ORIGINAL ARTICLE

Systematic review of human post-mortem immunohistochemical studies and bioinformatics analyses unveil the complexity of astrocyte reaction in Alzheimer's disease

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Abstract

Aims: Reactive astrocytes in Alzheimer's disease (AD) have traditionally been demonstrated by increased glial fibrillary acidic protein (GFAP) immunoreactivity; however, astrocyte reaction is a complex and heterogeneous phenomenon involving multiple astrocyte functions beyond cytoskeletal remodelling. To better understand astrocyte reaction in AD, we conducted a systematic review of astrocyte immunohistochemical studies in post-mortem AD brains followed by bioinformatics analyses on the extracted reactive astrocyte markers.

Methods: NCBI PubMed, APA PsycInfo and WoS-SCIE databases were interrogated for original English research articles with the search terms 'Alzheimer's disease' AND 'astrocytes.' Bioinformatics analyses included protein–protein interaction network analysis, pathway enrichment, and transcription factor enrichment, as well as comparison with public human -omics datasets.

Results: A total of 306 articles meeting eligibility criteria rendered 196 proteins, most of which were reported to be upregulated in AD vs control brains. Besides cytoskeletal remodelling (e.g., GFAP), bioinformatics analyses revealed a wide range of functional alterations including neuroinflammation (e.g., IL6, MAPK1/3/8 and TNF), oxidative stress and antioxidant defence (e.g., MT1A/2A, NFE2L2, NOS1/2/3, PRDX6 and SOD1/2), lipid metabolism (e.g., APOE, CLU and LRP1), proteostasis (e.g., cathepsins, CRYAB and HSPB1/2/6/8), extracellular matrix organisation (e.g., CD44, MMP1/3 and SERPINA3), and neurotransmission (e.g., CHRNA7, GABA, GLUL, GRM5, MAOB and SLC1A2), among others. CTCF and ESR1 emerged as potential transcription factors driving these changes. Comparison with published -omics datasets validated our results, demonstrating a significant overlap with reported transcriptomic and proteomic changes in AD brains and/or CSF.

Lucía Viejo and Ayush Noori contributed equally to this work.

Conclusions: Our systematic review of the neuropathological literature reveals the complexity of AD reactive astrogliosis. We have shared these findings as an online resource available at www.astrocyteatlas.org.

INTRODUCTION

Astrocytes are known to undergo profound morphological and functional changes in central nervous system diseases, collectively termed astrocyte reaction or reactive astrogliosis [1]. This astrocyte reaction has traditionally been depicted by an increased immunoreactivity for the cytoskeletal intermediate filament glial fibrillary acidic protein (GFAP). However, in recent years, transcriptomic studies of acutely isolated astrocytes or single nuclei from mouse models and post-mortem human brains have revealed that astrocyte reaction is heterogeneous, context-dependent (e.g., different in acute injuries vs chronic neurodegenerative diseases) [1, 2] and complex beyond cytoskeletal rearrangement (reviewed in previous studies [3–6]). Unfortunately, transcriptomic and proteomic approaches in the Alzheimer's disease (AD) brain are limited by lack of spatial information, which is relevant as there are layer-specific subtypes of astrocytes [7] and reactive (GFAP$^+$) astrocytes tend to localise near both dense-core neuritic amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) [8–10]. While ongoing efforts to develop spatial -omics methods will eventually overcome this constraint [11, 12], immunohistochemistry remains the gold-standard technique to capture spatial expression patterns of astrocytes in post-mortem tissue sections.

Here, we conducted a systematic review of post-mortem human brain neuropathological immunohistochemical studies describing potential markers of AD reactive astrocytes (ADRA). We hypothesised that compiling the neuropathological literature could provide a catalogue of dysregulated proteins in ADRA around plaques and tangles, shed light on the complexity of their associated functional changes, and inform the development of fluid (CSF and plasma/serum) and positron emission tomography (PET) imaging biomarkers to detect ADRA in patients.

MATERIAL AND METHODS

Systematic review

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [13] as explained in detail in the Supporting Information [14, 15].

Bioinformatics analyses

Additional details regarding the bioinformatics analyses can be found in the Supporting Information.

Key points

- This is a systematic review of human post-mortem immunohistochemical studies followed by bioinformatics analyses to define the functional changes of reactive astrocytes in Alzheimer's disease (AD).
- A total of 306 eligible articles rendered 196 markers of AD reactive astrocytes (ADRA), implicating inflammation, oxidative stress, lipid metabolism, proteostasis, extracellular matrix remodelling, neuromodulation and blood–brain barrier integrity, among other alterations.
- This ADRA protein set is catalogued in a new online resource available at www.astrocyteatlas.org.

Pathway enrichment analysis

We first classified the astrocyte proteins into one of 18 functional categories based on published evidence. The interconnectivity between these assigned functions was demonstrated via chord and Circos plots. In addition, to validate these functional pathways, we performed pathway enrichment analysis (PEA) on the ADRA markers against the Gene Ontology (GO) and Reactome databases available from the Molecular Signatures Database (MSigDB) [16–19].

Protein-protein interaction network analysis

To examine direct (physical) and indirect (functional) interactions between the ADRA protein set, a protein–protein interaction (PPI) network was constructed via the application programming interface (API) to the STRING biological database of known and predicted PPIs (version 11.0, *Homo sapiens*) [20].

Transcription factor enrichment analysis

We identified transcription factors potentially regulating the expression of the ADRA markers with two analogous tools: TFEA.ChIP [21] and Enrichr [22]. While sequence-based tools using position weight

matrices to predict transcription factor binding sites proximal to genes of interest have suboptimal specificity, TFEA.ChIP and Enrichr compute enrichment analysis based on databases of publicly available chromatin immunoprecipitation sequencing (ChIP-seq) experiments [23–26].

Comparison with transcriptomic and proteomic studies

To corroborate the results of our systematic review of AD neuropathological post-mortem studies, we compared the ADRA protein set with recent transcriptomic and proteomic studies on human control and AD brains [27–29]. First, enrichment analysis against differentially expressed genes or proteins (between control and AD individuals) was conducted with Fisher's exact test. Next, we created heatmaps to illustrate the expression levels of the overlapping transcripts and proteins.

RESULTS

Summary of systematic review results

Figure 1 depicts the PRISMA flowchart with the number of articles obtained after each step (i.e., identification, screening, eligibility and inclusion). A total of 306 articles were ultimately included in the systematic review and reviewed by either LV or AS-P to extract relevant data. Across these 306 articles, a total of 196 proteins were identified, which we refer to as the ADRA protein set. Table 1 summarises these 196 markers by functional category; a more detailed description of the neuropathological studies included in this systematic review is provided in Table S1.

Functional characterisation of markers of astrocyte reaction

As expected, an increase in GFAP immunoreactivity was the most frequently described hallmark of astrocyte reaction, even without considering studies that only used GFAP to colocalise their marker of interest with reactive astrocytes. However, the ADRA protein set revealed many other functional changes beyond cytoskeletal rearrangement, which we review below.

Cytoskeletal remodelling

Of note, while GFAP isoform 1 (full length) is the main isoform in the brain, less abundant splice forms [30, 31] and caspase-3-cleaved GFAP fragments [32] have also been reported to increase in ADRA. Additionally, the cytoskeletal remodelling that reactive astrocytes undergo in AD involves the upregulation of other intermediate filaments proteins such as vimentin (VIM) [31, 33]

and nestin (NES) [31, 34], as well as actin-interacting proteins such as the 280-kDa actin-binding protein filamin-A (FLNA) [35] and ankyrin-1 (ANK1) [36].

Inflammation

Multiple studies report increased immunoreactivity for a wide variety of inflammatory cytokines, including the inflammasome-activating enzyme caspase-1 (CASP1) [37]; interleukins such as interleukin-1 beta (IL1B) [37, 38], interleukin-6 (IL6) [37, 39, 40], interleukin-18 (IL18) [41], interleukin-33 (IL33) [42] and its receptor interleukin-1 receptor-like 1 (IL1RL1) [42], as well as tumour necrosis factor (TNF) [43]; chemokines such as C–C motif chemokine ligand 2 (CCL2) [40], C–C motif chemokine ligand 4 (CCL4) [44], C–X–C motif chemokine ligand 10 (CXCL10) [45] and stromal cell-derived factor 1 (CXCL12) [46]; cell adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) [47]; eicosanoid metabolism enzymes such as prostaglandin G/H synthase 2 (PTGS2, also known as cyclooxygenase-2) [48], cytosolic phospholipase A2 (PLA2G4A) [49] and haematopoietic prostaglandin D synthase (HPGDS) [50] (while the microsomal prostaglandin E synthase (PTGES) was reported to be decreased in ADRA [51]); and the immunomodulatory receptor sphingosine 1-phosphate receptor 3 (S1PR3) [52] but not sphingosine 1-phosphate receptor 1 (S1PR1) [53]. In addition, some studies have shown increased immunoreactivity for transcription factors known to mediate immune and inflammatory responses such as nuclear factor of activated T-cells, cytoplasmic 2 (NFATC2) [54]; nuclear factor of activated T-cells, cytoplasmic 4 (NFATC4) [54]; transcription factor p65 (RELA, also known as nuclear factor NF-kappa-B p65 subunit) [55, 56]; adipocyte enhancer-binding protein 1 (AEBP1) [57]; CCAAT/enhancer-binding protein delta (CEBPD) [58]; and glia maturation factor (e.g., GMFB) [59, 60]. Lastly, reactive astrocytes also express chitinase-3-like protein 1 (CHI3L1, also known as YKL-40) [61–64] and the 18 kDa translocator protein (TSPO) [65, 66]; although their function remains to be fully elucidated, these two proteins are commonly interpreted as evidence of inflammation when measured in CSF and detected via PET imaging, respectively.

Oxidative stress

Numerous studies implicate ADRA in oxidative stress, based on an increased immunoreactivity for advanced glycation end-products (AGEs) [67–69] and the AGE-specific receptor (AGER, also known as RAGE) [69, 70]; DNA [71–73] and protein [74] oxidative damage markers; and pro-oxidant enzymes such as myeloperoxidase (MPO) [75] and the three nitric oxide synthase isoforms: brain (NOS1) [76], inducible (NOS2) [43, 77] and endothelial (NOS3) [78]. In addition, ADRA exhibit decreased immunoreactivity for nuclear factor erythroid 2-related factor 2 (NFE2L2) [79]—the main transcription factor orchestrating the antioxidant response—and for solute carrier family 40 member 1 (SLC40A1, also known as
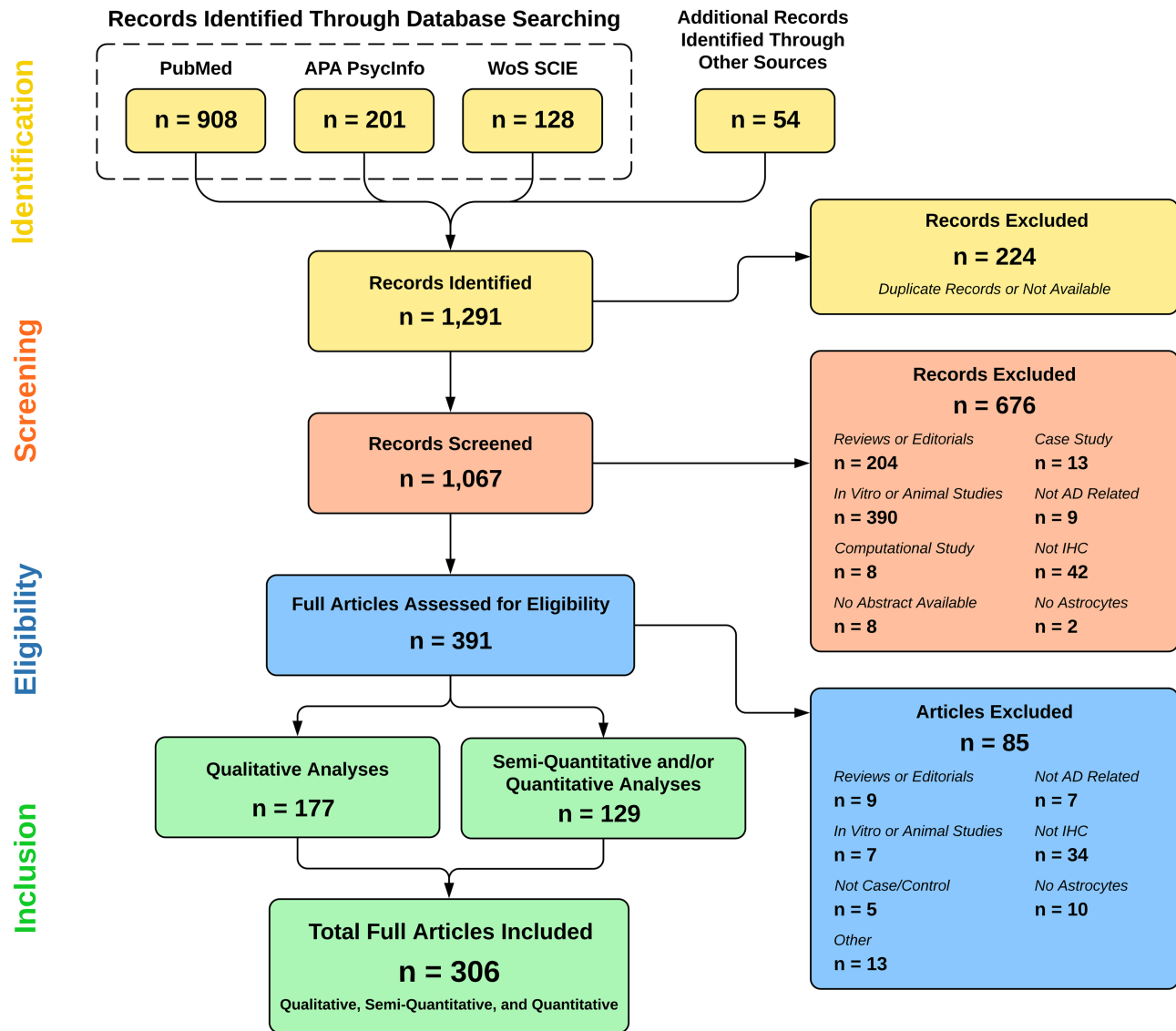
FIGURE 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart of the systematic review. The PubMed, APA PsycInfo and Web of Science-Science Citation Index Expanded (WoS-SCIE) databases were queried with the search terms 'Alzheimer's disease' AND 'astrocytes,' yielding 1237 records, plus 54 additional records identified by scanning reference lists. The titles and abstracts of the 1067 unique records were screened. Of these, 391 were assessed for eligibility based on prespecified inclusion and exclusion criteria. Finally, a total of 306 original articles were thoroughly reviewed to extract relevant information, including markers of Alzheimer's disease reactive astrocytes (ADRA)

ferroportin-1) and hepcidin (HAMP) [80], both of which regulate iron homeostasis. Conversely, there is also strong evidence for a role of ADRA in antioxidant defence, including increased immunoreactivity for antioxidant enzymes such as the metallothioneins -1 (e.g., MT1A), -2 (MT2A) and, to a lesser extent, -3 (MT3, also known as growth inhibitory factor or GIF) [73, 81–85], which are also important for zinc and copper homeostasis; the superoxide dismutases [Cu-Zn] (SOD1) and [Mn], mitochondrial (SOD2) [86, 87]; heme oxygenase 1 (HMOX1) [88]; thioredoxin (TXN) [89]; peroxiredoxin-6 (PRDX6) [90]; epoxide hydrolase 1 (EPHX1) [91]; and aflatoxin B1 aldehyde reductase member 2 (AKR7A2) [92].

Lipid metabolism

The lipid metabolism markers found in our systematic review can be classified as (1) apolipoproteins, which bind and transport cholesterol and phospholipids packed as lipoproteins; (2) lipoprotein receptors, which internalise these via endocytosis; and (3) enzymes that metabolise lipids. As expected, the most studied apolipoprotein is apolipoprotein E (APOE), followed by clusterin (CLU, also known as APOJ). Most studies investigating APOE report increased expression in ADRA [93–98], but some authors have described reduced expression restricted to the vicinity of amyloid plaques [99, 100] or unchanged

TABLE 1 Functional categorisation of 196 ADRA proteins resulting from the systematic review

Functional category	Protein markers	Count
Aβ metabolism	APP, BACE1, CAV3, IDE, MME, PSEN1, PSEN2, SHC1, YY1	9
Blood–brain barrier	CLDN11, CLDN2, CLDN5, EDN1, FGA, IGHA1, IGHG1, IGHM	8
Calcium homeostasis	CALB1, CALB2, KCNIP3, S100A6, S100B	5
Cytoskeleton	ANK1, FLNA, GFAP, NES, VIM	5
Extracellular matrix	A2M, CD44, HSPG2, ITIH1, LOX, MMP1, MMP3, NRG1, PLAU, SERPINA3, SERPINE1, TGM1, TGM2	13
Inflammation	AEBP1, CASP1, CCL2, CCL4, CEBPD, CHI3L1, CXCL10, CXCL12, GMFB, HPGDS, ICAM1, IL18, IL1B, IL1RL1, IL33, IL6, NFATC2, NFATC4, PLA2G4A, PTGES, PTGS2, RELA, S1PR1, S1PR3, TNF, TSPO	26
Insulin signalling	AMY2A, IGF1, IGF1R, IGF2R, IGFBP3	5
Intracellular trafficking	CLTA, CLTB, CPE, HOOK2, KIF21B, SCG3	6
Kinase/phosphatase	CSNK2A1, FYN, GSK3B, MAPK1, MAPK14, MAPK3, MAPK8, PPP1CA, PPP2CA, PPP3CA, PRKDC	11
Lipid metabolism	APOA1, APOC1, APOD, APOE, CETP, CLU, CYP46A1, LDLR, LRP1	9
Miscellaneous	ALDH1L1, ESR1, ESR2, FKBP1A, MAPT, PADI2	6
Neurotransmission	ADORA2A, CHAT, CHRM1, CHRNA7, DRD3, DRD5, GAD1, GLUL, GRM5, HTR2A, IDO1, MAOB, SLC1A2, SLC1A3, SLC6A11, SRR	16
Oxidative stress	AGER, AKR7A2, EPHX1, H2AX, HAMP, HMOX1, MPO, MT1A, MT2A, MT3, NFE2L2, NOS1, NOS2, NOS3, PRDX6, SLC40A1, SOD1, SOD2, TXN	19
Phagocytosis	C3, CR1, MFGE8, SCARB1	4
Proliferation/apoptosis	APC, BCL2, CASP3, CCNC, CDK1, CDK7, CDK8, CDKN2A, DCX, E2F1, EEF1A2, ETS2, FAS, FASLG, FOXO3, GLB1, MYC, MYCN, PTEN, RB1, RBL2, TP53	22
Proteostasis	BECN1, CAPN10, CD68, CRYAB, CTSB, CTSD, CTSH, CTSL, HEXA, HSPB1, HSPB2, HSPB6, HSPB8, PARK7, PREP, PRKN, SYVN1	17
Trophic factors	EGR1, FGF1, FGF2, FGFR1, GAP43, HGF, HGFAC, PEA15, TGFB2, TGFB3, TGFBR2	11
Water/K^+ homeostasis	AQP1, AQP4, KCNJ11, KCNN4	4

Note: The 196 AD reactive astrocyte (ADRA) proteins were classified into one of 18 functional categories based on published evidence. The constituent proteins of each functional category are shown here.

Abbreviation: ADRA, Alzheimer's disease reactive astrocyte.

[101] expression compared with normal brain astrocytes. In general, all other apolipoproteins including CLU [102, 103] and the apolipoproteins A-I (APOA1) [98], C-I (APOC1) [104] and D (APOD) [105] have been shown to increase in ADRA. Among the lipoprotein receptors, low-density lipoprotein (LDL) receptor-related protein 1 (LRP1) is the most frequently studied and is increased in ADRA according to the majority of studies [39, 95, 106, 107] (but unchanged in the basal ganglia [101]). In contrast, the LDL receptor (LDLR) is unchanged in ADRA vs normal brain astrocytes [95]. Finally, two cholesterol enzymes, cholesteryl ester transfer protein (CETP) [108] and cholesterol 24-hydroxylase (CYP46A1) [109, 110], have been shown to be increased in ADRA, while two immunohistochemical studies have shown accumulation of ceramide in ADRA [52, 111].

for secreted proteases such as the matrix metalloproteinases interstitial collagenase (MMP1) [112] and stromelysin-1 (MMP3) [113] as well as urokinase-type plasminogen activator (PLAU) [107]; protease inhibitors such as alpha-1-antichymotrypsin (SERPINA3, also known as ACT) [114–116], the inter-alpha-trypsin inhibitors (e.g., ITIH1) [117] and plasminogen activator inhibitor 1 (SERPINE1) [107]; protein-lysine 6-oxidase (LOX), which oxidises ECM proteins [118, 119]; the protein-glutamine gamma-glutamyltransferases K (TGM1) and 2 (TGM2), which crosslink ECM proteins [120, 121] (but are unchanged in ADRA based on [122]); and cell surface and extracellular matrix adhesion receptors and ligands such as CD44 antigen (CD44) [64, 123, 124], the heparan sulphate proteoglycans (e.g., HSPG2) [125], neuregulin 1 (NRG1) [126, 127] and the ganglioside GM1 [128].

Extracellular matrix

Reactive astrocytes in AD also play an essential role in reorganisation of the extracellular matrix, as judged by their increased immunoreactivity

Proteostasis

Evidence for an activation of protein degradation systems in ADRA is indicated by increased immunoreactivity for lysosomal enzymes such

as the cathepsins B (CTSB) [129], D (CTSD) [94, 129, 130], H (CTSH) [130] and L (CTSL) [130], beta-hexosaminidase subunit alpha (HEXA) [130] and lysosomal membrane proteins including macrosialin (CD68) [131] and Beclin-1 (BECN1) [132]; small chaperones such as alpha-crystallin B chain (CRYAB) [133–135], the heat shock proteins beta-1 (HSPB1, also known as HSP27) [134–136], beta-2 (HSPB2) [134, 135], beta-6 (HSPB6) [134, 135], beta-8 (HSPB8) [135] and Parkinson disease protein 7 (PARK7, also known as DJ-1) [137]; the E3 ubiquitin-protein ligases Parkin (PRKN) [138] and synoviolin (SYVN1, also known as HRD1) [139]; and proteases such as calpain-10 (CAPN10) [140] and prolyl endopeptidase (PREP) [141].

Neurotransmission

The fine processes from protoplasmic astrocytes are structural components of excitatory synapses and modulate glutamatergic transmission by taking up glutamate via the membrane-bound excitatory amino acid transporters 1 (SLC1A3, better known as EAAT1 or GLAST-1) and 2 (SLC1A2, better known as EAAT2 or GLT-1) and converting glutamate into glutamine via glutamine synthetase (GLUL). GLUL immunoreactivity has been reported to be increased [142, 143], decreased [144, 145] and unchanged [146] in ADRA, whereas SLC1A2 levels have more consistently been documented as reduced [147–149], and SLC1A3 levels appear to be stable [147, 148]. In addition, metabotropic glutamate transporter 5 (GRM5) [56, 150, 151] and serine racemase (SRR, which converts L-serine into D-serine, a gliotransmitter that modulates neuronal NMDA receptors) [152] are increased in ADRA. Besides glutamatergic neurotransmission, other neurotransmitter systems have also been associated with ADRA based on increased immunoreactivity for specific markers, including (1) GABAergic: γ-aminobutyric acid (GABA) [153, 154], the 67-kDa glutamate decarboxylase 1 (GAD1, also known as GAD-67) [153] and sodium- and chloride-dependent GABA transporter 3 (SLC6A11) [153]; (2) cholinergic: neuronal acetylcholine receptor subunit alpha-7 (CHRNA7) [155–159], choline O-acetyltransferase (CHAT) [158] and muscarinic receptors (e.g., CHRM1) [160]; (3) catecholaminergic: amine oxidase (flavin-containing) B (MAOB) [154] (but see Pugliese et al. [34] as well) and D(1B) dopamine receptor (DRD5) (but not D (3) dopamine receptor [DRD3], which is unchanged) [161]; (4) serotoninergic: 5-hydroxytryptamine receptor 2A (HTR2A) [162]; (5) kynurenergic: quinolinic acid and indoleamine 2,3-dioxygenase (IDO1) [163]; and (6) purinergic: adenosine receptor A2a (ADORA2A) [164].

Trophic factors

Although homeostatic astrocytes have traditionally been considered as ancillary cells providing trophic support to neurons for neuronal development and survival, remarkably, multiple trophic growth factors have been reported to increase in ADRA by immunohistochemistry. These trophic factors include hepatocyte growth factor (HGF) [165, 166] and its activator (HGFAC) [167]; fibroblast growth factors 1 (FGF1, also known as acidic FGF) [168, 169] and 2 (FGF2, also known as basic FGF) [170–173] and FGF receptor 1 (FGFR1) [174]; transforming growth factors beta-2 (TGFB2) [175–177] and beta-3 (TGFB3) [177]; and neuromodulin (GAP43) [178]. By contrast, TGF-beta receptor type-2 (TGFBR2) has been reported to be reduced in ADRA [177], whereas both early growth response protein 1 (EGR1) [179] and astrocytic phosphoprotein PEA-15 (PEA15) [180] are unchanged.

Proliferation and apoptosis

Several studies have suggested that ADRA are actively proliferating based on an increased immunoreactivity for proliferative markers including the proto-oncogenes apoptosis regulator Bcl-2 (BCL2) [181, 182], Myc (MYC) [183], N-myc (MYCN) [183] and protein C-ets-2 (ETS2) [184], as well as the cell cycle proteins cyclin C (CCNC) [185] and the cyclin-dependent kinases -1 (CDK1) [186] (although CDK1 is unchanged according to [187]), -7 (CDK7) [185] and -8 (CDK8) [185]. Conversely, multiple studies argue against ADRA proliferation based on increased immunoreactivity for tumour suppressors such as cellular tumour antigen p53 (TP53) [188] (although unchanged based on [72]), the hyperphosphorylated form of retinoblastoma-associated protein (RB1, also known as pRb) [189], retinoblastoma-like protein 2 (RBL2, also known as p130) [190], adenomatous polyposis coli protein (APC) [191], transcription factor E2F1 (E2F1) [189], Forkhead box protein O3 (FOXO3) [143], phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (PTEN) [192] and cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16^{INK4a}) [112, 193], together with decreased immunoreactivity for the microtubule-associated neuronal migration protein doublecortin (DCX) [194]. Lastly, ADRA apoptosis has been suggested due to an increased immunoreactivity for activated caspase-3 (CASP3) [32, 195] (but unchanged according to Simpson et al. [72]), caspase-cleaved actin (ACTB, where the caspase-cleaved form is known as fragment of actin, or fractin) [72] and caspase-cleaved GFAP [32], as well as the death receptor TNF receptor superfamily member 6 (FAS) [188, 196, 197] and its ligand, TNF ligand superfamily member 6 (FASLG) [197]. Similarly, increased DNA fragmentation has been shown using dUTP nick-end labelling by many authors [131, 182, 197, 198] but not others [199].

Kinase/phosphatase activity

ADRA have been shown to upregulate multiple kinases and phosphatases. Among these kinases, besides the cyclin-dependent kinases described above, there is also evidence of increased immunoreactivity for the mitogen-activated protein kinases 1 (MAPK1, also called extracellular signal-regulated kinase 2, or ERK2) [200] (but unchanged based on Webster et al. [201]), 3 (MAKP3, also called ERK1) [200, 201], 8 (MAPK8, also called SAPK1c or JNK1) [200] and 14 (MAPK14, also called p38) [200]; glycogen synthase kinase-3 beta

(GSK3B) [200]; casein kinase II (e.g., CSNK2A1, also known as CK II) [202]; and tyrosine-protein kinase Fyn (FYN) [203], whereas DNA-dependent protein kinase (PRKDC) is unchanged [72]. Regarding phosphatases, besides PTEN listed as a tumour suppressor above, there is evidence of increased immunoreactivity for the serine/threonine-protein phosphatases 2A (PPP2CA) [204] and 2B (PPP3CA, also known as calcineurin) [56, 204–206], whereas serine/threonine-protein phosphatase PP1-alpha (PPP1CA) remains unchanged [204].

Insulin signalling

ADRA appear to mobilise their energy metabolism, as indicated by an increased immunoreactivity for the limiting enzyme in glycogenolysis, pancreatic alpha-amylase (AMY2A, also called amylase alpha 2A) [207]. In addition, ADRA exhibit increased immunoreactivity for insulin-like growth factor I (IGF1) [208, 209] and its receptor (IGF1R) [210], as well as for cation-independent mannose-6-phosphate receptor (IGFR2, which is the receptor for insulin-like growth factor II) [211] and for insulin-like growth factor-binding protein 3 (IGFBP3) [205].

Intracellular trafficking

ADRA feature active intracellular trafficking, as assessed by an increased immunoreactivity for clathrin light chains A (CLTA) and B (CLTB) [212], which are critical for clathrin-mediated endocytosis; protein Hook homolog 2 (HOOK2), which is a microtubule-binding protein that participates in endosomal transport [213]; kinesin-like protein KIF21B (KIF21B) [214], a motor protein that transports cargo along microtubules; carboxypeptidase E (CPE) [215], which functions as a sorting receptor for processing of pro-peptides and secretion of the resulting peptides via the regulated secretory pathway; and secretogranin-3 (SCG3) [215], which is also involved in the sorting of peptides within secretory granules in the regulated secretory pathway.

Blood–brain barrier integrity

Astrocytes are a key structural element of the blood–brain barrier, with their vascular endfeet wrapping capillaries as well as small arteries and veins. ADRA have increased levels of the vasoconstrictor endothelin-1 (EDN1) [216, 217] and of the tight junction proteins claudin-2 (CLDN2) and claudin-11 (CLDN11), but not claudin-5 (CLDN5) [218]. Moreover, ADRA surrounding leaky capillaries take up plasma proteins such as fibrinogen (e.g., FGA) [219] and immunoglobulins A (e.g., IGHA1), G (e.g., IGHG1) and M (e.g., IGHM) [220].

Calcium homeostasis

Increased immunoreactivity for cytosolic calcium (Ca^{2+})-binding proteins, which buffer any excess of Ca^{2+}, provides indirect evidence of Ca^{2+} dyshomeostasis in ADRA. Indeed, protein S100-A6 (S100A6, also known as calcyclin) [221], calsenilin (KCNIP3) [222], calbindin (CALB1) [223] and calretinin (CALB2) [223] have all been shown to increase in ADRA by immunohistochemistry. The Ca^{2+}-binding protein S100-B (S100B) has been reported to be increased by some studies [38, 224–227] but not others [148, 149].

Water/K^+ homeostasis

Astrocytes regulate brain water and potassium (K^+) homeostasis through specific membrane channels. ADRA show increased immunoreactivity for aquaporin-1 (AQP1) [228, 229], whereas aquaporin-4 (AQP4) levels have been reported to be unchanged [228, 230, 231], decreased [232] and elevated [233] by different authors. Moreover, ADRA exhibit increased immunoreactivity for K^+ channel subunits, including intermediate conductance calcium-activated potassium channel protein 4 (KCNN4, also known as $K_{Ca}3.1$) [234] and ATP-sensitive inward rectifier potassium channel 11 (KCNJ11, also known as $K_{ir}6.2$) [235].

Phagocytosis

ADRA have increased immunoreactivity for the opsonin complement C3 (C3) [152, 236] and for scavenger receptor class B member 1 (SCARB1) [237]. One study reported that the C3b/C4b receptor complement receptor type 1 (CR1)—which is a genome-wide association study AD risk locus—is expressed by astrocytes but unchanged in AD vs control brains [238]. Another study reported reduced immunoreactivity for lactadherin (MGFE8) in ADRA, which has been involved in Aβ phagocytosis by astrocytes [239].

Aβ and tau

Numerous studies have reported Aβ immunoreactivity in ADRA, especially in subpial cortical astrocytes in close proximity with extracellular diffuse Aβ deposits [69, 101, 158, 240–252], supporting effective Aβ phagocytosis by astrocytes. Of note, Aβ within ADRA has been described as granules or dot-like staining and is best observed with antibodies against its mid-segment and C-terminus (Aβ40 or Aβ42) due to prominent N-terminal truncation [241–244, 247, 250–252]. Aβ oligomeric species have also been shown in ADRA using a conformation-specific antibody [251]. Remarkably, amyloid-beta precursor protein (APP) [223, 253–255], beta-secretase 1 (BACE1) [256, 257], the BACE1-cleaved soluble APP ectodomain fragment (i.e., sAPPβ) [258] and the presenilins -1 (PSEN1) [35, 255, 259–261] and -2 (PSEN2) [255, 259] have all been reported to increase in ADRA as well, raising the possibility that ADRA contribute to the production and secretion of Aβ. Indeed, APP and PSEN1/2-positive ADRA exhibit enhanced immunoreactivity for caveolin-3 (CAV3), which has been implicated in APP

cleavage by BACE1 [255], and ADRA also have increased immunoreactivity for the transcriptional repressor protein YY1 (YY1, also known as Yin Yang 1), which can activate BACE1 transcription [262]. In addition, ADRA are immunoreactive for the adaptor protein SHC-transforming protein 1 (SHC1, also known as ShcA), which is known to interact with APP C-terminal fragments and may mediate its intracellular signalling [263, 264]. Conversely, the Aβ-degrading enzymes neprilysin (MME) and insulin-degrading enzyme (IDE) have also been reported to increase in ADRA [265].

Similar to Aβ, multiple studies have reported the existence of the microtubule-associated protein tau (MAPT, hereafter referred to as tau) in ADRA [200, 266–271] with two morphologies: thorn-shaped astrocytes and granular fuzzy astrocytes, collectively termed ageing-related tau astrogliopathy (ARTAG). ARTAG tau species are misfolded based on immunoreactivity for the conformation-specific mouse monoclonal antibodies Alz50 [200, 267, 268, 270] and MC1 [200] and hyperphosphorylated based on positive staining with antibodies against pSer202, pSer202/205 (AT8), pSer214, pSer396, pSer396/404 (PHF1), pSer422, pThr181 and pThr231 [200, 267–269, 271]. One study also reported nitration of tau in ADRA at Tyr18 [270].

Miscellaneous

Lastly, ADRA have been reported to display increased immunoreactivity for the oestrogen receptors alpha (ESR1) [272] and beta (ESR2) [273], peptidyl-prolyl cis-trans isomerase FKBP1A (FKBP1A, also known as 12-kDa FK506-binding protein or FKBP12) [274] and protein-arginine deiminase type-2 (PADI2) [275], which catalyses the citrullination of GFAP and VIM. On the other hand, cytosolic 10-formyltetrahydrofolate dehydrogenase (ALDH1L1, better known as aldehyde dehydrogenase 1 family member L1) was described as unchanged in ADRA [146]. Finally, one immunohistochemical study found deficient DNA methylation and hydroxymethylation in ADRA [276], whereas other authors observed no change [277].

Bioinformatics analyses

Pathway enrichment analysis

To further evaluate the functional changes associated with astrocyte reaction in AD, we applied PEA on the 196 ADRA proteins against the curated GO and Reactome pathway databases available from MSigDB [16–19]. The most salient enriched functional pathways included inflammatory cytokines and innate immune response (e.g., MAPK, toll-like receptor and interleukin signalling, as well as inflammasomes), response to nitrosative and oxidative stress, lipoprotein metabolism, extracellular matrix organisation, protein degradation, signalling by nuclear receptors (including oestrogen receptor and ERBB4-mediated signalling) and trophic factors (e.g., FGF) (Figure 2, Table S2).

Protein-protein interaction network analysis

PPI network analysis on the ADRA protein set ($n = 196$) using the STRING database [20] rendered a highly connected functional network with 193 nodes, 2331 edges (expected 836), an average node degree of 24.2, an average local clustering coefficient of 0.563 and a PPI enrichment p value of $< 1.0e-16$ (Figure 3). Based on centrality scores, IL6, TP53, CASP3, TNF, MAPK3, MAPK8, MAPK1, MYC, PTGS2, IGF1, APP, IL1B, CCL2, FGF2 and ESR1 were the top 15 hub proteins. The remarkable interconnectivity between the individual markers of the ADRA protein set was visualised with a network plot (Figure 3A), a Circos plot (Figure S1) and a network heatmap (Figure S2). Similarly, a chord plot (Figure 3B) illustrated the high interconnectivity of these ADRA proteins at the functional pathway level, with inflammation as the most prominent functional alteration in AD reactive astrogliosis.

Transcription factor enrichment analysis

To explore the main transcription factors driving the expression changes associated with ADRA, we performed transcription factor enrichment analysis (TFEA) against publicly available datasets of ChIP-seq studies in a wide variety of cell lines [21, 22, 278] (see Methods). TFEA using two separate bioinformatics tools—namely, TFEA.ChIP and Enrichr—uncovered transcriptional repressor CTCF (CTCF, also known as CCCTC-binding factor) and ESR1 as novel transcription factors potentially implicated in astrocyte reaction (Figure 4). The antioxidant defence orchestrator NFE2L2 reached statistical significance in Enrichr but not in TFEA.ChIP, whereas RELA (the catalytic subunit of NF-kappa-B) and signal transducer and activator of transcription 3 (STAT3) were mostly not significantly enriched (Table S3).

Comparison with transcriptomic and proteomic studies

Lastly, we aimed to compare the ADRA markers with publicly available human -omics datasets, including a microarray study of laser-capture microdissected GFAP$^+$ astrocytes in the temporal neocortex [27], the astrocyte subset of a single-nuclei RNA-sequencing (RNA-seq) study of the entorhinal cortex [28], the AMP-AD Consortium bulk brain proteomic dataset (total $n = 419$) [29] and Cohort 1 of the AMP-AD CSF proteomic study (total $n = 297$) [29] (see Supporting Information). We found a substantial representation of ADRA proteins in each of the four datasets examined. Enrichment analysis of the 196 ADRA markers against all genes or proteins differentially expressed between control and AD dementia in Simpson et al. [27], Grubman et al. [28] and Johnson et al. (bulk brain) [29] revealed that this overlap was consistently significant, with p values of 1.55e-2, 3.45e-12 and 2.25e-13, respectively (calculated by Fisher's exact test). Cross-validation with the two astrocyte-specific transcriptomic studies listed above indicated that the expression of the genes encoding ADRA proteins correlates with disease progression as measured by
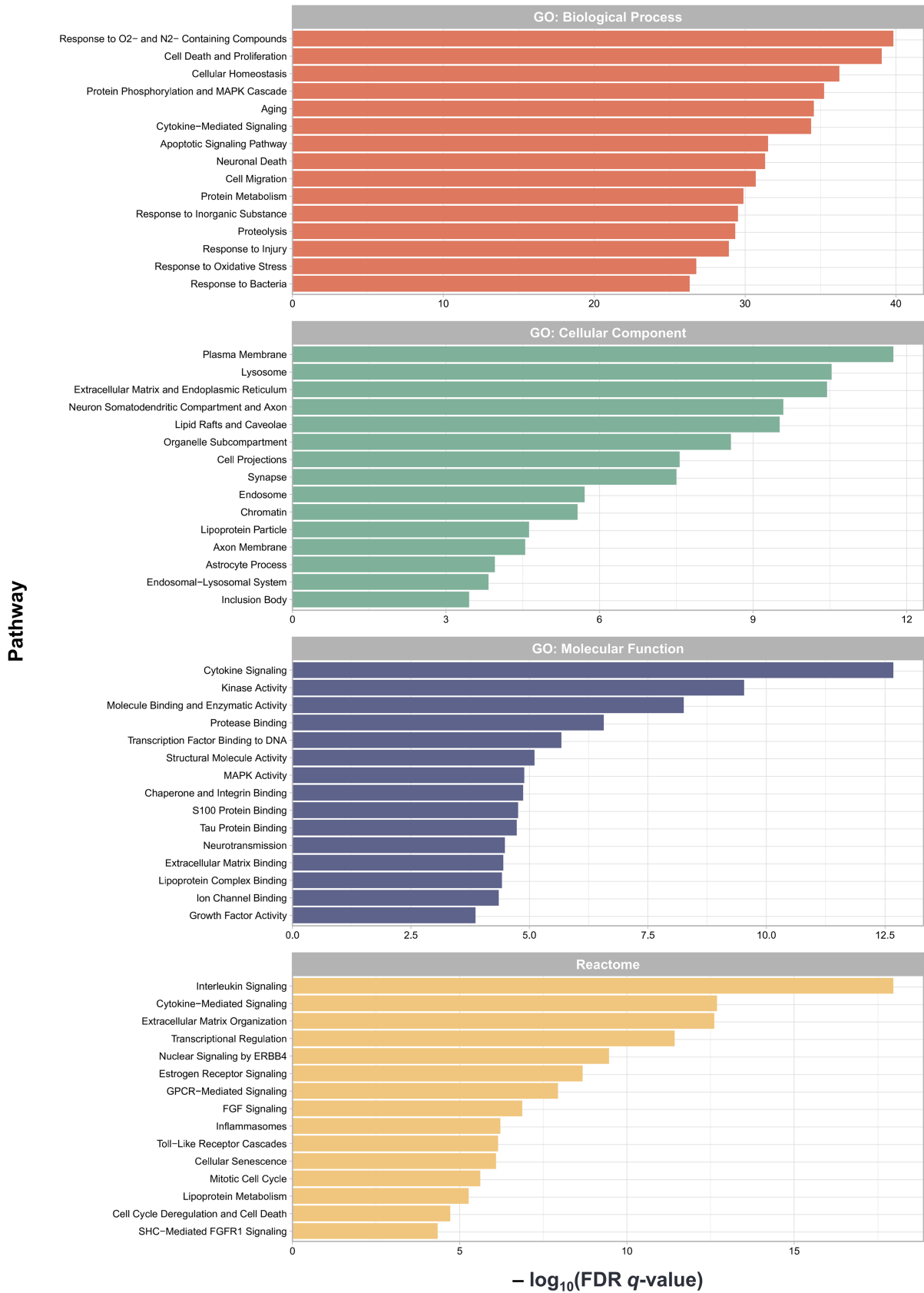
FIGURE 2 Pathway enrichment analysis (PEA) highlights neuroinflammation, oxidative stress, lipid metabolism and extracellular matrix in Alzheimer's disease reactive astrocytes (ADRA). PEA was performed on the 196 ADRA proteins against the following curated pathway databases: Gene Ontology (GO): Biological Process (BP); GO: Cellular Component (CC); GO: Molecular Function (MF); and Reactome. Bar graphs illustrate the statistical significance of enrichment (i.e., $-\log_{10}$[FDR q value]) for the top 15 pathways in each database
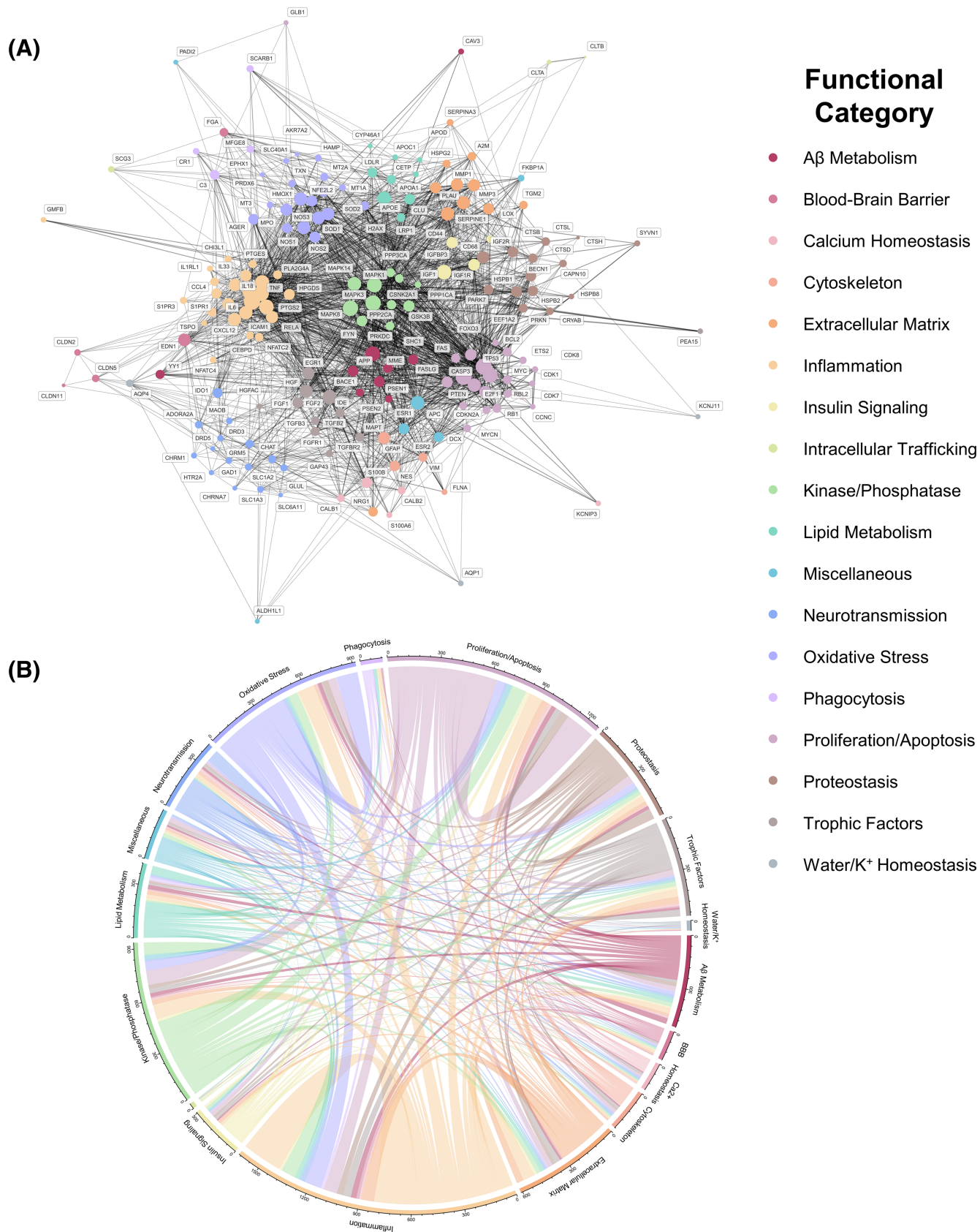
FIGURE 3 Protein–protein interaction (PPI) network analysis reveals complex functional changes in Alzheimer's disease reactive astrocytes (ADRA). (A) STRING PPI functional network analysis on the 196 ADRA proteins resulting from our systematic review demonstrates a highly connected network with IL6, TNF and MAPK 1, 3, and 8 as top hub proteins. (B) Chord diagram based on expert annotation of the 196 ADRA markers in one of 18 functional categories shows the high interconnectivity of the functional alterations of ADRA

FIGURE 4 Transcription factor enrichment analysis (TFEA) reveals novel potential drivers of astrocyte reaction. (A) Volcano plot represents the effect size (i.e., log[fold-change] on the x-axis) against the statistical significance (i.e., −log[p value] on the y-axis) of the TFEA.ChIP enrichment analysis for each of the ChIP-seq experiments. The horizontal red line corresponds to p value = 0.05, whereas the vertical blue lines represent fold-changes of +1.5 and −1.5. (B) Bar graph represents the statistical significance (nominal p values; adjusted p values are available in Table S3) of the Enrichr TFEA results. The vertical red line corresponds to p value = 0.05. Both methods showed CTCF and ESR1 as novel transcription factors potentially implicated in astrocyte reaction

TFEA.ChIP Analysis

Enrichr Analysis
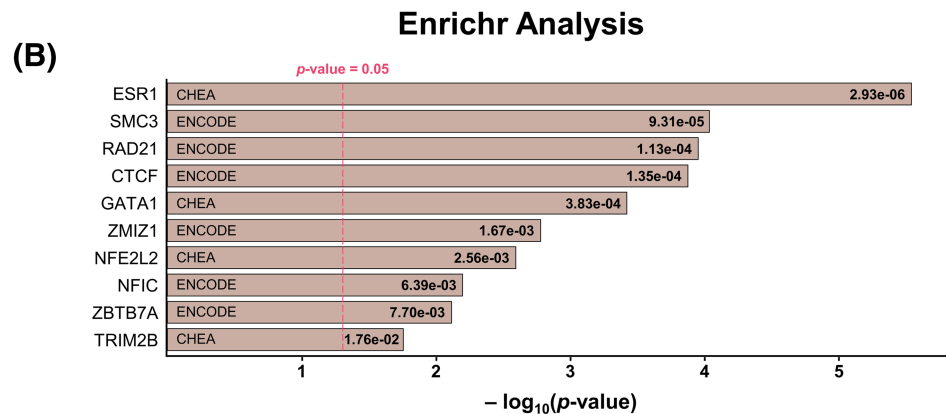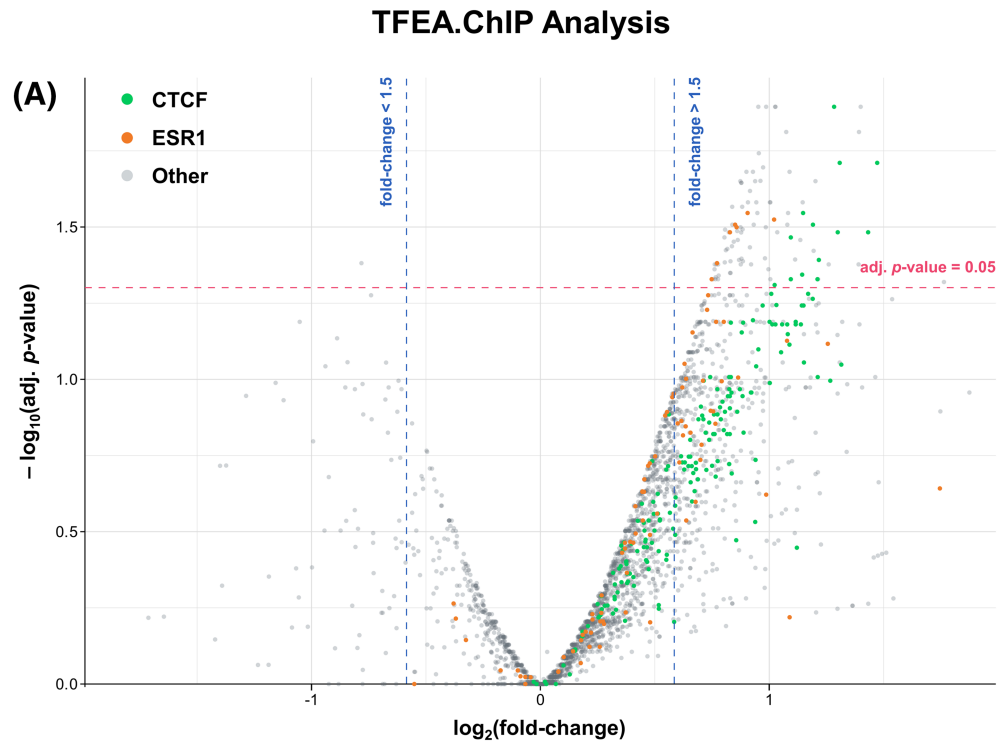
Braak NFT stage [27] (Figures 5A and S3) and/or with AD vs control diagnosis [28] (Figures 5B and S4). Notably, cross-validation with the AMP-AD CSF proteomics dataset showed that the levels of many ADRA proteins in CSF correlate with the $A\beta_{42}$/p-tau ratio, which is a surrogate biomarker of the severity of brain AD neuropathological changes [29] (Figure 5C). Similarly, cross-validation with the AMP-AD bulk tissue proteomics dataset revealed that a considerable proportion of ADRA proteins change in parallel to the diagnostic group (control vs asymptomatic AD vs AD dementia) and/or Braak NFT stage [29] (Figures 5D and S5).

DISCUSSION

We demonstrate the feasibility and validity of a systematic review of post-mortem human neuropathological studies to characterise the

protein expression changes associated with astrocyte reaction in the AD brain. Some limitations of this approach should be acknowledged in advance. Systematic reviews are inherently affected by a risk of publication bias; in this case, increased immunoreactivity indicating protein upregulation is typically more obvious to the examiner (and likely more readily reported) than decreased immunoreactivity associated with protein downregulation; therefore, loss of normal astrocyte functions might be underreported. Moreover, the possibility of missing studies because they are published in non-indexed journals, rarely cited and therefore less likely to be captured from reference lists, inappropriately indexed in databases or simply not encompassed by our search strategy should be considered. Other limitations are inherent to post-mortem neuropathological studies: antibody specificity was not always tested; the effects of ante-mortem agonal period and post-mortem interval on marker immunoreactivity were usually not investigated; most studies compared healthy control brains with
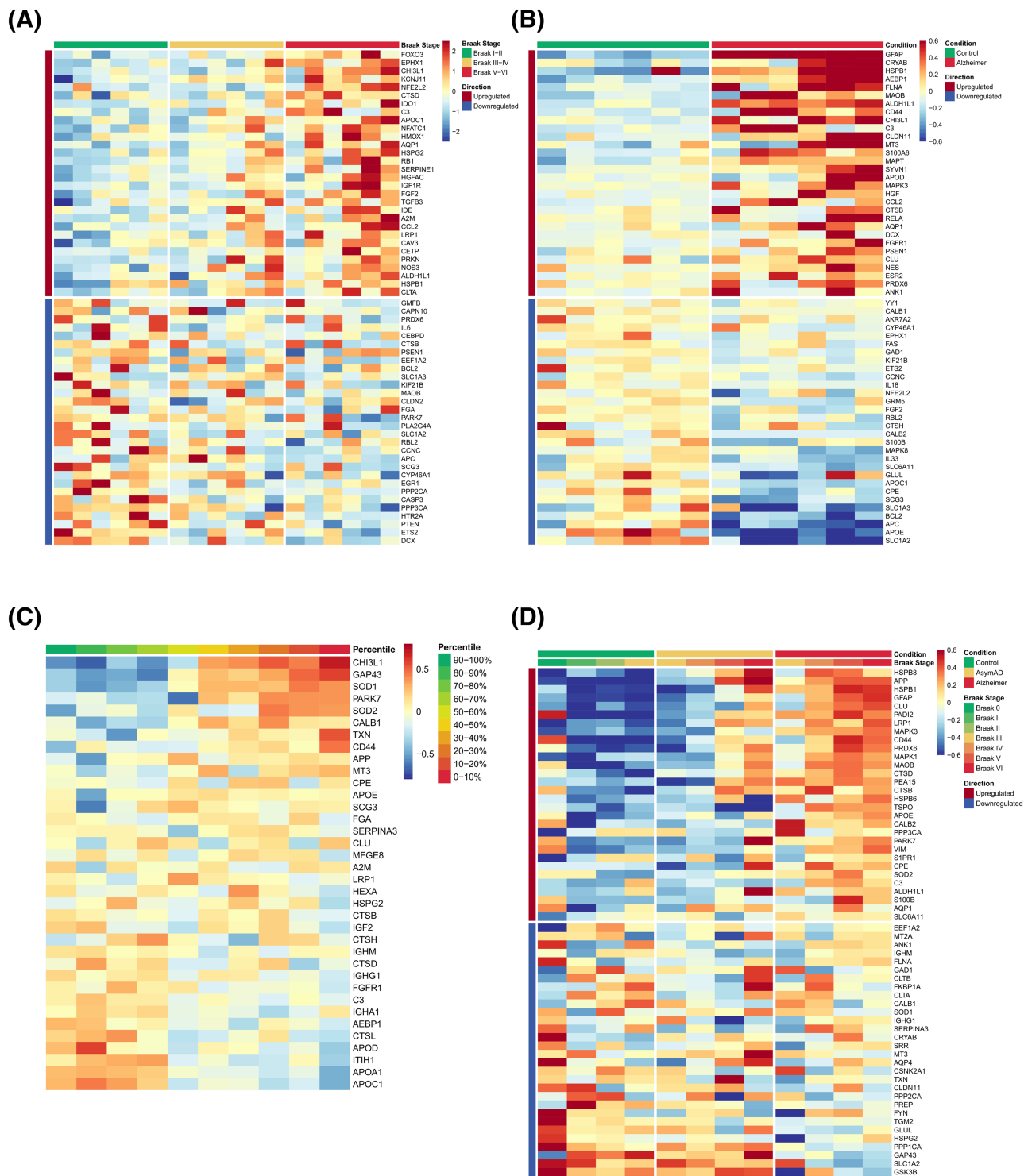
FIGURE 5 Comparison of Alzheimer's disease reactive astrocytes (ADRA) protein set with publicly available human -omics datasets. (A) Heatmap shows the z scores of gene expression of the top 30 upregulated and downregulated ADRA markers across all 18 subjects ($n = 6$ Braak I/II, $n = 6$ Braak III/IV and $n = 6$ Braak V/VI) included in a microarray study of laser-capture microdissected GFAP$^+$ astrocytes from the temporal neocortex (Simpson et al. [27]). (B) Heatmap shows the z scores of gene expression of the top 30 upregulated and downregulated ADRA markers across all 12 subjects ($n = 6$ control and $n = 6$ AD) included in a single nuclei RNA-sequencing (RNA-seq) study from the entorhinal cortex (Grubman et al. [28]). (C) Heatmap represents the z scores of protein expression of all available ADRA markers averaged by deciles of cerebrospinal fluid (CSF) Aβ_{42}/p-tau ratio across the $n = 147$ control and $n = 150$ AD subjects from Cohort 1 of the Accelerating Medicines Partnership-Alzheimer's Disease (AMP-AD) Consortium CSF proteomic study (Johnson et al. [29]). (D) Heatmap illustrates the z scores of protein expression of the top 30 upregulated and downregulated ADRA markers averaged by Braak neurofibrillary tangle (NFT) stage within each diagnostic group ($n = 91$ control, $n = 98$ asymptomatic AD and $n = 230$ AD dementia subjects) described in the AMP-AD Consortium bulk brain proteomic dataset (Johnson et al. [29])

advanced AD brains, hence, the described changes in immunoreactivity may only reflect end-stage status and may differ at earlier stages of disease; and qualitative and semi-quantitative reports could be affected by examiner subjectivity. Nevertheless, some of these biases are offset by the significant overlap demonstrated between the ADRA protein set and various human -omics datasets, which have different inherent methodological biases. Therefore, our thorough bioinformatics analyses on the ADRA protein set provide important clues about the physiological changes central to astrocyte reaction in AD.

PEA on the 196 ADRA proteins revealed that astrocyte reaction is a complex process involving multiple astrocyte functions beyond cytoskeletal remodelling (viz., beyond the classic upregulation of GFAP). These include neuroinflammation (e.g., the cytokines IL1B, IL6 and TNF and the stress kinases MAPK 1, 3 and 8), oxidative stress (e.g., the metallothioneins MT1A, MT2A and MT3; the nitric oxide synthases NOS1, NOS2 and NOS3; and the superoxidase dismutases SOD1 and SOD2), lipid metabolism (e.g., APOE, CLU and LRP1), extracellular matrix (e.g., CD44, SERPINA3, the matrix metalloproteinases MMP1 and MMP3 and the tissue transglutaminases TGM1 and TGM2) and proteostasis (e.g., CRYAB, HSPB1 and the cathepsin protease family).

PPI network analysis of the ADRA protein set revealed an extraordinary interconnectivity across many of these functions, suggesting that one or more parallel pathogenic cascades occur within the reactive astrocyte (i.e., domino effect). This analysis identified IL6 as the protein with the highest connectivity by eigenvalue centrality score. Importantly, mouse models genetically engineered to overexpress IL6 in astrocytes (i.e., *Gfap-Il6* transgenic mice) exhibit a neurodegenerative phenotype with loss of cortical synapses and cerebellar atrophy [279], indicating that IL6 secretion by reactive astrocytes could be neurotoxic. Although our TFEA on the 196 ADRA genes did not detect STAT3, IL6 signalling is known to activate the JAK/STAT pathway, which is thought to be key for astrocyte reaction in transgenic mouse models of β-amyloidosis [280, 281]. IL6 signalling could be blocked by repurposing IL6 inhibitors (e.g., siltuximab), IL6 receptor inhibitors (e.g., sarilumab and tocilizumab) and JAK inhibitors (e.g., bariticinib, tofacitinib and ruxolitinib). Interestingly, a recent machine learning study to identify candidates for drug repurposing in AD concluded that Food and Drug Administration-approved JAK inhibitors could be beneficial in AD [282]. Taken together, these observations strongly support the design of clinical trials aimed at inhibiting the IL6/JAK/STAT signalling axis to attenuate astrocyte reaction in AD.

Intriguingly, CTCF and ESR1 emerged as potential novel transcription factors involved in astrocyte reaction according to two separate TFEA tools. While these methods use curated databases of ChIP-seq experiments and outperform methods based on ascertaining binding motifs within the DNA sequence, most ChIP-seq experiments correspond to tumour cell lines of various organs; hence, any extrapolation to astrocytes in the human brain should be taken with caution. However, CTCF was also enriched in the ChIP-seq experiments conducted on primary astrocytes and astrocytoma cell lines in our TFEA. ChIP analysis (Table S3). CTCF regulates 3-D genome architecture and facilitates enhancer–promoter interactions across multiple cell types in the brain [283]; mutations in this transcription factor cause a neurodevelopmental disorder with intellectual disability, possibly by deregulating the expression of multiple genes [284]. Conditional deletion of CTCF from excitatory glutamatergic neurons in mice impairs synaptic plasticity, learning and memory and causes both neurodegeneration and reactive gliosis [285–287]. Further, upon TGF-beta stimulation of astrocytes in vitro, CTCF enhances APP expression [288]; of note, APP ranked 11th in our PPI network by centrality score and is significantly upregulated in AD vs controls in both CSF and bulk brain proteomic studies [29]. The oestrogen receptors ESR1 and ESR2 are present in the plasma membrane and are trafficked to the nucleus, where they act as transcription factors regulating gene expression. Consistent with the results of our PEA and TFEA, ESR1 ranked 15th in our PPI network by centrality score. Further, ESR1 and ESR2 have been reported as upregulated in reactive astrocytes in AD [272, 273], in male primates after transection of the fimbria fornix [289] and in male rats after kainic acid-induced status epilepticus [290]. Hence, together with our findings, the available literature suggests that CTCF and ESR1 warrant further investigation as regulators of AD astrocyte reaction.

Finally, a significant subset of the ADRA protein set was also altered in AD vs control individuals in several reference transcriptomics and proteomics studies. For example, in their microarray study of laser-capture microdissected GFAP-immunoreactive astrocytes, Simpson et al. highlighted actin cytoskeleton, proliferation/apoptosis and proteostasis, as well as stress and immune responses as the main dysregulated astrocyte functions, and implicated the MAPK signalling pathway [27]. The pro-inflammatory astrocyte phenotype has also been shown in aged wild-type mice and APP/PS1 AD transgenic mice [2, 291, 292]. These similarities are remarkable given the large technical and biological discrepancies frequently observed between these methods [293]. Moreover, these findings have two important implications. First, astrocyte-specific transcriptomic studies and bulk brain or CSF proteomics studies could be validated by cyclic multiplex immunohistochemistry methods in post-mortem brain sections [294]. Second, our ADRA protein atlas may inform ongoing efforts to discover serum/plasma, CSF and PET imaging biomarkers of reactive astrocytes, which may assist the early diagnosis and prognostication of AD. For example, many of the overlapping proteins between the ADRA protein set and a public CSF proteomic dataset strongly correlated with the levels of AD CSF biomarkers, which are used for the clinical diagnosis of AD and as a proxy for the severity of AD neuropathological changes (Aβ plaques and NFTs) in the brain.

In summary, our systematic review of the neuropathological literature reveals the complexity of AD-associated astrocyte reaction, which has been increasingly recognised [1, 3–6]. Besides biomarker discovery, these findings could inform future astrocyte-centric single-cell and single-nuclei RNA-seq studies as well as spatial transcriptomic and proteomic investigations. To this end, we have shared these results as a web-based resource available at www.astrocyteatlas.org.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to the content of this article.

AUTHOR CONTRIBUTIONS

Lucía Viejo performed the data curation, investigation and writing—original draft; Ayush Noori performed the formal analysis, software, visualisation and writing—original draft; Emily Merrill provided resources; Sudeshna Das performed the funding acquisition, supervision and writing—review and editing; Bradley T. Hyman performed the funding acquisition, supervision and writing—review and editing; Alberto Serrano-Pozo performed the conceptualisation, data curation, funding acquisition, investigation, methodology, supervision and writing—original draft.

ETHICS STATEMENT

This research did not involve interaction with living human subjects or animal models. Data in this article were extracted from published neuropathological studies, which only included deidentified post-mortem brain specimens.

CODE AVAILABILITY

The source code for all bioinformatics analyses is available on GitHub at https://github.com/serrano-pozo-lab/astrocyte-review (DOI: 10.5281/zenodo.5140749).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The source code for all bioinformatics analyses is available on GitHub at https://github.com/serrano-pozo-lab/astrocyte-review (DOI: 10.5281/zenodo.5140749).

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