1. Introduction

Alzheimer’s disease (AD) is a progressive neurological disorder and is the most common form of dementia amongst older people. Although the precise mechanisms of AD pathogenesis are not completely understood, increasing evidence suggests AD pathology is driven by metabolic dysfunction in the brain (de la Monte and Tong, 2014). Recent studies have demonstrated that changes in the metabolic profiles of microglia, the primary innate immune cells of the central nervous system (CNS), are important in controlling their activation and effector functions (Keren-Shaul et al., 2017; Shippy and Ulland, 2020; Ulland et al., 2017). Furthermore, dietary regimens, such as the ketogenic diet, are being investigated as potential treatments for AD (Brownlow et al., 2013; Fortier et al., 2019; Kashiwaya et al., 2013; Shippy et al., 2020). Therefore, identifying genes involved in nutrient uptake and utilization by microglia could be valuable in further understanding AD pathogenesis.

Zinc is the second most abundant trace element found in the human body and is required by all living organisms. Zinc is used extensively in many biological processes, and alterations in zinc levels are implicated in the pathogenesis of numerous diseases, including AD. Since small fluctuations in brain zinc levels appear to affect AD progression, we investigated the zinc-related transcriptional responses in an AD versus non-AD state using microarray and RNA-sequencing (RNA-seq) datasets from cultured cells, mice, and humans. We identified 582 zinc-related differentially expressed genes (DEG) in human dorsolateral prefrontal cortex samples of late-onset AD (LOAD) versus non-AD controls, 146 zinc-related DEG in 5XFAD versus wild-type mice, and 95 zinc-related DEG in lipopolysaccharide (LPS)-stimulated N9 microglia versus unstimulated control cells, with 19 zinc-related DEG common to all three datasets. Of the 19 common DEG, functional enrichment and network analyses identified several biological processes and molecular functions, such as mRNA destabilization and nucleic acid binding, which may be important in neuroinflammation and AD development. Furthermore, therapeutic drugs targeting zinc-related DEG in the human dataset were identified. Taken together, these data provide insights into zinc utilization for gene transcription during AD progression which may further our understanding of AD pathogenesis and could identify new targets for therapeutic strategies targeted towards AD.
brain zinc levels appear to affect AD progression (Maret and Sandstead, 2006; Portbury and Adlard, 2017; Rivers-Auty et al., 2021), we investigated the zinc-related transcriptional responses in an AD versus non-AD state using microarray and RNA-sequencing (RNA-seq) datasets from cultured cells, mice, and humans. We identified 582 zinc-related differentially expressed genes (DEG) in human dorsolateral prefrontal cortex samples of late-onset AD (LOAD) versus non-AD controls, 146 zinc-related DEG in SXFAD versus wild-type mice, and 95 zinc-related DEG in lipopolysaccharide (LPS)-stimulated N9 microglia versus unstimulated control cells, with 19 zinc-related DEG common to all three datasets. Of the 19 DEG, functional enrichment and network analyses identified several biological processes, molecular functions, and gene interactions which may be important in AD development. Additionally, therapeutic drugs targeting zinc-related DEG in the human dataset were identified suggesting new targets for therapeutic interventions directed towards AD.

2. Materials and methods

2.1. N9 RNA-seq

The N9 microglial RNA-seq dataset was published previously by our group (GSE183038) (Shippy et al., 2022). Briefly, immortalized murine N9 microglia were cultured as previously described (Righi et al., 1989). N9 microglia were seeded at a cell density of 250,000 cells/well in a 24-well tissue culture plate. Cells were stimulated with LPS (1 µg/ml) from Escherichia coli O111:B4 (InvivoGen) for 6 h. Three biological assays with technical replicates (n = 3) were used. RNA was extracted using a RNeasy Plus Mini Kit (Qiagen, Cat. No. 74134). Quality and quantity of RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and a Nanodrop spectrophotometer (Thermo Scientific). All samples had an RNA integrity number (RIN) of 9.7 or higher. RNA library preparation and transcriptome sequencing were performed by Novogene using the Illumina NovaSeq 6000 Sequencing System. Bioinformatics analysis was performed by Novogene with differential expression analysis performed using the DESeq2 R package (1.20.0). Genes with false discover rate (FDR)-adjusted P-values < 0.05 and fold change (FC) > 1 were considered differentially expressed.

2.2. Mouse microarray

The mouse microarray has been published in a previous study (Wang et al., 2015) and the publicly available dataset (GSE65067) was used. Briefly, microglia from female 8 month old wild-type (n = 3) and SXFAD (n = 5) mice (The Jackson Laboratory) were FACs-sorted directly into RTL-plus lysis buffer. RNA extraction from microglia was performed using an RNAsy Plus Micro Kit (Qiagen, Cat. No. 74034). Microarray hybridization (Affymetrix MoGene 1.0 ST array) and data processing were performed at the Washington University Genome Center. Raw data were normalized using the Robust Multi-Array (RMA) method and genes were pre-filtered for expression value greater than or equal to 120 expression units. This method provides a cut-off above which genes have a 95% chance of expression demonstrated in Immgen dataset, which uses the same array platform (Wang et al., 2015). P-values were calculated using a Student’s t-test and FDR-adjusted P-values were calculated using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Genes with FDR-adjusted P-values < 0.05 and FC > 1 were considered differentially expressed.

2.3. Human microarray

To better understand the role of zinc in AD pathogenesis, we examined DEG in a large human cohort studying AD using publicly available data (GSE44770) (Zhang et al., 2013). The Harvard Brain Tissue Resource Center cohort consists of 230 human dorsolateral prefrontal cortex samples which were separated by clinical diagnosis of LOAD (n = 129) and non-AD controls (n = 101). Postmortem pathological examination was used to confirm the clinical diagnosis (Zhang et al., 2013). LOAD versus non-AD control DEG were identified using GEO2R. Genes with FDR-adjusted P-values < 0.05 and FC > 1 were considered differentially expressed.

2.4. Gene analyses

The 19 zinc-related DEG common to all three datasets were selected for biological function analysis. The gene list was uploaded into the Database for Annotation, Visualization and Integrated Discovery (DAVID, v. 6.8) (Huang da et al., 2009a, 2009b) for Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) gene ontology (GO) analyses. Each GO with a P-value ≤ 0.05 was considered significant. Gene-drug interactions of the zinc-related DEG in the human dataset were identified using the drug-gene interaction database (DGIdb) (Cotto et al., 2018; Freshour et al., 2021) using the default settings.

Scatterplots were created using Prism 9.0.0 (GraphPad). Venn diagrams demonstrating overlap in zinc-related DEG amongst the three datasets were generated using InteractiVenn (Heberle et al., 2015). Gene constellations identifying genes in the Zc3h12a regulatory network were created with ImmGen (Heng et al., 2008) using the myeloid cells reference populations option.

3. Results

3.1. AD promotes differential expression of zinc-related genes

Analysis of publicly available RNA-seq data from LPS-stimulated N9 microglia versus non-stimulated control cells revealed a total of 95 DEG with zinc-related functions (FC > 1, FDR-adjusted P-value < 0.05). Of these 95 DEG, 59 were up-regulated and 36 were down-regulated (Fig. 1A). Zinc finger CCCH type containing 12 A (Zc3h12a) was the most up-regulated zinc-related gene (FC = 11.78) and zinc finger protein 395 (Zfp395) was the most down-regulated zinc-related gene (FC = −4.51).

We identified 146 zinc-related DEG (FC > 1, P < 0.05) in publicly available transcriptional data from sorted microglia from 8-month-old SXFAD mice, a mouse model of AD which accumulates Aβ plaques (Oakley et al., 2006), versus wild-type mice (Wang et al., 2015). Of the 146 zinc-related DEG 18 were up-regulated and 128 were down-regulated (Fig. 1B). Zinc finger, NFX1-type containing 1 (Znfx1) was the most upregulated zinc gene (FC = 2.43) and solute carrier family 39 (zinc transporter), member 12 (Slc39a12) was the most down-regulated zinc gene (FC = −2.87).

Finally, we analyzed publicly available microarray data of human dorsolateral prefrontal cortex samples from LOAD versus non-AD controls (Zhang et al., 2013). A total of 582 zinc-related DEG (FC > 1, FDR-adjusted P-value < 0.05) were identified with 314 up-regulated and 268 down-regulated zinc-related genes (Fig. 1C). Solute carrier family 39 (zinc transporter), member 12 (Slc39a12) was the most up-regulated zinc-related gene (FC = 1.28) and zinc finger, CCHC domain containing 12 (Zcchc12) was the most down-regulated zinc-related gene (FC = −1.31).

In total, 19 zinc-related DEG overlapped between the three datasets (Fig. 1D). A complete list of the 19 zinc-related genes and their fold change values for all three datasets is shown in Table 1. A complete list of all the zinc-related DEG found in all three datasets is shown in Supplementary Table 1.

3.2. Enrichment analysis of altered zinc-related genes

In order to further understand the potential biological relevance of the zinc-related DEG in AD, GO analyses were performed on the 19 DEG common to the three datasets. BP GO indicated the zinc-related DEG...
participated in regulation of transcription, DNA-templated (Cas1, Zbtb43, Zfp287, Zfp467, Zfp64, Zfp703, Znfx1, Zhx3), transcription, DNA-templated (Cas1, Zbtb43, Zfp287, Zfp467, Zfp64, Zfp703, Zhx3) 3’-UTR-mediated mRNA destabilization (Zc3h12a, Zfp36), negative regulation of transcription, DNA-templated (Zbtb38, Zfp703, Zfp706, Zhx3), positive regulation of mRNA catabolic process (Zc3h12a, Zfp36), positive regulation of fat cell differentiation (Zc3h12a, Zfp36), and multicellular organism development (Cas1, Zc3h12a, Zfp64, Znym3) (Fig. 2A). CC GO indicated the zinc-related DEG were located in the nucleus (Cas1, Zc3h12a, Zbtb38, Zbtb43, Zfp287, Zfp36, Zfp395, Zfp467, Zfp64, Zfp703, Zfp706, Zfand2a, Znym3, Znfx1, Zhx3) (Fig. 2B). MF GO indicated the zinc-related DEG were involved in metal ion binding (Cas1, Zc3h12a, Zc3h12c, Zbtb38, Zbtb43, Zfp287, Zfp36, Zfp395, Zfp467, Zfp608, Zfp64, Zfp644, Zfp703, Zfp706, Zfand2a, Znym3, Znfx1, Zhx3), DNA binding (Cas1, Zc3h12a, Zbtb43, Zfp287, Zfp36, Zfp395, Zfp467, Zfp64, Znym3, Zhx3), nucleic acid binding (Zbtb43, Zfp287, Zfp467, Zfp64, Zfp644, Zfp703), and mRNA 3’UTR AU-rich region binding (Zc3h12a, Zfp36) (Fig. 2C).

Since Zc3h12a was a common zinc-related DEG in the three datasets, and involved in many of the biological processes described above, expression network analysis was performed to identify genes positively and negatively correlated with Zc3h12a. In the positive correlation map, several genes involved in inflammation including Stat1 (0.744), Eif2ak2 (0.780), and Jbj214 (0.834) were positively correlated with Zc3h12a (Fig. 3A). In the negative correlation map, several genes encoding zinc finger proteins including Zfp958 (~0.738) and Zfp322a (~0.770) were negatively correlated with Zc3h12a (Fig. 3B).

In order to determine zinc-related gene targets for therapeutic drugs, we performed gene-drug interactions in DGIdb (Cotto et al., 2018; Freshour et al., 2021) using the 582 zinc-related DEG in the human dataset. Initially, the analysis was performed on the 19 zinc-related DEG common to all three datasets, but no drugs were found to target these genes. For the human dataset, a total of 19 zinc-related DEG had interactions with therapeutic drugs (Fig. 4). Bromodomain adjacent to zinc finger domain, 2B (Baz2b) had the most interactions (343 drugs). Of the 343 drugs, some of the drugs are used to treat hypertension (benzthiazide, methyldopa, reserpine, nifedipine), depression (mianserin hydrochloride), and Parkinson’s disease (remacemide hydrochloride). Idebenone was also identified as interacting with Baz2b, and this drug was originally developed for AD and other cognitive impairments, and is now under investigation as a treatment for neuromuscular diseases. A complete list of the zinc-related DEG and their associated drugs is shown in Supplementary Table 2.

4. Discussion

Using cell culture, mouse, and human gene expression datasets, we identified 19 common zinc-related DEG which may be important in AD disease processes. Several previous studies report lower zinc serum levels in AD patients (Li et al., 2017; Ventriglia et al., 2015). Other evidence suggests excessive zinc supplementation could lead to AD progression by enhancing amyloid (Flinn et al., 2014) and tau (Craven et al., 2016) pathology. Based on this evidence, it appears zinc accumulation in the brain is a tightly controlled process, with small fluctuations in zinc concentrations having large effects on disease status and other biological processes (Maret and Sandstead, 2006; Portbury and Adlard, 2017; Rivers-Auty et al., 2021). Therefore, the identification of genes involved in zinc transport and utilization could be important in further understanding AD pathogenesis.

Neuroinflammation plays a major role in AD pathogenesis by exacerbating both amyloid and tau pathologies (Heppner et al., 2015). Zinc homeostasis is crucial for maintaining a healthy immune system, and zinc deficiency often elevates the inflammatory response (Gammoh and Rink, 2017). Activation of nuclear factor kappa-light-chain-enhancer of...
activated B cells (NF-κB) and the nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome are important mechanisms in chronic neuroinflammation that significantly increase AD pathology (Granic et al., 2009; Heneka et al., 2013). Zinc deficiency has been shown to exacerbate cognitive decline by enhancement of the NLRP3 inflammasome in a mouse model of AD (Bales et al., 1998; Rivers-Auty et al., 2001). In our study, nine genes encoding ZFP were differentially expressed, RNA packaging, and protein folding and assembly (Laity et al., 2021). In this study, tau mRNA was significantly reduced, leading to rescued neuronal damage around Aβ plaques in a mouse model of AD (Wegmann et al., 2021). Taken together, these studies support the continued investigation of zinc for potential therapies of AD.

AD drug development has been especially arduous, with approximately 99% of trials showing no difference between the treated and placebo groups (Cummings et al., 2017, 2014). To date, many of the therapeutic agents developed for AD have focused on amyloid and tau reduction (Congdon and Sigurdsson, 2018; Pinheiro and Faustino, 2019). In our study, gene-drug analysis of the altered zinc-related genes in the human dataset identified drugs targeting zinc-related DEG in our study. The 2azb gene had the most interactions (343 drugs) with several drugs used to treat hypertension. Hypertension is one of the most prevalent medical conditions with over 1.3 billion people diagnosed worldwide (Bloch, 2016). Several animal studies show a direct relationship with hypertension and neuroinflammation and amyloid deposition (Carnevale et al., 2012; Kruyer et al., 2015) while postmortem studies in humans have shown mid-life hypertension increased amyloid and neurofibrillary tangles (Petrovitch et al., 2000; Shah et al., 2012). Idebenone was also identified as a target of 2azb in our study. Idebenone was initially developed as an AD drug (Weyer et al., 1997), but is now being investigated for the neuromuscular diseases Friedreich’s ataxia and Duchenne muscular dystrophy (Artuch et al., 2002; Buyse et al., 2015). Overall, there is a clear need for the development of new drugs for AD, and these data could assist in the identification of new targets for therapeutic strategies directed towards AD.

As with most gene expression analyses, there are strengths and weaknesses associated with our study identifying zinc-related DEG in AD. A major advantage of our study is that common zinc-related DEG were identified from three distinct AD datasets (cell culture, mouse, and human) which strengthens the probability of zinc being involved in AD progression. In contrast, most of the zinc-related DEG, especially in the human dataset, were not greatly altered. Furthermore, there was no strong consensus amongst the 19 zinc-related DEG common to the three datasets, as only eight of the 19 showed the same directional change in expression. This could possibly be due to the tightly controlled regulation of zinc in the human brain, making it plausible that small alterations in the zinc-related genes could lead to large biological effects. Also, although not in the N9 cell culture dataset, and thus not in the list of common zinc-related DEG, Slc39a12 was the most down-regulated gene in the mouse dataset, while being the most up-regulated gene in the human dataset. Differences in collection methods between the two studies could be a possible explanation for the contrasting results, as the mouse dataset is from RNA isolated from microglia, and the human dataset is from RNA isolated from dorsolateral prefrontal cortex samples. A previous study, however, indicates humans have higher densities of microglia in the dorsolateral prefrontal cortex during AD (Edler et al., 2018), and the alteration of Slc39a12 in the human dataset used in our study is similar to a study using single-cell RNA-seq from a microglia cluster in human dorsolateral prefrontal cortex samples (Zhou et al., 2020). Overall, studies comparing the transcriptional signature of microglia in AD in mice and humans have been mixed, with some indicating that the response may be similar (Sobue et al., 2021; Ulland et al., 2017) and some studies indicating that the response may be different (Olah et al., 2020; Zhou et al., 2020). These results are anticipated, as human disease consists of both neurofibrillary tangles and Aβ plaques compared to the 5XFAD mouse model in which only plaques are aperted, as human disease consists of both neurofibrillary tangles and Aβ plaques compared to the 5XFAD mouse model in which only plaques are aperted. Therefore, it is important to consider all models of AD when identifying gene network mechanisms in AD progression.

### 5. Conclusions

Overall, we used publicly available datasets to identify 582 zinc-related DEG in human dorsolateral prefrontal cortex samples of LOAD versus non-AD controls, 146 zinc-related DEG in 5XFAD versus wild-type mice, and 95 zinc-related DEG in LPS-stimulated N9 microglia.
versus unstimulated control cells, with 19 zinc-related DEG common to all three datasets. Of the 19 zinc-related DEG, functional enrichment and network analyses identified several biological processes, molecular functions, and gene interactions which may be important in AD development. Furthermore, therapeutic drugs targeting zinc-related DEG in the human dataset were identified. Taken together, these data provide insights into zinc utilization during AD progression which may further our understanding of AD pathogenesis and could identify new targets for therapeutic strategies for AD.

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**CRediT author contribution statement**

Daniel C. Shippy: Conceptualization, Investigation, Data curation, Formal analysis, Methodology, Writing – original draft. Tyler K.
Ulland: Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Conflicts of interest

None.

Data Availability

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus ( GEO ) repository, GSE65067, GSE183038, and GSE44770.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jibnre.2022.06.002.

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D.C. Shippy and T.K. Ulland

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