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Revealing cell vulnerability in Alzheimer's disease by single-cell transcriptomics

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder that by affecting specific brain cell types and regions cause severe pathological and functional changes in memory neural circuits. A comprehensive knowledge of the pathogenic mechanisms underlying AD requires a deeper understanding of the cell-specific pathological responses through integrative molecular analyses. Recent application of high-throughput single-cell transcriptomics to postmortem tissue has proved powerful to unravel cell susceptibility and biological networks responding to amyloid and tau pathologies. Here, we review single-cell transcriptomic studies successfully applied to decipher cell-specific gene expression programs and pathways in the brain of AD patients. Transcriptional information reveals both specific and common gene signatures affecting the major cerebral cell types, including astrocytes, endothelial cells, microglia, neurons, and oligodendrocytes. Cell type-specific transcriptomes associated with AD pathology and clinical symptoms are related to common biological networks affecting, among others pathways, synaptic function, inflammation, proteostasis, cell death, oxidative stress, and myelination. The general picture that emerges from systems-level single-cell transcriptomics is a spatiotemporal pattern of cell diversity and biological pathways, and novel cell subpopulations affected in AD brain. We argue that broader implementation of cell transcriptomics in larger AD human cohorts using standardized protocols is fundamental for reliable assessment of temporal and regional cell-type gene profiling. The possibility of applying this methodology for personalized medicine in clinics is still challenging but opens new roads for future diagnosis and treatment in dementia.

1. Introduction

Neurodegenerative diseases are incurable brain disorders that affect millions of people, causing enormous medical, societal and economic impacts. Alzheimer's disease (AD) is the most common cause of dementia affecting currently 57 million cases worldwide [1]. These neurological diseases are characterized by abnormal conformation, aggregation and accumulation of pathological proteins in particular degenerating neuronal and glial populations through still unclear mechanisms. The presence of amyloid plaques containing amyloid-β (Aβ) peptides, neurofibrillary tangles (NFTs) formed by paired-helicament filaments (PHFs) of phosphorylated microtubule-associated protein tau (MAPT), inflammatory responses, and synapse and neuron loss are characteristic of AD patients' brain [2,3]. Among these hallmarks, amyloid plaques are widely distributed, whereas tau pathology appears first in the medial temporal lobe regions (i.e., hippocampus, entorhinal cortex and amygdala) and then spreads to limbic and association cortices [4]. Tau pathology correlates tightly with cognitive decline and propagates spatially in parallel with microglial activation, whereas Aβ potentiates the effects of microglial activation on tau spreading in the neocortex [5]. Despite major research efforts to decipher the genetic and pathological causes of AD, our understanding of the molecular mechanisms underlying this regional and cellular vulnerability is still poor, which has limited the development of effective therapies to ameliorate or reverse progression of dementia.

One of the major scientific challenges to understand neurodegenerative diseases is the complexity of the human brain that contains a vast diversity of cells, including billions of neurons of distinct (sub)types. The presence of neurons and non-neuronal cells displays even further diversity of molecular, morphological and functional properties of brain network connectivity. Precisely, understanding the complexity of physiological changes occurring in the dysfunctional degenerative brain

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requires advanced systems-level analyses of biological, pathological, neuroimaging and clinical data integrated through cross-modal computational modeling, as recently applied to define a mechanistic framework of the mammalian brain [6]. Moreover, comprehensive analyses accounting for cellular heterogeneity, novel cell subtypes and responses to pathological changes are required for a deep understanding of the molecular mechanisms occurring in brain diseases. In this scenario, bulk transcriptomics, single-cell transcriptomics and genomics coupled with gene network analysis represent ideal tools for investigating cell-specific molecular mechanisms of disease (Fig. 1). It is conceivable that these powerful technologies will unravel pathogenic mechanisms underlying cell vulnerability while providing novel molecular targets for drug discovery in neurodegenerative diseases. In this review, we focus on single-cell transcriptomic studies that have shifted our current understanding of the cellular diversity and pathways affected in AD. We finally discuss current limitations and future challenges of applying cell transcriptomics to understand the biological complexity of AD brain.

2. Transcriptome changes in Alzheimer's disease brain

Brain transcriptomics is useful to decipher gene networks and transcriptional mechanisms affected during biological and pathological aging. Pioneering microarray-based transcriptomic analyses uncovered multiple differentially expressed genes (DEGs) in vulnerable AD brain regions affecting, among others, energy metabolism, transcriptional regulation, inflammation and synaptic function [7–10]. In CA1 hippocampus, altered genes related to metabolic, synaptic, immune response and myelination pathways, correlate with clinical and NFT scores, suggesting that these pathways are tightly associated with disease progression [10,11]. Particularly, genes related to metabolism, endocytosis, and synaptic function are upregulated in mild cognitive impairment, and then decline as the disease progresses [12–15], which is relevant considering that deregulation of synaptic genes/proteins can lead to synapse dysfunction and cognitive decline in AD and mouse models [16, 17]. Recently, bulk RNA sequencing (RNA-seq) and digital pathology using machine learning algorithms identified five genes (e.g., *SERPINA5*, *RYBP*, *SLC38A2*, *FEM1B*, and *PYDC1*) associated with neuropathological changes and hippocampal vulnerability in AD [18]. In the prefrontal cortex, elevated synaptic function and plasticity genes are detected at early pre-symptomatic stages, and their expression decrease coinciding with the appearance of pathological traits and clinical impairment [19]. However, cognitive decline correlates tightly with changes in synaptic transmission/plasticity and myelin genes in the entorhinal cortex and hippocampus but not in neocortical regions [15]. Therefore, despite multiple gene changes ongoing in the degenerating brain, some gene modules are conserved among brain regions. These studies indicated temporal and regional vulnerability of synaptic and activity-dependent genes during AD pathology and clinical symptoms ([20], for a review), but they did not clarify the contribution of individual cell transcriptomes to regional vulnerability in AD brain.

More recently, bulk transcriptomics and co-expression network analysis showed AD-associated neuronal and glial gene modules (e.g., cell survival/death, immune response) [21–25], and highlighted oxidative phosphorylation as a cluster that predicts AD [26]. Gene co-expression analysis by combining publicly available AD transcriptomic datasets has allowed the identification of cell-specific gene signatures associated with pathological and cognitive status [23,27,28]. For instance, large scale gene expression and network analyses across nineteen AD brain regions identified selective regional vulnerability and abnormal gene modules in astrocytes, neurons and oligodendrocytes [29]. Additionally, a transcriptome-wide association study (TWAS) combining genomic and transcriptomic data identified twenty-four hippocampal genes (*APOE*, *DMPK*, *ERRC2*, *EXOC3L2*, *KAT8*, *PCDHA4*, *PTPN9*, *QPCTL*, *SNRPD2*, *TOMM40*, etc.) clustered in five functional modules related to AD cellular processes, including amyloid processing, tau phosphorylation, neuronal apoptosis, neurogenesis, chaperone-mediated autophagy, transcription and telomerase regulation [30]. Combining genome-wide association studies (GWAS), transcriptome and proteome analyses has further revealed a set of genes related to translational repression by miRNAs in the lateral amygdala of the Religious Order Study/Rush Memory and Aging Project (ROSMAP) donors with anxiety traits phenotypes [31]. Bulk brain transcriptomics has been useful to decipher gene biological networks in cells of a homogenous material, but it does not allow the identification of vulnerable cell subpopulations and to elucidate transcriptional changes at single cell level, particularly in less abundant cell types. Alternatively, advanced molecular profiling of individual cells (i.e., cell transcriptomics) using single-cell (sc; intact cells) and single-nucleus (sn; only nuclei) RNA-seq in fresh and frozen postmortem tissue has allowed unprecedented molecular characterization of cell types and tissue responses to physiological and pathological changes (Fig. 1).

3. Cell-specific vulnerability in Alzheimer's disease

Single-cell transcriptomics has revealed substantial cellular heterogeneity and vulnerability, and cell-type specific but also coordinated gene expression responses in AD brain. Subcluster analysis of individual cell types in the mammalian brain using snRNA-seq shows subcellular heterogeneity, comprising multiple subpopulations of neurons, including excitatory and inhibitory neurons, microglia, astrocytes, oligodendrocytes, oligodendrocyte precursor cells (OPCs) and endothelial cells, affected in AD brain [32–36] (Fig. 2). These cellular subpopulations are not static or quiescent as changes in cell subtypes occur

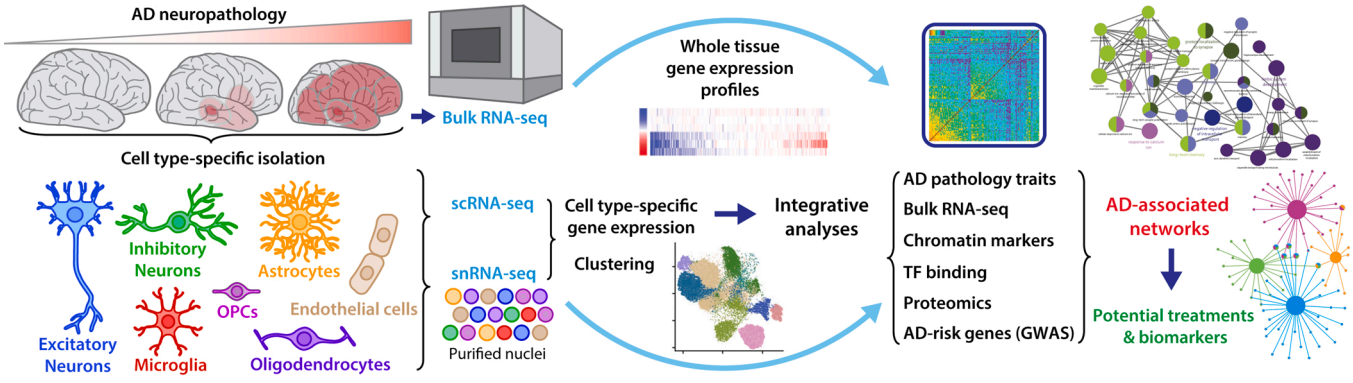

Fig. 1. Experimental design of cell transcriptomic analysis of human AD brain. Schematic illustration of the experimental transcriptomic workflow, including the design, sample processing and integrative bioinformatic analysis of bulk, single-cell (sc) and single-nucleus (sn) RNA sequencing (RNA-seq) of control and AD brains. Darker red shades in the cartoon brains indicate increase of AD neuropathology. GWAS: genome-wide association studies; TF: transcription factor.

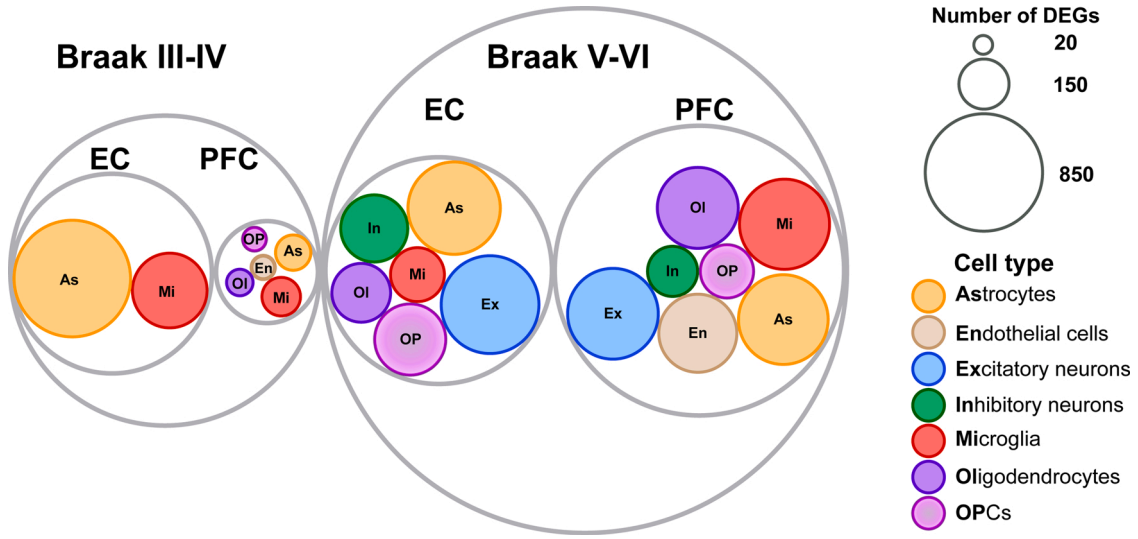

Fig. 2. Changes in gene expression in vulnerable cell types in AD brain. Diagram showing the average number of differentially expressed genes (DEGs) per cell type, region, and AD pathological stage (Braak III-IV/V-VI) in human brain. The average of DEGs for each cell type and brain region and/or pathological stage is proportional to the circle size. Affected cell types are depicted in different colors and letter codes as indicated at the bottom right of the image. EC: entorhinal cortex; PFC: Prefrontal cortex.

in vulnerable AD brain regions during the disease process, including decreased neurons and increased number of microglia, astrocytes and endothelial cells [23]. Accordingly, reduced excitatory neuron subtypes and increased microglia are detected first in the entorhinal cortex and later in the superior frontal gyrus [33,36]. In AD prefrontal cortex, microglial and astrocytic subpopulations increase and excitatory neurons tend to decrease, whereas oligodendrocytes suffer little changes during disease progression [22,23,34,37]. On the contrary, inhibitory neurons subpopulations are not globally affected in the entorhinal cortex, superior frontal gyrus and prefrontal cortex [36]. Specific cell-type subpopulations change depending on pathology, cognitive status and sex, which suggests a strong association of specific cell subpopulations and AD pathological stage [32]. Surprisingly, when cell subtypes from the same origin are analyzed as unique populations, the proportion of cell types is not apparently affected in AD prefrontal cortex, except for an increase of endothelial cells [35]. This discrepancy in cell type abundance could be explained, among others, by methodological differences in: sample dissociation and preparation; ages of the cohorts (mean age, [32]: 86.7 in AD and 87.1 in controls; [35]: 74.6 in AD and 85.4 in controls); and cell-type annotation, which is based exclusively on cell markers and transcriptomes rather than morphological and functional features. In conclusion, temporal changes in cell-type composition involving selective regional vulnerability of excitatory neurons, and appearance of microgliosis and astrocytosis occur in AD brain.

The differential composition of cell subclusters and gene expression profiles indicate disease-associated segregation of transcriptional changes across cell types. The global trend of gene changes seems cell specific as indicated by the fact that a vast majority of DEGs in the prefrontal cortex of the ROSMAP cohort are downregulated in excitatory and inhibitory neurons, and upregulated in astrocytes, microglia and oligodendrocytes [32]. In cerebral vasculature of AD patients, 61–78% of deregulated genes are downregulated in endothelial cells, fibroblasts and astrocytes [38]. Similarly, excitatory neurons but not inhibitory neurons show the most striking gene differences in AD entorhinal cortex, but the most coordinated gene expression changes occur in astrocytes, endothelial cells and microglia [33,36]. The fact that some gene changes are more pronounced in entorhinal and temporal cortices compared with frontal cortex is consistent with the temporal pathological progression from the medial temporal lobe to frontal cortex in AD. Interestingly, a recent single-cell genomics study found age-dependent accumulation of somatic mutations in hippocampal and prefrontal cortical neurons from AD subjects, which affect coding exons and are predicted to induce further transcriptional alterations [39]. These results are important because imply that transcriptomic analyses are useful to follow region-dependent cell-specific transcriptional vulnerability in AD.

The regional vulnerability of cell subpopulations is accompanied by cell-type transcriptional changes affecting distinct but also overlapping biological networks (Table 1; Fig. 3). This implies that specific and common biological pathways are regulated simultaneously in distinct cell types in the degenerating brain. Some overlapping gene clusters are simultaneously increased in a variety of AD cell types, particularly those associated with protein folding, autophagy, apoptosis, cell stress, immune response and mRNA regulation [32,33,35,40]. AD brain contains enrichment of cell death-related genes in astrocytes, oligodendrocytes, OPCs, and endothelial cells, and upregulation of glial differentiation, myelination, inflammatory responses and mitochondrial/cellular respiration/metabolic genes in neurons, astrocytes and/or oligodendrocytes [33,35,41]. Alternatively, synapse function and learning/cognition genes are downregulated in most cell types, including astrocytes, endothelial cells, microglia, neurons, oligodendrocytes and OPCs [33,35]. Other pathology-related biological pathways are shared mainly between two cell types in the prefrontal cortex, including those related to synaptic signaling in neurons and astrocytes, myelination/axonal integrity/immune system in endothelial cells and oligodendrocytes, and immune response genes in microglia and endothelial cells [32,35] (Fig. 3). Together, cell transcriptomics studies indicate that: 1) cell-type transcriptional changes affecting shared biological pathways occur coordinately in response to AD pathology; and 2) cell-specific disease-associated gene signatures implicate multiple cell types in AD pathophysiology. In conclusion, coordinated protective and compensatory responses against protein aggregation and cell damage occur in distinct cell types of AD brain.

4. Cell transcriptome changes in Alzheimer's disease

Cell transcriptomics show that most DEGs affect a single cell type whereas very few genes are simultaneously affected in several cell types in AD brain. Despite this minimal overlap of DEGs among cell subtypes, numerous biological processes are affected across cell types (Table 1, Fig. 3).

Table 1

Biological pathways differentially affected in cell types of the AD human brain revealed by single-cell transcriptomics. Specific gene network modules upregulated or downregulated in human brain at distinct Braak pathological stages. The information is collected only from published studies using single nucleus/cell transcriptomics and shorted by alphabetical name. Biological pathways are named according to the specific description in the original studies, The original manuscripts from which the biological pathway information has been extracted are indicated with numbers using superscripts as follows: 1: [33]; 2: [35]; 3: [36]; 4: [32]; 5: [34]; 6: [40]; 7: [38]; 8: [37]. The number of individuals of each study are: [33]: 8 AD and 8 control [1]; [35]:12 AD and 9 control[2]; [36]: 3 AD and 3 control[3]; [32]: 24 AD and 24 control[4]; [34]: 11 AD and 7 control[5]; [40]:6 AD and 6 control[6]; [38]: 9 AD and 8 control[7]; [37]: 11 AD and 11 control[8]. The data in reference [38] were obtained from both frontal cortex and hippocampal samples. The following abbreviations are used: EC: entorhinal cortex. PFC: prefrontal cortex. OPCs: Oligodendrocytes precursor cells.

Cell type	Region	Biological pathway			
		Upregulated		Downregulated	
		Braak III-IV	Braak V-VI	Braak III-IV	Braak V-VI
Astrocytes	**EC**	Chaperone mediated protein assembly[6] Inflammatory processes[6] Mitochondrial oxidation phosphorylation[6] Nrf2 activation pathways[6] Response to metal ions[6]	Actin filament based process[1] Cellular response to stress[1] Glial cell differentiation and myelination[1] Negative regulation of cell death[1] Regulation of immune response and response to cytokine[1] Response to topologically incorrect protein[1]	Glutamate transporters[6]	Cognition[1] Glutamate-glutamine uptake and metabolism[3] Neuronal system[3] Reuptake of GABA[3] Synapse organization and transmission[1,3]
	PFC	Extracellular matrix molecules[8]	Action potential[5] Anterograde trans-synaptic signaling[5] Chaperone mediated protein folding[2,4] Presynaptic membrane assembly[5] Protein localization to ER[4] Response to stress and mechanical stimulous[2,4] Translation initiation[4]	Lipid and oxidative metabolism[8]	BMP signaling pathway[5] Glutamate neurotransmission[2,4,5] Negative regulation of glial cell differentiation[5] Neurogenesis[2] Post synapse organization and synaptic signaling[2] Regulation of membrane potential[4] Regulation of PI3K signaling[5]
Endothelial cells	**PFC**		Angiogenesis[1,2] Cell motility[2] Cellular responses to stress[1] Cytokine secretion and immune response[1] IFN α/β signaling[7*] IL-1 regulation of ECM[7*] IL-2 signaling[7*] Immune signaling[7*] Myeloid leukocyte activation[2] Negative regulation of cell death[1] Response to topologically incorrect protein[1] Ribosomal processes[1]		Cation transport[1] Cognition[1] Metal ion transport[1]
Neurons (general)	**EC**		Cellular response to stress[1] Glial cell development[1] Regulation of cell death[1] Regulation of cytokine production[1] Response to topologically incorrect protein[1]		Synapse organization and cognition[1]
Excitatory neurons	**PFC**		Glycosaminoglycan metabolism[3] Learning, memory, and cognition[2,4] Myelination, axonal outgrowth, and regeneration[4] Negative regulation of inclusion body assembly[5] Negative regulation of protein phosphorylation[4] Negative regulation of protein ubiquitination[5] Neuron cell-cell adhesion[5] Neuron death[4] Protein phosphorylation[2] Regulation of IGF transport and uptake[3] Regulation of protein catabolic process[4] Synaptic transmission and signaling[4,5]		Amyloid fibril formation[5] Axon development and guidance[2,5] CREB phopphorylation[3] Ion homeostasis[3] Mitochondrial respiratory chain assembly[4] Organelle transport along microtubule[5] Pre- and pot- synaptic proteins[2,3] Protein folding[4] Ribonucleoprotein complex biogenesis[4] Wnt/calcium signaling pathway[5]
Inhibitory neurons	**PFC**		Cellular protein complex disassembly[4] Cellular respiration[4] ERBB2 signaling pathway[5] Learning, memory, and cognition[2] Neuronal death[4,5]		Cell-cell adhesion[2,4,5] Membrane repolarization[5] Protein dephosphorylation[5] Synaptic membrane adhesion, organization and signaling[2,4,5]

(*continued on next page*)

Table 1 (*continued*)

Cell type	Region	Biological pathway			
		Upregulated		Downregulated	
		Braak III-IV	Braak V-VI	Braak III-IV	Braak V-VI
			Phosphorylation[2] Positive regulation of glucose transport[5] Regulation of mRNA processing[5] Response to metal ion[4]		
Microglia	**EC**	Carbohydrate metabolic processes[6] IL-1 related pathway[6] MAPK cascade[6] Microglia pathogen pahgocytosis[6] Response to unfolded protein[6] Selective autophagy[6]	Cellular response to stress[2] Response to topologically incorrect protein[2] Ribosomal processes and translation initiation[2]		Behavior and cognition[2] Cell-cell adhesion[2] G-protein coupled receptor signaling[2] Homeostatic processes[2] Regulation of cytokine production[2] Response to lipid[2]
	PFC	ERK1 and ERK2 cascade[8] NFκβ signaling pathway[8] Regulation of glial cell migration[8] Regulation of lipid metabolic process[8] Response to IL-6[8]	Cellular respiration related processes[2] Cytoplasmic translation[4] Inflammatory response[4] Lipid metabolic process[4] Pathways associated with β-amyloid clearance[4] Regulation of apoptotic process[5] Vesicle targeting through ER to cis-Golgi[5]	Cellular response to ionizing radiation[8] Defense response to other organism[8] Metal-ion homeostasis[8] Negative regulation of protein phosphorylation[8] Response to IFN-γ[8]	IFN-γ mediated signaling pathway[5] Immune system process[2] Membrane organization[2] Negative regulation of immune effector process[5] Phospholipid transport[5] Regulation of GTPase activity[4] Regulation of Phosphatidylinositol 3-kinase signaling[4] Response to cytokine[2] T cell activation[4]
Oligodendrocytes	**EC**		Glial cell proliferation and myelination[1] Mitochondrion[1] Negative regulation of cell death[1] Regulation of cytokine production[1] Response to topologically incorrect protein[1]		Synapse organization and cognition[1]
	PFC	Lipid accumulation[8] Osmotic imablance[8] Oxidative stress[8] pH and electrolites control[8]	Amyloid-β metabolic process[5] Axon guidance, outgrowth, and regeneration[4,5] Cell-cell adhesion via plasma membrane[5] Cellular respiration processes[2] Chaperone mediated protein folding[5] Lipid biosynthetic process[5] Protein stabilization[5] Regulation of cellular response to heat[5] Regulation of inclusion body assembly[5] Regulation of synaptic assembly[5]	Differentiation of precursor cells into mature myelin-forming cells[8] Myelination[8]	Cellular response to catecholamine stimulus[4] Dicarboxylic acid transport[4] Myelination[2] Negative regulation of protein polymerization[5] Nervous system development[5] Neuron projection morphogenesis[5] Neuronal and axonal ensheathment[2] Postsynaptic membrane assembly[5] Regulation of cholesterol biosynthetic process[5] Regulation of GTPase activity[4] Regulation of release of sequestered calcium[5]
OPCs	**EC**		Cellular response to stress[1] Negative regulation of cell death[1] Response to topologically incorrect protein[1]		Synapse organization cognition[1]
	PFC		Neuron development and neuron projection morphogenesis[4,5] Ionotropic glutamate receptor signaling pathway[4] Regulation of membrane potential[4] Synapse organization[4]		Granulocyte activation[4] Myelination[5] Positive regulation of cell proliferation[5] Protein folding[4]

4.1. Neuronal transcriptomes in Alzheimer's disease

Single-cell transcriptomic analyses demonstrate differential neuronal susceptibility in response to AD pathology depending on the pathological Braak stage, cognitive status and sex [32,33,36]. Excitatory neuronal subpopulations are reduced but inhibitory neurons are unchanged in vulnerable AD brain regions, such as entorhinal and prefrontal cortices, and superior frontal gyrus [32,36]. These results agree with recent evidence indicating major susceptibility of excitatory neurons in AD pointing to the use of glutamatergic system modulators as a promising therapeutic strategy [42]. Other studies have failed to find differences in the total number of neurons [35]. Such discrepancy could be due to technical differences, divergence of ages between groups (control: 87 years vs AD: 71.5 years) and/or that specific neuronal subtypes could be more susceptible to AD pathology. For instance, three excitatory neuron subpopulations expressing the RAR-related Orphan Receptor B (RORB) are more vulnerable to tau pathology and cell death in entorhinal cortex but not in the superior frontal gyrus at early pathological stages [36]. It seems that region-specific vulnerability is linked with strong transcriptional changes in neurons in response to AD pathology.

Neurons show, together with astrocytes, the most dramatic gene signatures changes compared with other cell types (Table 1, Fig. 2). In AD prefrontal cortex, a high percentage of deregulated genes in

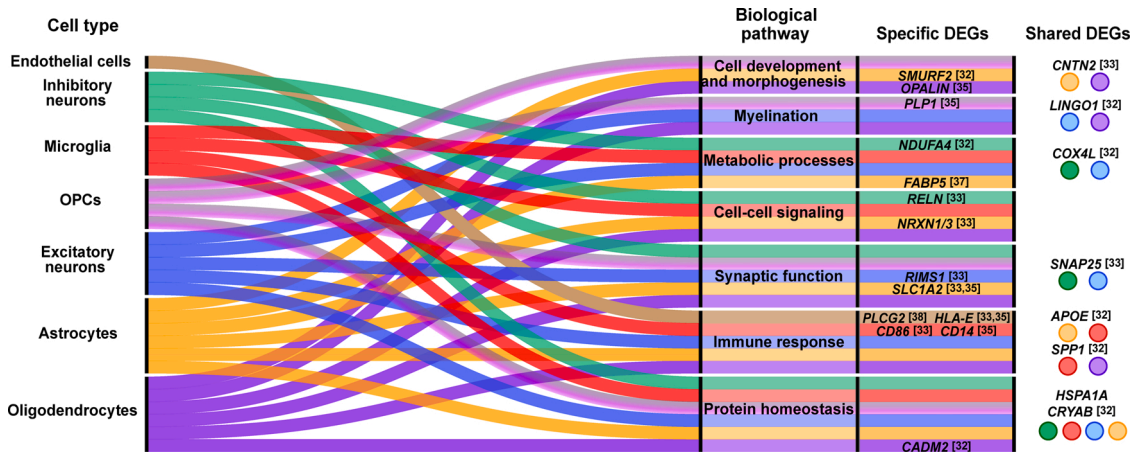

Fig. 3. Cell specific and common biological pathways and deregulated genes affected in AD. Alluvial plot depicting the link of each cell type with the most significant biological pathways affected in AD according to Table 1. For simplicity, altered biological networks reported in AD -irrespective of stage or region- were grouped into seven main terms. Cell types are depicted in different colors, and cell type-specific genes and those shared by several cell types are indicated in the right. The original studies from which the gene information was extracted are indicated in brackets.

excitatory (75%) and inhibitory (95%) neurons are downregulated [32], whereas several gene modules from excitatory and inhibitory neurons correlate significantly with AD diagnosis [34]. AD excitatory and inhibitory neurons exhibit differential expression of genes involved in proteostasis, including protein folding, autophagy, apoptosis and stress response pathways [32–34] (Table 1, Fig. 3). Interestingly, genes involved in axon outgrowth, regeneration and myelination are the most deregulated genes in AD prefrontal cortex excitatory neurons, but not inhibitory neurons [32]. This is surprising because although myelin ensheathes mainly excitatory axons in neocortical regions it also covers inhibitory axons, specially of parvalbumin interneurons [43]. In addition, excitatory neurons show downregulated genes related to amyloid fibril formation, Wnt/calcium signaling pathway, and axon guidance and transport, and increased expression of synaptic transmission/memory, protein ubiquitination/phosphorylation, and cell death/adhesion [34]. The similar transcriptomes of affected excitatory neurons in distinct vulnerable brain regions suggest the possibility for common regional mechanisms of excitatory vulnerability in response to AD pathology. In inhibitory neurons, the ERBB2 signaling, glucose transport, mRNA processing regulation and neuron death pathways are upregulated, whereas genes involved in synapse organization and signaling, cell adhesion, protein dephosphorylation and membrane repolarization are downregulated [32,34].

In agreement with previous bulk transcriptomic analyses [12,13,15, 19,44], expression of synaptic genes are deregulated in excitatory and inhibitory neurons in AD prefrontal and entorhinal cortices [33,35,36] (Table 1). Neuronal subpopulations enriched for axon, ion and vesicle transport, synapse and neurotransmitter signaling genes are decreased in AD [33,35]. Particularly, excitatory and inhibitory neurons of AD entorhinal cortex show decreased expression of genes regulating excitatory synaptic transmission (*SNAP25*, *RIMS1*), and ion transport and memory/cognition (*CCK*, *SST*, *RELN*, *VIP*, *KCNIP4*), respectively [33]. Changes of synaptic genes are relevant considering the possible role of excitatory/inhibitory imbalance in the cognitive decline in AD [45]. Indeed, excitatory/inhibitory disbalance is associated with altered excitatory and inhibitory postsynaptic proteins as well as degeneration/dysfunction of specific inhibitory neurons [46–48]. Despite the distinct vulnerability of neuron subtypes in AD brain, it remains unclear the specific neuronal subtypes and individual or sets of excitatory and inhibitory neuronal genes that contribute to excitatory/inhibitory transmission imbalance, memory circuit dysfunction and neurodegeneration in AD.

4.2. Astrocyte transcriptomes in Alzheimer's disease

Cerebral inflammation, a common pathological feature of aging and neurodegenerative diseases, is characterized by the presence of inflammatory molecules, reactive astrocytes and activated microglial cells [49]. Astrocytes maintain brain homeostasis and function by supporting metabolic and trophic factors to neurons. They also play active roles in pathology and cognitive impairment in AD, for instance by engulfing dystrophic neurites and synapses, and causing hippocampal dysfunction [50–52]. Astrocytes adopt a diversity of pathological and cellular changes depending on pathological and clinical AD stages [32,33]. snRNA-seq analysis of AD prefrontal cortex shows astrocyte subpopulations with elevated levels of stress response (*CRYAB, GFAP*, *LINGO1*) and synaptic assembly/signaling genes, and downregulation of glial differentiation and glutamate transport/metabolism genes (*SLC1A2*, *GLUL*) [32,35]. Similar analysis in astrocytes from AD entorhinal and somatosensory cortices show enrichment of genes associated with proteostasis (*CRYAB*, *HSPB1*, *HSPH1*, *HSP90AA1*), mitochondrial oxidation, inflammatory, glial cell differentiation and myelination pathways, and decreased homeostatic genes related to glutamate transport/metabolism and synapse remodeling (*SLC1A3*, *SLC1A2*, *CSF1R*) [33,36,40] (Table 1, Fig. 3). These astrocytic gene changes are associated with AD pathological and clinical markers suggesting that astrocytes respond to pathological and network changes by modulating their transcriptomes. It is currently unclear which specific astrocytic subtypes or transcriptomic responses contribute to neurodegeneration.

It is intriguing that *APOE*, the major genetic risk factor associated with late onset AD, is downregulated in AD astrocytes, oligodendrocytes and OPCs but upregulated in microglia [32,33]. A balance of *APOE* levels or isoforms seems critical for astrocyte function as suggested by recent studies showing that astrocytic APOE4 promotes neuronal activation, disrupts clearance of Aβ and cerebrovascular integrity, while selective inactivation of *APOE3* and *APOE4* in astrocytes reduces plaque deposition and microglial reactivity[53–56]. Moreover, differential increase of calcium signaling due to *APOE4* vs *APOE3* expression may lead to changes of activity-regulated transcriptional alterations in astrocytes [57,58]. Other genes simultaneously increased in astrocytes and neurons or oligodendrocytes include: the Bridging Integrator 1 (*BIN1*), the second largest genetic risk factor for sporadic AD associated with tau and amyloid pathologies; *LINGO1*, a negative regulator of myelination; and *NEAT1*, a regulatory long non-coding RNA [33,34]. Interestingly, the AD risk genes *CLU* and *IQCK*, found in an astrocytic gene module enriched in metal ion homeostasis and proteostasis, are upregulated in reactive astrocytes and associate with tau and/or amyloid pathologies

[32,40]. These results indicate that astrocytes acquire a reactive immunological phenotype characteristic of astrocytosis in AD brain. Accordingly, a subcluster of astrocytes expressing high levels of GFAP in AD entorhinal cortex show reduced levels of transcripts associated with glutamate/GABA homeostasis and synaptic adhesion/maintenance, suggesting that these are reactive astrocytes with compromised homeostasis [36]. Whether astrocyte dysfunction is due homeostatic gene changes is unclear, but a recent bulk RNA-seq meta-analysis of astrocyte-specific gene sets identified altered astrocytic genes associated with endolysosomes and mitochondrial dysfunction in AD [27]. Future studies should discern the relevance of these astrocytic subpopulations and gene changes in the pathogenesis and clinical progression of AD.

4.3. Microglia transcriptomes in Alzheimer's disease

Microglia are immune cells of the central nervous system that respond to pathogens and degenerating cells and contribute directly to synapse and cell loss in AD [5,59]. Disrupted microglial transcriptional signatures are associated with Aβ/tau pathologies and phagocytosis in AD brain [25,40], which suggests that microglia may adapt to cerebral changes in aging and neurodegeneration through deep transcriptomic changes [60]. The increase of microglial cells in AD brain should be considered when analyzing microglia transcriptomes [22,23,36]. According to transcriptome profiles, and depending on the region and physiological state, the human brain contains from 4 to 14 microglia subpopulations, some containing disease-associated microglia (DAM) signatures [32,35,36,61]. Particularly, three microglia subpopulations, one of them exhibiting reduced expression of homeostatic, synaptic pruning and cytokine genes, contribute significantly to microglial transcriptomic changes in AD prefrontal cortex [35]. An independent single-cell study of prefrontal cortex revealed genes changes in several microglial clusters enriched in genes related to Aβ and PHFs [61]. However, microglia cluster 7, the most enriched for DAM, MHC antigen presentation and endosomal/vacuolar genes, is the only cluster whose genes are downregulated in relation to pathological and clinical diagnosis of dementia [61].

This raises the possibility that subpopulations of DAM participate in inflammatory responses and synaptic pruning imbalance in AD. Accordingly, microglia exhibit reduced homeostatic genes and age-independent increase in inflammatory gene signatures in AD brain [32, 33, 62–64] (Table 1, Fig. 3). In AD entorhinal cortex, microglia show enrichment in autophagy, phagocytosis, inflammation and proteostasis pathways, and downregulation of homeostatic (*CX3CR1*, *P2RY12*, *P2RY13*), cell adhesion (*CD86*, *CD83*), lipid response (*LPAR6*) and G-protein receptors (*GPR183*, *LPAR6*) genes [33,40]. Loss of basal microglia functions, indicated by reduced homeostatic microglial genes, could represent a microglial response to AD pathology [62]. It is important to consider that technical differences, including age/-postmortem delay, sample preparation, sequencing approaches, cell annotation, and downstream data analysis pipelines (see below), may directly affect the results, which can explain some discrepancies of gene profiles among these studies. For example, prominent artifactual gene signatures reported in microglia are caused by enzymatic dissociation of human and mouse brains [65], whereas in some cases snRNA-seq methods fail to detect changes in microglial activation and DAM genes [64].

The identification of microglial-specific AD risk genes supports not only a causative role of microglia in AD but also provides a strong link between genetic risk factors and microglial responses in AD [49]. Gene association studies have identified several genetic risk factors (e.g., *APOE*, *TREM2*, *MEF2C*, *PICALM*, *HLA-DRB*, *HLA-DRB5*) in microglial gene modules of AD prefrontal cortex [32]. Expression of microglial genes linked with AD (*APOE*, *MS4A6A*, *PILRA*) or other neurodegenerative diseases (*LRRK2*, *SNCA*, *GPNMB*, *GRN*) are positively correlated with Aβ and pTau in AD entorhinal cortex [40]. Notably, microglia from AD patients harboring *TREM2* variants show upregulation of genes related to ERK and NFκB signaling, homeostasis (*CX3CR1*, *P2RY12*, *TMEM119*) and DAM (*APOE, CD68*, *CH13L1*, *SORL1*, *TREM2*), whereas the metal-ion homeostasis gene cluster is the top downregulated pathway in microglia [37] (Table 1). Among these genetic factors, *APOE* is consistently upregulated across studies in microglia of AD brain [32, 33,40]. This result is relevant considering that immune responses and Aβ clearance are affected in human *APOE4*-expressing iPSCs-derived microglia [55]. Future investigations should discern the functional and pathological consequences of altered *APOE* isoforms in microglia activity, phagocytosis and dysfunction.

4.4. Oligodendrocyte transcriptomes in Alzheimer's disease

Oligodendrocytes are the unique source of axonal myelin that allows electrical transmission and metabolic coupling in the adult brain. Gross dysregulation of genes profiles and hubs in oligodendrocyte subpopulations suggests a pathogenic involvement of oligodendrocytes and OPCs in this disease (Table 1, Fig. 2). In AD prefrontal cortex, oligodendrocyte subtypes show downregulation of ribosomal subunits and myelination genes (*MAG*, *CNP*, *PLP1*), and upregulation of protein folding, translation and cell death genes (*CADM2*, *CRYAB*, *QDPR*, *NLGN1*) [32,34]. Transcriptional changes in specific oligodendrocyte subpopulations are also observed in AD entorhinal cortex [33,36]. These results reinforce the idea that oligodendrocytes and myelination-related pathways may be affected across vulnerable brain regions leading to age-dependent demyelination and memory loss in mild cognitive impairment, AD and vascular dementia [66,67].

Biological pathways affecting oligodendrocyte proliferation, differentiation and myelination are consistently detected across studies. OPCs show increased expression of glutamatergic receptor signaling, synaptic and neural development genes, and downregulation of protein folding, mitochondrial complex, cell proliferation and myelination genes [32, 34] (Fig. 3). AD oligodendrocytes show enrichment of protein folding, metabolic, oxidative phosphorylation/stress and cell death genes, and dysregulation of myelination genes, involving upregulation (*BIN1*, *CNTN2*) or downregulation (*CTNNA2*, *OPALIN, SLIT2*) [33–35, 37] (Fig. 3). Interestingly, cell subcluster analysis reveals increased number of oligodendrocytes expressing remyelinating genes and reduced mature oligodendrocytes enriched in myelination genes [33–35]. Whether these transcriptional changes result from oligodendrocyte dysfunction and/or a shift from OPC differentiation to oligodendrocytes in response to pathology is still unclear. Nonetheless, increased oligodendrocytes subtypes and reduced OPCs at late pathological stages strongly supports a role of differentiation of OPCs into mature oligodendrocytes with increasing pathology [22]. In fact, a gene set enriched in oligodendrocyte differentiation and myelination pathways correlates positively with amyloid and NFT pathologies in prefrontal cortex [32], whereas oligodendrocyte gene markers (*MAG*, *MBP*, *MOBP*, *CLDN11*, *CNP*) are reduced at Braak III-IV stages in AD precuneus, a parietal cortex region vulnerable to early amyloid deposition [62]. Of relevance, a spatial transcriptomics study showed that amyloid plaques alter expression of oligodendrocytes genes (e.g., *CRYAB*, *QDPR*) resulting in gene changes in inflammation, oxidative stress and lysosome pathways [68]. These results likely reflect oligodendrocyte compensatory responses to neuron and/or myelin loss occurring in AD. Together, these transcriptomic analyses further support a role of oligodendrocytes on neuronal demyelination in AD. Whether changes in oligodendrocyte maturation or myelination genes mediate the effects of Aβ and/or tau on axonal demyelination and degeneration requires further investigation.

4.5. Endothelial cells transcriptomes in Alzheimer's disease

Endothelial cells, fibroblasts and pericytes, together with neurons and glial cells, are part of the cerebrovasculature of the blood-brain barrier (BBB) that controls entry of pathogens and delivery of nutrients and oxygen to the brain. Dynamic magnetic resonance imaging

shows that BBB damage and permeability in the hippocampus is exacerbated in people with mild-cognitive impairment likely contributing to early memory impairment during aging [69,70]. Single-cell transcriptomics revealed selective vascular cell vulnerability, comprising reduction of endothelial cells, fibroblasts, pericytes and arterial smooth muscle cells, in hippocampal and frontal cortex vasculature of AD brain [38], which likely contributes to the neurovascular damage in dementia [71]. It is interesting that the abundance of specific endothelial cells subpopulations increases in AD brain at early and late pathological stages [35,36]. Particularly, three endothelial cell subpopulations -e1, e3, and e4- that vastly contribute to upregulation (89%) of endothelial transcriptomic changes double in prefrontal cortex of AD brain [35]. The biological relevance of the selective vulnerability of specific vascular cell subpopulations is still unclear, but it may represent the cellular basis or a response to brain vasculature damage during neurodegeneration.

The most representative gene networks affected in endothelial cells are related to angiogenesis, cell motility/adhesion, stress cell death, inflammation, ion transport, cellular respiration and rRNA processing (Table 1, Fig. 3). Endothelial cells in AD prefrontal cortex adopt angiogenic and immune response features characterized by upregulation of angiogenic factors and receptors (*EGR*, *FLT1*, *VWF*), and antigen presentation (*B2M, HLA-E*) genes [35]. Similarly, endothelial cells in AD entorhinal cortex overexpress genes related to cytokine secretion and immune responses (*HLA-E*, *MEF2C*, *NFKB1A*), and ribosomal processes and translation initiation (*RPS19*, *RPS28*) [33]. Genes related to immunological response (interleukin-1, interleukin-2 and IFN signaling) are upregulated in endothelial cells in hippocampal and cortical vasculature of AD patients [38]. Considering the established relationship between cerebral inflammation and angiogenesis in dementia [71], it is plausible that inflammatory factors may contribute to changes in activation and/or proliferation of endothelial cells in AD and other neurological disorders [72]. Indeed, activation of the innate immune system in endothelial and glial cells, and disruption of neurovascular proteins, characterize Huntington's disease brains [73]. More recently, deregulated genes related to vasoconstriction and blood flow in mural cells and fibroblasts, and selective vulnerability of endothelial cells, pericytes and arterial smooth muscle cells were detected in AD cerebral vasculature [38]. These results provide potential molecular mechanisms for cerebrovascular damage and cerebral blood flow dysfunction in AD [74]. Considering that cerebrovascular disruption is a common feature of neurodegenerative diseases, it may be relevant to integrate transcriptomic information from several neurodegenerative diseases to discern whether common biological pathways and genes in endothelial cells are affected in distinct brain pathologies.

5. Conclusions and future challenges

Single-cell transcriptomics has uncovered diverse cellular heterogeneity and dynamics of the aging and degenerating brains, particularly of postmortem AD samples. The identification of disease-related gene networks and pathways in multiple cell types has further unraveled unique and shared biological responses to pathology in the degenerating brain, shifting the neurocentric view of brain diseases. Gene regulatory network analyses of scRNA-seq data have allowed the identification of potential -not validated yet- transcription factors mediating transcriptional disturbances in specific cell subtypes of AD brain [33,34,36,40]. For instance, the transcription factor RORB was identified in vulnerable excitatory neurons of AD preferentially affected by NFTs [36], but its role in neurodegeneration and pathology is still unknown. Although transcriptome dysregulation in AD brain differs from normal aging [22], the fact that several cellular pathways are shared between aging and AD suggests that some transcriptomic changes could be due to the aging process [75]. This may explain why gene expression profiles and subpopulations of microglia do not significantly differ in cortical regions in non-demented elderly people and dementia patients [25,76]. It is also possible that changes in the relative abundance of cell subtypes may contribute to apparent changes of transcriptome profiles, as suggested by a recent study showing that altered microglia and astrocyte gene modules arise from cell-type composition changes [23].

Single-cell transcriptomics has satisfactorily demonstrated multiple transcriptomic and molecular changes occurring in distinct cell populations of vulnerable anatomical regions of AD brain (Fig. 2). However, it is noteworthy that the number of overlapping DEGs in specific cell subtypes is relatively low across studies, which may be caused by differences in the experimental and data analysis methods. Due to difficulties for isolating undamaged cells from fixed or frozen post-mortem brain, a step required for optimal gene profiling, most studies employ snRNA-seq that does not represent exactly the whole cellular transcriptional state [77]. As recently reported in microglia [64], differential analysis by snRNA-seq or scRNA-seq can directly affect the transcriptional cell profiles. Other important constrains of snRNA-seq studies are the limited number of sequenced cell types and analysis of nuclear transcripts that are not necessary a direct measure of translational profiling. Other technical issues, particularly related to sample preparation, can severely impact on gene profiling [77]. Fortunately, artifactual gene signatures in glial cells due to enzymatic dissociation can be overcome using alternative protocols [65]. Differences and limitations of the experimental designs and protocols, particularly regarding small sample sizes, sample preparation, restriction to specific brain areas and pathological stages, may lead to discrepancies in the absolute transcriptional profiles (i.e., number of DEGs). Despite this, most studies have revealed consistent cell-specific gene expression alterations, at least within similar brain regions. Intriguingly, single-cell analyses performed in entorhinal cortex, an early pathological affected region, highlighted deregulation of synaptic-related genes in neurons and glial cells [33,36], whereas changes in glial-related pathways with minor or unreferenced disturbances in synaptic pathways are detected in the prefrontal cortex [32,34,37], consistent with previous network-based microarray analysis [24]. This is surprising, given the roles that glial cells play on regulating neuronal and synaptic activities in health and disease, and it may suggest that synaptic dysfunction may not be properly captured at the transcriptional level at distal cortical areas affected during late pathological stages.

Single-cell sequencing applied to postmortem human brain has multiple applications but still important caveats, such as low number of brain specimens, properly represented cell types and detected DEGs, and lack of spatial resolution. Indeed, a limitation of currently available single-cell technologies, reflected in the reviewed literature, is the difficulty to account for human biological variability, which in the case of a complex age-dependent brain disease such as AD requires large cohort studies. The quality and validity of single-cell transcriptomics data depend on considering biological variability, and methods that ignore this are biased and result in false discoveries [78]. Compared to general scRNA-Seq, another important limitation of snRNA-seq from postmortem tissue is that some cell subtypes are under- or over-represented due to the dissociation process or artificial filtering, which negatively affects gene profiling [77]. Optimization of cell isolation methods from postmortem brain will undoubtedly increase the number and quality of sequenced cell types. Standardization of cell clustering annotation methods (computational, manually curated, machine learning) can facilitate the classification of cell types for raw datasets comparison, which will reduce discrepancies in cell classification between studies [77]. Finally, a major weakness of most of the reported scRNA-seq studies is the lack of tissue spatial information within an anatomical region. Incorporating spatial cell transcriptomics is critical for dissecting the complexity of cellular interactions in the context of local pathological changes occurring in the degenerating brain [77]. Overall, this reveals the need for reliable studies using rigorous experimental designs and validation protocols directed at assessing brain regions affected at early AD stages when diagnosis and therapeutic interventions are critically needed.

Thus, considering the marked protocol differences among the

reviewed studies, which directly affect data analysis and interpretation, future studies should consider benchmarking, standardization and quality control of experimental and computational pipelines [79]. We think that integrating multiple single-cell omics modalities (genomics, transcriptomics and proteomics) in larger human cohort studies is critical to reveal disease-associated alterations, especially at early AD stages. Finally, future studies should include spatial gene profiling in early vulnerable brain regions at different pathological stages to identify relevant cell-specific mechanisms of therapeutic and diagnostic value. It is expected that a deeper understanding of the cell-specific mechanisms mediating neurodegeneration will facilitate the development of novel therapeutic strategies effective to combat this devastating disorder.

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Declaration of interests

The authors declare no financial competing conflicts of interest in relation to this work.

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