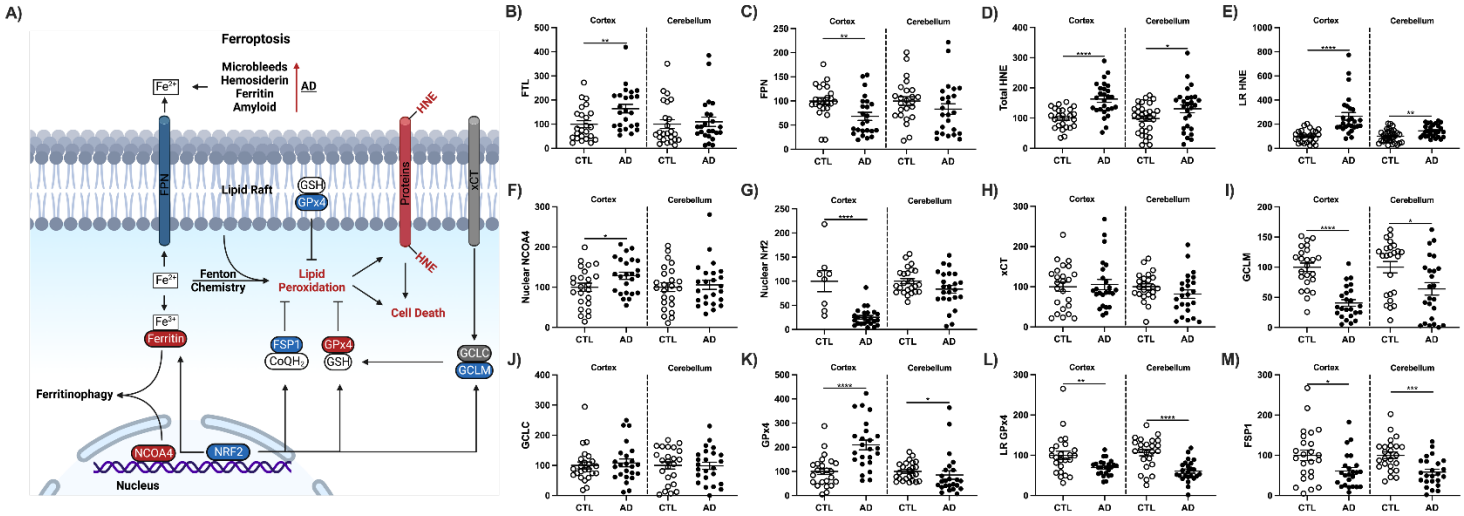
0
-0.5
-1.0
GSH
GSSG
GSH Cycle
GPx
GSR
NADP+
NAPDH
Pentose Phosphate Pathway

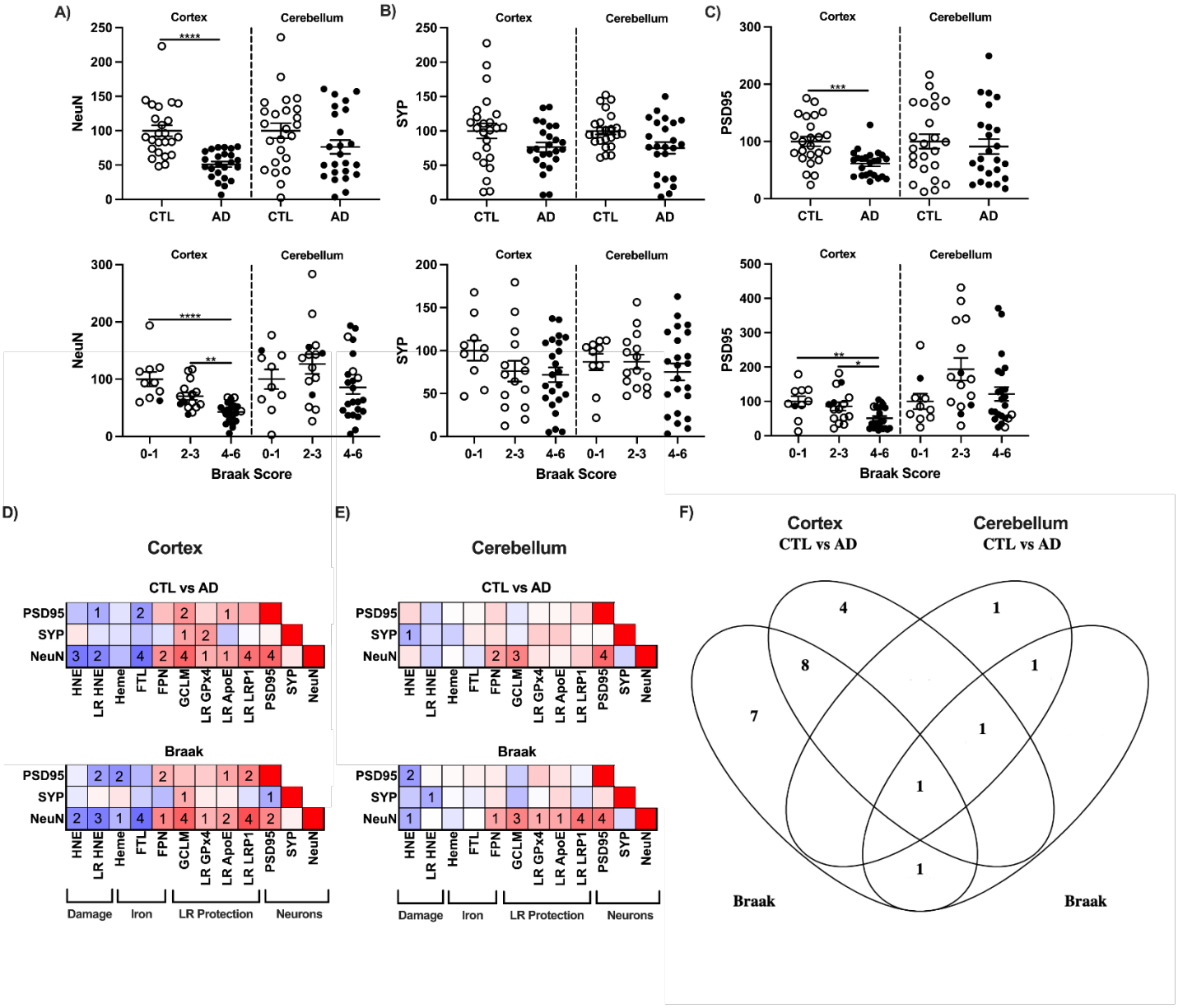
Extended Figure 3: Enzymes that mitigate lipid peroxidation and facilitate GSH production. Western blot data is shown as relative fluorescent units (RFUs) for prefrontal cortex or cerebellum from whole tissue lysate for antioxidants **A)** GPx4, **B)** GPx1, **C)** Prdx6, **D)** FSP1, **E)** GSTA4, and **F)** ALDH2. **G)** Enzymatic repair of lipid peroxidation. GSH cycle enzyme levels by Western blots, as relative fluorescent units (RFU). **H)** GCLM, **I)** GCLC, **J)** xCT/SLC7A11, **K)** LAT1, **L)** GSS, **M)** GSR, **N)** G6PD, and **O)** PGD. Correlation matrix for GSH cycle enzymes for **P)** prefrontal cortex **Q)** cerebellum and **R)** Schema of GSH metabolic enzymes and pentose phosphate pathway. AD increase (red), decrease (blue), no change (grey), relative to cognitively normal controls; not measured (white). Significance by 2-tailed t-test (A-H) or Spearman correlation (I): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



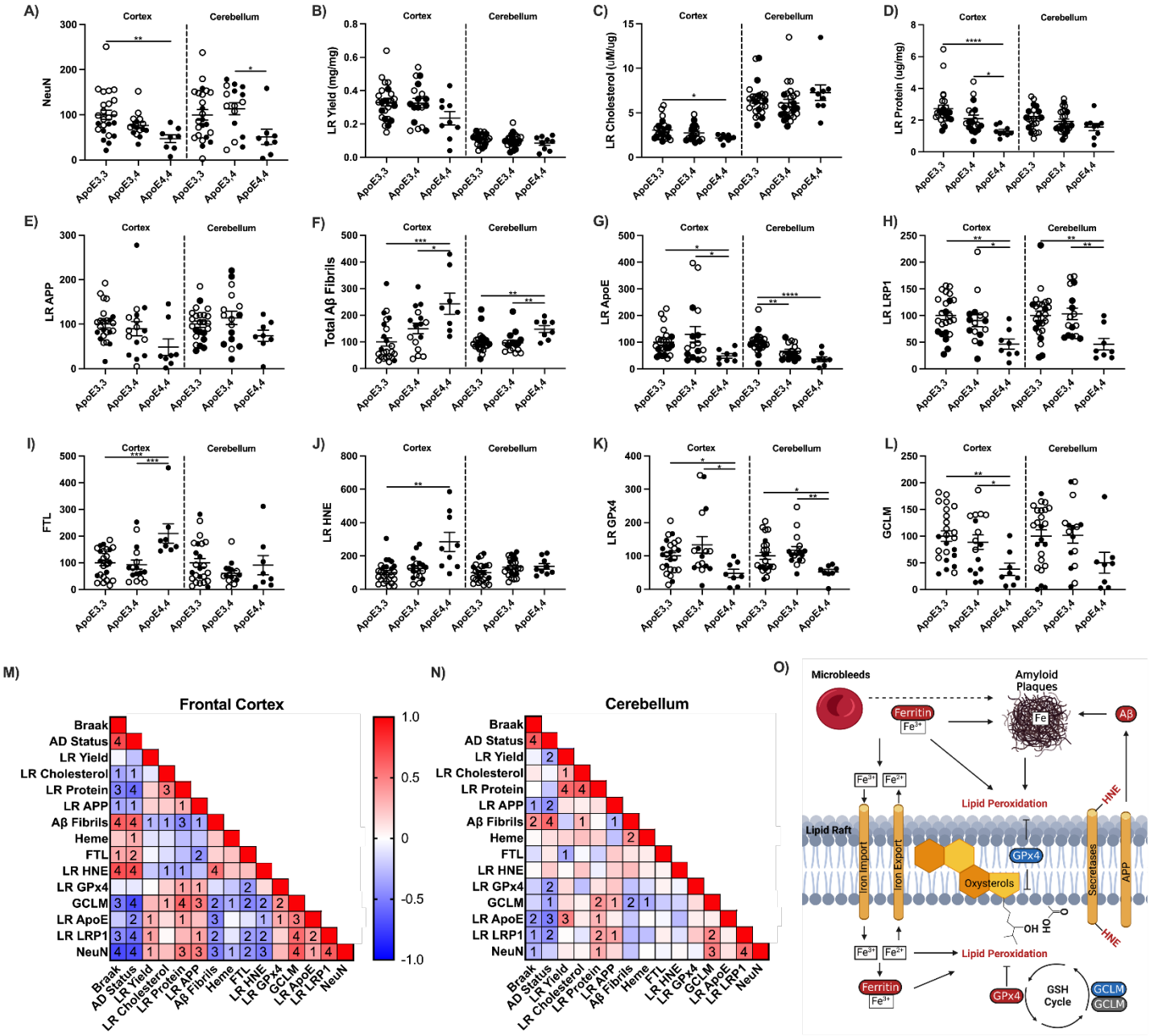
Extended Figure 4: A) Schema of lipid raft damage, protective mechanisms, and cholesterol shuttling in AD prefrontal cortex and cerebellum. Western blots as relative fluorescent units (RFUs) of lipid raft **B)** GPx4, **C)** GPx1, **D)** ALDH2, **E)** ApoE, **F)** LRP1, **G)** NT, and HNE presented as **H)** CTL vs AD, **I)** ApoE allele, and **J)** sex. B-I) Significance by 2-tailed t-test or J-K) one-way ANOVA with Tukey's posthoc:*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



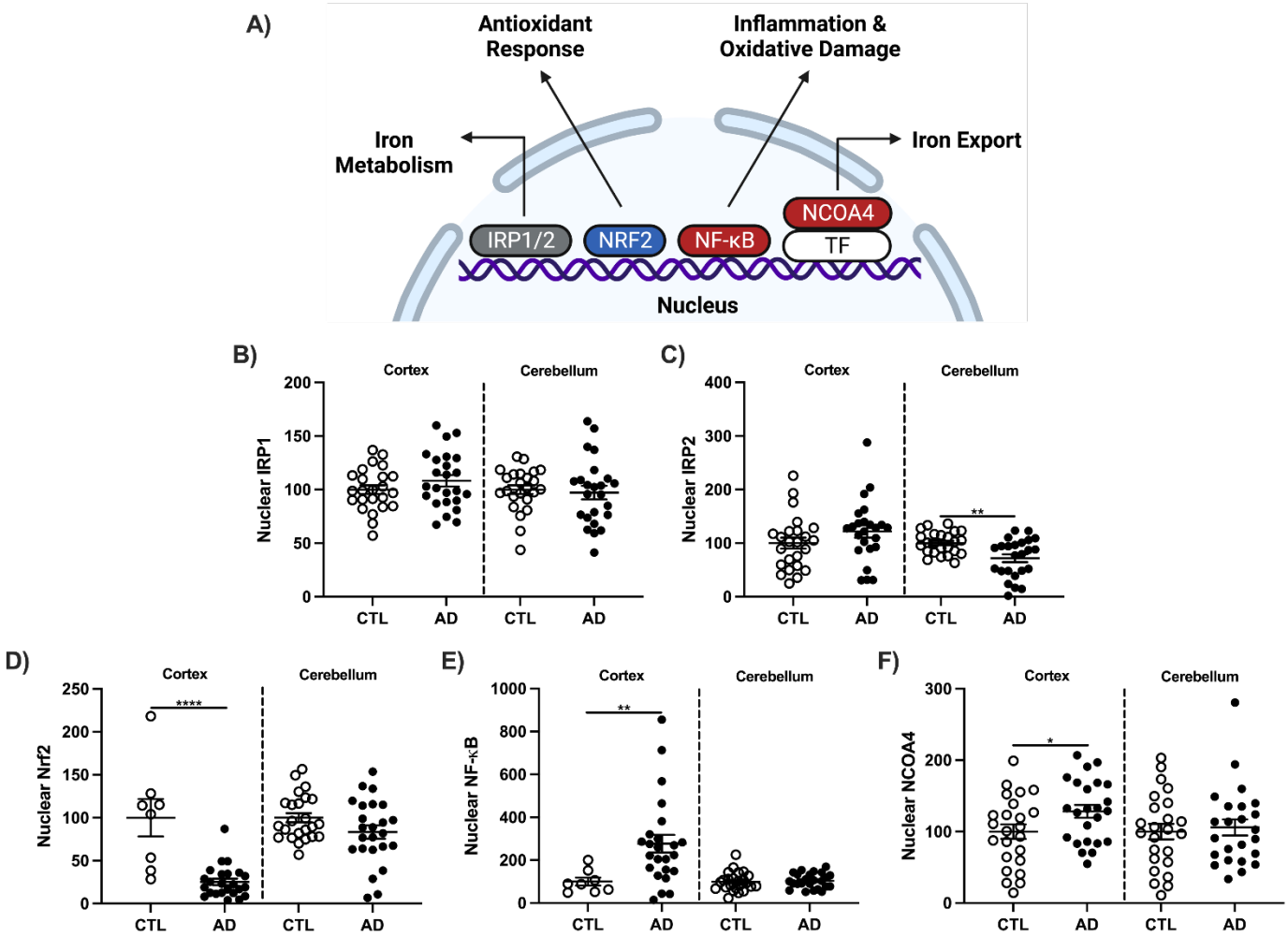
Extended Figure 5: Ferroptosis schema which consolidates data from prior figures. **A)** Schema of ferroptosis markers related to iron transport, lipid rafts, and oxidative damage mitigation. **B)** FTL, **C)** FPN, **D)** HNE, **E)** lipid raft HNE, **F)** NCOA4, **G)** Nrf2, **H)**, xCT, **I)**, GCLM, **J)** GCLC, **K)** GPx4, **J)** Lipid raft GPx4, and **M)** FSP1. Significance by 2-tailed t-test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Extended Figure 6: Neuronal loss in AD. Western blots as relative fluorescent units (RFU) of neuronal markers presented as CTL vs AD or Braak stage[32]. **A)** neuronal nuclei antigen (NeuN), **B)** synaptophysin 1 (SYP), **C)** postsynaptic density protein 95 (PSD95). Correlation matrixes for variables with significant relationships to NeuN for **D)** prefrontal cortex and **E)** cerebellum. **F)** Venn diagram comparison of correlations observed in both brain region. Significance by 2- tailed t-test (CTL vs AD), one way ANOVA (Braak) with Tukey's posthoc, or by Spearman correlation: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Correlation matrix: 1, p<0.05; 2, p<0.01; 3, p<0.001; 4, p<0.0001.



Extended Figure 7: ApoE allele differences are associated with lipid rafts, antioxidant defense, and amyloid. Levels of **A)** NeuN, **B)** LR yield, **C)** LR cholesterol, **D)** total LR protein, **E)** LR APP, **F)** total Aβ fibrils, **G)** LR ApoE, **H)** LR LRP1, **I)** FTL, **J)** LR HNE, **K)** LR GPx4, and **L)** GCLM in prefrontal cortex and cerebellum of cognitively normal (open circles) and demented (closed circles). Correlation matrix of proteins analyzed by ApoE allele in **M)** frontal cortex and **N)** cerebellum. **O)** Hypothesis linking oxidized lipid rafts to amyloid processing. One way ANOVA with Tukey's posthoc, or by Spearman correlation: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Correlation matrix: 1, p<0.05; 2, p<0.01; 3, p<0.001; 4, p<0.0001.



Extended Figure 8: A) Transcription factors of genes for antioxidant defense and iron signaling in human prefrontal cortex of AD and CTL. IRP, iron regulatory protein; Nrf2, nuclear factor erythroid 2–related factor 2; NF-κB p65, nuclear factor κ-light-chain-enhancer of activated B cells; NCOA4, nuclear receptor coactivator 4; TF, transcription factor. Western blot data is relative fluorescent units (RFU) for **B)** IRP1, **C)** IRP2, **D)** Nrf2, **E)** NF-κB p65, and **F)** NCOA4 in prefrontal cortex and cerebellum. Significance by 2-tailed t-test: *p<0.05, **p<0.01, ****p<0.0001.

Discussion

Iron is increasingly implicated in brain oxidative damage during AD[10,11,21], but the sources of iron and contributions to region-selective neurodegeneration during AD remain unresolved. Ferritin protein was strongly correlated with membrane oxidation (increased HNE) and loss of neurons and synapses in AD prefrontal cortex. These data were obtained with minimization of blood in the vasculature. We addressed the confound of vascular blood in frozen samples from unperfused human brains by a simple washing procedure. Besides iron, erythrocytes contain ferritin (FTL and FTH1) and GSH enzymes including GPx4.

Washed brains from AD and controls had the same levels of total iron in prefrontal cortex. In contrast, heme iron was 23% higher in AD than controls (**Fig 1C**). Because the washing procedure cannot completely remove intravascular blood, these data should be considered upper limits to heme and ferritin from microbleeds, stroke, and superficial siderosis. The number and size of microbleeds, brain location, and size differ 10-fold between studies[44–47], possibly due to differences in methodology and diversity of clinical samples. Artifactual microbleeds can arise postmortem and may confound

correlations of PET imaging for microbleeds with postmortem analysis[48]. Because microbleeds arise sporadically during 'usual aging' as well as in clinical AD, their contribution to brain iron is undefined. Mice transgenic for familial AD genes have increased hemoglobin from microbleeds by age 6-months[47,49] when AD pathology manifests[50]. Microbleed numbers also correlate with Aβ load[49]. Microbleeds and associated hemosiderin deposits may be major sources of total iron in AD brains.

Ferritin deposits store non-heme iron. FTL was increased 50% in AD prefrontal cortex. The 30% decrease of several iron import/export proteins in AD may contribute to increased brain iron. Plaques and NFT contain ferritin protein and multivalent iron[10,51–53]. In addition to its redox activity, iron can modify aggregation of amyloid peptides[54]. Iron also directly influences amyloid peptide production by the iron-response element in the APP primary transcript[55]; moreover, iron can enhance alpha-secretase cleavage[56]. Ferric iron also promotes NFT aggregation, whereas NFTs are solubilized by ferrous iron[57]. Furthermore, neurite dystrophy occurs near amyloid plaques, facilitating microtubule disruption[58]. Hippocampal CA1 neurons that contain NFTs have 2-fold higher iron than the same-cell cytoplasm, shown by laser-guided mass spectrometry[12]. Neighboring CA1 neurons without NFT had 50% less cytoplasmic iron (**Supplemental Table 2**). Given the association of heme with HNE shown here, we predict that NFT-containing neurons have elevated HNE. The hippocampus merits cell-by-cell analysis of HNE in relation to NFT, particularly for the most AD vulnerable CA1 neurons.

Little is known of brain cell types for AD changes in proteins related to iron transport and membrane oxidation. Microglia have high levels of ferritin in AD that are presumed to be phagocytosed from amyloid plaques and NFT[53,59,60]. Endogenous cell ferritin merits consideration because microglia and astrocytes in young rat brain have 2-fold more ferritin than neurons, while oligodendroglia have the highest ferritin levels[61] that may contribute to white matter degeneration during AD in hippocampus and cerebral cortex[26]

Relationships of neurodegeneration to increased brain iron included increased FTL, a major protein of ferritin in senile plaques and NFT. Moreover, AD decreased the four main iron transport proteins: transferrin (TF), its receptor TfR, divalent metal transporter 1 DMT1, and ferroportin FPN. The 40% decrease of ferroptosis suppressor protein FSP1 was not expected because of its protective role in mitigating lipid peroxidation. Oxidative damage was assayed by downstream markers of HNE and NT, which were associated with 2-fold higher GPx4 in whole prefrontal cortex tissue.

Lipid rafts, the site of neuronal amyloid peptide production, also showed oxidative damage and repair. Paralleling whole brain tissue, lipid rafts from prefrontal cortex had 50% higher HNE in AD. However, unlike its increase in whole tissue of prefrontal cortex, lipid raft GPx4 was 50% lower, suggesting subcellular specificity. Cytosolic GPx4 is 20kDa, while the 23kDa isoform with a leader sequence is cleaved after mitochondrial import[62]. Because lipid rafts are derived from all brain cells, the loss of GPx4 may include mitochondrial; alternatively, leader sequences may be needed for membrane localization. Lipid raft composition from prefrontal cortex was defined for glutathione peroxidases (GPx1-8) and contained only GPx1 and GPx4 but not Prdx6. The absence of Prdx6 in the lipid raft fraction is consistent with its transient interactions with membranes[63,64]. Prdx6 and GPx1 also reduce lipid hydroperoxides[65] released from membrane lipids[66] and may therefore play a secondary role in defense against ferroptosis.

Oxidation may enhance APP processing by the secretases, shown for γ-secretase in rat cortex neurons and human neuronal cells exposed to 1-10 μM HNE or 100 μM $FeSO_4$[67]. Moreover, H_2O_2 increased BACE1 activity in mouse neuronal N2a cells transgenic for human APP_{695}[68]. Oxidizable amino acids that can be conjugated by HNE and NT are abundant in APP processing enzymes BACE1 (57/501) or PSEN1 (50/467), while ADAM10 (142/748) has 3-fold more vulnerable residues. BACE1 activity increases with AD in prefrontal cortex[69]. Recognizing that HNE and NT can increase or decrease enzyme activities [68,70], biochemical analysis is needed for secretase activity in relation to membrane oxidation in AD brains. The decreased NeuN and the lower yield of lipid rafts shown by us[32] from AD in prefrontal cortex, but not cerebellum, is consistent with the regional specificity of neurodegeneration. The concurrent loss of neurons, decreased lipid raft yield, and increased

membrane oxidation are correlated with fibrillary amyloid. AD increases of BACE1 activity in brain and serum[71,72] parallel the increased peroxidation of serum and tissue lipids[73,74].

The ApoE4 allele, a major AD risk factor, is associated with higher HNE in prefrontal cortex but not cerebellum. Whole tissue and lipid raft HNE in prefrontal cortex were higher in E4 carriers than E3, while NT did not differ by ApoE allele. ApoE4 prefrontal cortex had 35% higher HNE than E3; the absence of E4 differences in cerebellum correspond to its lesser AD neurodegeneration. The E4 allele was also associated with less FSP1 and nuclear Nrf2 in prefrontal cortex which protect against oxidative damage. The current sample of 52 brains is underpowered to evaluate differences by ApoE allele-sex interactions.

The transcription factor Nrf2 induces phase II gene response that mitigate oxidative damage including GPx4[75,76]. Nrf2 mediated antioxidant defense declines with age in cells and tissues including cerebellum[77,78]. We suggest that the age-related decline of Nrf2 defense facilitates higher membrane oxidation and amyloid peptide production (**Fig. 7N**). Increased nuclear p65 NF-kB may inhibit Nrf2 gene response[79,80] because both require binding of the CBP/P300 transcriptional co-activators[80]. The 3-fold increases in nuclear p65 NF-κB in AD suggest increased activity of genes that promote oxidative damage (**Fig.1)**.

Genomic damage during AD involves iron-derived oxidants. Heme from microbleeds and microhemorrhages may contribute to oxidants via iron from heme degradation by heme oxygenases[81]. AD brains have 2-fold increased double-strand DNA breaks (DSB) in neurons and astrocytes[82]. The link of iron to DSBs in neurons was shown experimentally with chelation of iron by deferoxamine[22], which decreased DSB in mice injected with hemolyzed blood and in cultured neurons exposed to hemin. Unspecified oxidative damage is implied for exomic somatic mutations in the AD hippocampus with the 'oxidative mutational signature' C>A[83]. Iron is given little attention in the growing literature of genomic instability in AD.

Chelation therapy for AD was first attempted in 1991 to remove aluminum by deferoxamine, which slowed cognitive decline in subjects during 24-months[84]. While cognitive benefits were attributed to chelation of brain aluminum, Gleason and Bush[85] noted that deferoxamine has 6-fold greater affinity for ferric iron than aluminum, which implies that cognitive improvements were due to iron chelation, rather than aluminum. The present brains also did not differ by aluminum for AD vs CTL (**Supplemental Fig. 1**). This early chelation study was not pursued because of weakening evidence for the environmental role of aluminum, despite growing evidence for the role of iron in amyloid aggregation. Decades later, a new chelation trial has begun, 'Deferiprone to Delay Dementia'. The '3D Study' (NCT03234686) is a randomized study of prodromal and mild AD patients by ApoE genotype for cognition and brain iron levels, with expected completion by September 2023. Whatever is learned from the 3D Study, future therapies may consider chelation in combination with amyloid removal and include direct measures of brain synapse density by PET. Little is known about the turnover of iron in plaque or NFT. A new concern arises: do monoclonal therapies that decrease amyloid plaque size also cause by-stander damage from released redox active iron?

Air pollution may also enhance iron-mediated neurodegeneration, suggested by ferritin deposits in the olfactory bulb that are contiguous with foreign particulate material[86]. Connections of the olfactory pathways throughout the brain merit analysis for iron-associated damage from air pollution. For example, air pollution derived nanoparticles (nPM) caused membrane oxidation in lipid rafts in an ADtg mouse[87]. Acute exposure to nPM rapidly increased HNE and NT in mouse olfactory bulb in wildtype mice[88].

In summary, this analysis of iron metabolism and membrane oxidation in AD is the first to comprehensively document the ensemble of proteins that mediate iron transport and GSH-mediated neuroprotection. AD pathway specificity was shown by the greater change in prefrontal cortex than cerebellum. Prior studies on subsets of these proteins had lower statistical strength from smaller sample size. These data substantiate reports of lipid peroxidation during AD, and identify new alterations in iron related transport, clearance, and oxidative repair mechanisms. Taken together, this

suite of findings supports the concept of ferroptosis during AD. Future studies will analyze neuronal and glial membrane damage in relation to the cellular localization of iron deposits in brain pathways from the prefrontal cortex to the hippocampus.

Methods

Human Samples Postmortem prefrontal cortex and cerebellum with Braak scores were provided by three Alzheimer's Disease Centers: University of Southern California (Dr. Carol Miller), University of California Irvine (UCI MIND), and University of Washington (BRaIN). Samples were genotyped for ApoE alleles by PCR for SNP variants rs429358 and rs7412. All human subjects provided informed consent. Human tissue use was approved through IRB protocol #UP-20-00014-EXEMPT. Details for individual brains are in **Supplementary Table 1**.

Mice C57BL/6 mice from Jackson laboratories were housed by the University of Southern California's Department of Animal Resources. Mice had ad libitum access to Purina Lab Chow (LabDiet, Hayward, CA) and water. Mice were housed in groups of 5 at 22°C/ 30% humidity, with standard nesting material and light cycles of 0600-1800 hr. Studies were approved by the USC Animal Use and Care Committee (IACUC# 20417).

Mouse Tissue 6-month old mice were anesthetized with isoflurane and euthanized by cardiac puncture. Mouse cortex was collected as non-perfused, with transcardial PBS perfusion, or PBS perfusion + 10U/mL heparin (Sigma-Aldrich, St. Louis, MO). Tissues were stored at -80°C.

Tissue Washing Human and mouse brain tissue was thawed on ice and minced with surgical scissors for heme or ceramic scalpel for iron measurements. PBS (10 volumes) was placed in each tube and the samples were gently washed by two cycles of vortexing and collected by gentle centrifugation 800g/30 sec/4°C. PBS was retained on a small subset of samples and further spun at 4,000g/10 min/ 4°C. The pellet was sonicated 10 sec at 50% power in PBS. Resuspended pellet and lysate were assayed for heme (**Table 1; Supplemental Fig. 2**).

Amyloid Measurements Human tissue was processed for amyloid measurements as previously described. Briefly, tissue was homogenized in RIPA without SDS and spun for 10,000g for 1h at 4°C. Supernatant was collected and analyzed for soluble Aβ fibrils using M78 (Conformation specific antibody for Aβ fibrils[89], a gift from Dr. Charles Glabe, UCI). The residual pellet was resuspended in 70% formic acid and sonicated. The resuspension was nutated at room temperature for 2h and neutralized with 20 volumes of 1M Tris and concentrated with a vacuum concentrator (Labconco, Kansas City, MO).

Lipid Raft The lipid raft fraction was isolated from 40 mg homogenates of frozen prefrontal cortex (Brodmann area 8, 9 or 10) and cerebellum using a column-based isolation kit (Invent Biotechnologies, Plymouth, MN). These findings were verified by sucrose gradient ultracentrifugation[87] (**Supplemental Fig. 2**). Lipid raft fractions were resuspended in PBS+1% triton x-100 and sonicated 10 seconds at 50% power. Protein was assayed by the copper-based 660 nm assay (ThermoFisher Scientific, Waltham, MA). Lipid raft enrichment and purity were determined for cytosolic GAPDH, lipid raft marker flotillin1, and nuclear marker H3.

Nuclear Isolation 20 mg frozen brain was homogenized in 125uL of sucrose buffer for nuclear and cytosolic fractions . The protocol[92] was modified for brain tissue by adding 1% NP-40 to the first wash step, resuspending the pellet, and incubating on ice for 30 min. Nuclear and cytosolic extractions were cross probed for purity against both H3 and GAPDH by Western blot.

Western Blot Proteins from lipid rafts (5 ug), whole cell lysate (20ug), or nuclear fractions (20 ug) were boiled at 75°C under denatured conditions and resolved on 4-20% gradient gels. Proteins were electroblotted using a Criterion blotter (Bio-Rad Laboratories, Hercules, CA) and transferred onto 0.45um polyvinyl difluoride membranes. Membranes were stained using Revert 700 fluorescent protein stain and imaged prior to blocking with LI-COR Intercept blocking buffer (LI-COR Biosciences, Lincoln, NE), followed by primary antibodies. Membranes incubated with IRDye 800CW and/or 700CW secondary antibodies and visualized by Odyssey (LI-COR Biosciences). Western blot data was quantified with ImageJ and normalized by total protein per lane and/or loading control protein.

Biochemical Assays Lipid rafts were assayed for total cholesterol (Cell Biolabs, San Diego, CA). Tissue hemoglobin or heme quantified by Quantichrom assay (BioAssay Systems, Hayward, CA). α-Hemoglobin was quantified by ELISA (ThermoFisher Scientific, Waltham, MA).

ICP-MS 50mg of brain tissue was cut with a ceramic scalpel and placed in metal free test tubes. Tissue was washed twice with PBS and homogenized with sterilized disposable plastic pestles in Chelex 100 (sigma, St. Louis, MO) with purified water (18.2 MΩ; Millipore, Burlington, MA). Homogenates were desiccated by vacuum centrifuge at 95°C for 90 min. and solubilized in trace metal free 70% HNO_3 overnight. 30% H_2O_2 was then added followed by drying under heat. After resuspension in 2% HNO_3, samples were analyzed by Agilent 7500ce ICP-MS in hydrogen mode with a practical detection limit of 10ppb and a relative standard deviation (RSD) of replicate measures between 0.2 to 5%. Total iron concentration was normalized to wet weight tissue.

Statistics GraphPad Prism 9 software (GraphPad, San Diego, CA) for t-test or one-way ANOVA with Tukey's HSD ($p<0.05$). Correlation matrixes used Spearman's correlation for non-linear data. Data that were not normally distributed were analyzed by Kruskal-Wallis with Dunn's test.

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