Neurodegeneration cell per cell.

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Abstract

The clinical definition of neurodegenerative diseases is based on symptoms that reflect terminal damage of specific brain regions. This is misleading as it tells little about the initial disease processes. Circuitry failures that underlie the clinical symptomatology are themselves preceded by clinically mostly silent, slowly progressing multicellular processes that trigger or are triggered by the accumulation of abnormally folded proteins such as Aβ, Tau, TDP-43, α-synuclein, among others. Methodological advances in single-cell omics, combined with complex genetics and novel ways to model complex cellular interactions using induced pluripotent stem (iPS) cells, make it possible to analyze the early cellular phase of neurodegenerative disorders. This will revolutionize the way we study those diseases and will translate into novel diagnostics and cell-specific therapeutic targets, stopping these disorders in their early track before they cause difficult-to-reverse damage to the brain.

Main text

Neurodegenerative diseases (NDs) are affecting more people worldwide than cancer, and healthcare systems are not prepared to deal with the threefold increase of cases expected by 2050. Research into NDs is still building on concepts and insights from more than a century ago. The definitions of different NDs remain descriptive, and the borders between disorders are blurred. Amyloid plaques, neuronal tangles, Lewy bodies, and TDP-43 inclusions are hallmarks of different NDs; however, such pathologies are frequently present at the same time in the same patient, and they are associated with different clinical symptoms and different diagnoses.[1] The ALS-FTD spectrum of diseases is an excellent example where the same genes and similar biochemistry are leading to primary motor neuron disease (ALS) or to frontotemporal dementia (FTD). Furthermore, TDP-43 pathology is associated with amnestic dementia when localized to the hippocampus and is then called limbic-predominant age-related TDP-43 encephalopathy.[2] Another example is posterior cortical atrophy (PCA) which manifests with visual problems but is, in fact, a form of Alzheimer's disease (AD).[3]

To provide disease-modifying therapies, we need to move away from the century-old clinical classifications toward molecular descriptions at cellular resolution.[4] This is already well underway for AD, which is neuropathologically defined by Aβ and Tau aggregates; yet the adoption of biomarkers for *in vivo* biological disease definition as laid out by the National Institute on Aging and Alzheimer's Association Research Framework is not yet broadly adopted in routine clinics.[5] PD remains clinically defined through symptoms,[6] blurring the molecular definition and stratification of this syndrome.

Neurodegenerative disorders are complex, decade-long processes of cellular action and reaction, triggered in many - but not all - cases by the accumulation of abnormal proteins. We also must surpass the neuro-centric view and consider the mounting evidence pointing to different glial cell types and vasculature at early disease stages. Unfortunately, these initial cellular reactions are characterized poorly. Real progress and solutions will ultimately come from understanding this early cellular phase of disease and identifying drug targets that operate at this stage.

When the concept of the cellular phase of AD was proposed, it predicted that advances in single-cell biology would revolutionize the understanding of AD.[4] Six years later, this 'single-cell

revolution' has yielded tremendous insights into ways the different cell types, including neurons and glia, deal with amyloid plaque stress and how their molecular profiles adapt and are involved in the initiation and chronic propagation of disease. Similar breakthroughs are made in the field of Parkinson's disease (PD) and other NDs.

The cellular phase in disease can be hypothesized as (i) An initial trigger, called the biochemical phase (Aβ in AD or alpha-synuclein in PD or Tau in primary Tauopathies, amongst others), and some cell types are more prone to these reactions than others. The buildup of these toxic species causes cellular reactions and homeostatic imbalance (for instance, neuronal hyperactivity in AD) (ii) This evokes protective responses from glial cells. These protective responses become chronic, creating pathological stress in both neurons and glial cells, broadly described as "neuroinflammation" but better called "glial reactivity." (iii) Non-cell autonomous factors derived from the glial cells evoke or reinforce pathological cellular mechanisms in the neurons (Figure-1). This process runs over many years and ultimately leads to the failure of brain functional homeostasis and the clinical symptomatology that we currently use to define the diseases. The link between symptoms and the initial triggering events is very indirect and blurred by the complex cellular cascades that precede the collapse of brain function. Moreover, it is evident that upstream of the biochemical triggers, other alterations might play a role in the initiation of these diseases. These environmental and genetic factors upstream of the biochemical phase are important to understand and may affect some cell types more profoundly than others. However, the pathogenic aggregates and biochemical alterations of the biochemical phase are here considered to trigger specific pathways of neurodegeneration. The fact that Mendelian inherited mutations in those proteins are sufficient to trigger the full disease pathways provide a strong scientific rationale for this point of view.

Over the last six years, enormous progress in single-cell omics has helped to start to map these cellular disease processes. The initial push for this novel thinking comes from the immense flow of genetic risk factors identified in genome-wide association studies (GWAS) and other genetic studies. The complex information from those studies can be understood in the context of the cellular phase of these disorders. We discuss progress in AD and PD research.

Complex genetics interpreted in a cellular context

Rare, 'familial' forms of AD, PD, and other ND are caused by pure Mendelian inherited mutations. They link disease directly to specific proteins such as presenilin, APP, α-synuclein, PINK-1, LRRK-2, etc., making these findings highly informative to further functional research. But also, non-familial, confusingly called "sporadic", forms of ND are highly loaded by genetic risk: 36% in PD,[7] 60-80% in AD.[8–11] Despite enormous progress, mainly via GWAS, a large part of the inheritance remains unexplained.[12]

GWAS yield long lists of single nucleotide polymorphisms (SNPs) in the genome with their associated abundance and frequencies in disease cases compared to controls. An identified SNP can have direct effects on the functionality of a risk gene (for instance regulating its expression) or can be a signpost of another (functional) SNP with which it is in linkage disequilibrium. Frequently, the SNPs are in non-coding regions, and candidate genes are selected "by association": genes most closely to the SNP giving their name to the risk locus.[7–9,13,14] This is - often misleadingly - used to prioritize candidate genes and pathways. For instance, the PICALM locus was initially identified as a top hit in AD GWAS,[15] but in the latest and largest iteration, the major risk gene in this locus became the EED gene,[8] the product of which has a very different function than PICALM.[16]

It is challenging to link putative regulatory DNA elements in which a SNP is located to actual function and gene expression in specific cells. One powerful strategy is to identify, across the entire genome, the genes whose expression levels associate with the GWAS SNPs. This can be done for entire tissues, but also cell type per cell type, combining single cell expression profiling for each locus ("expression quantitative trait loci (eQTL)") with GWAS signals. This defines which genomic variant associates with (nearby) gene expression changes (cis-eQTLs).[17–20] Currently, this is done with mRNA eQTLs, but in the (near) future, it will be more powerful to use protein QTLs. Using massively parallel reporter assays Cooper et al. screened 5706 variants from AD and PSP GWAS loci to identify 320 functional regulatory variants, which they then linked to driver genes by CRISPR validation. This represents an important step towards pinpointing the causal SNPs amongst a plethora of variants co-inherited in linkage disequilibrium blocks.[21]

Further compelling developments combine GWAS and eQTL with snATACseq (single-nuclei Assay for Transposase-Accessible Chromatin sequencing) and ChIP-seq (chromatin immunoprecipitation sequencing). This integrates cell-specific open or active chromatin and promoter data,[22] and correlates each cell type, gene expression, and open chromatin with the GWAS SNPs.[23] Evidently, this type of information is essential to study the risk genes in the relevant cell types and context. Furthermore, they also identify the cell types in which the GWAS SNP heritability is most pronounce.[22,23] A major problem here is that we lack good models for NDs to put these predictions to the test. Possibly chimeric models where human cells grow in the mouse brain can provide part of a solution.[24–28]

To date, ˜75 AD risk loci have been identified from 111,326 cases and 677,663 controls.[8,9,14,29] The putatively associated genes strongly imply microglia and hint at endocytosis, lipid metabolism, immunity, and to APP and Tau processing as the cellular processes involved in this disease.[8] In addition to microglia, also endothelia,[30] and probably other cell types express risk genes, clearly supporting the idea that AD is a multicellular disorder.[4] In PD, 90 risk variants are identified from 37,688 PD cases, 18,618 proxy-cases, and 1,417,791 controls.[7] There is intriguingly little overlap between the risk genes for AD and PD, providing genetic evidence for different disease onset and progression mechanisms (Table 1). The putative PD GWAS genes are expressed in Substantia nigra (SN) dopaminergic neurons and cortical excitatory neurons,[31–35] consistent with the known vulnerability of the nigro-cortico-striatal system, and possibly explaining the emergence of dementia in PD patients. There are also significant signals from peptidergic neurons when mapped to neurons of the mouse brain,[36] that possibly explain some of the sleep disturbances seen in patients,[37] and also from cholinergic neurons, monoaminergic neurons (dopamine and serotonine), and enteric neurons in the gut, that correlate with the known problems of constipation in PD.[6] Interestingly, almost all these cells are known to degenerate in PD.[38–41] The putative PD GWAS genes point to endo-lysosomal, autophagy or mitochondrial systems with evidence of lysosomal enrichment in astrocytes, microglia, and oligodendrocytes.[42,43] While these genetic studies do not reveal how these cells cooperate to cause disease, the fact that gene expression alterations associated with GWAS SNPs occur across cell types highlights the need to consider and analyze AD and PD as systemic diseases of multiple faulty cellular interactions.

Microglia

Microglia play a pivotal role in maintaining CNS homeostasis.[44,45] The first reports involving microglia in AD and PD are decades old.[46,47] Despite this earlier work, microglia only took center stage when GWAS suggested that a significant part of the genetic risk of AD is located in loci expressed in microglia,[48] and with the identification of rare TREM2 variants,[49,50] that increase risk of AD. TREM2 (Triggering Receptor Expressed on Myeloid cells 2) is expressed in tissue-resident macrophages and microglia. TREM2 binds to various ligands, including Aβ oligomers (reviewed in[51]). TREM2 signaling activates phagocytosis, lysosomal function, and lipid metabolism.[52,53] Patients with complete loss of TREM2 function present an autosomal recessive disorder called Nasu–Hakola disease, characterized by cystic bone defects and severe presenile dementia. Interestingly, these patients do not develop AD-related pathology.[54] This shows that the loss of function TREM2 variants associated with AD should not be considered causal to the disease, but as risk factors, which modulate the response to initial Aβ accumulation. This puts TREM2 dysfunction central in the cellular phase of Alzheimer's disease.

Single-cell studies in mouse models of AD as well as in postmortem human brains reveal that the transition from homeostatic to "disease-associated microglia" (DAM) microglia depends critically on TREM2 signaling.[52,55] Notably, TAM receptors that aid the phagocytosis of Aβ plaques by microglia are downstream of TREM2 signaling.[56] It has become increasingly clear that changes in the microglial response toward accumulating proteopathic lesions precede overt clinical symptoms in AD,[53,57] and that a large part of the genetic risk of AD translates into altered microglia responses. Evidence also indicates that microglia might be involved in the spreading of Tau pathology,[58–60] and may, in some cases, even be upstream in the cascade, altering cell state even before amyloid plaques appear.[61] Also, aging has been shown to directly impact mouse and human microglia, characterized by an accumulation of lipid droplets, a phenotype that is also triggered by amyloid plaques.[62,63]

Microglia adapt their phenotype in response to changing environments,[64] such as injury, dying neurons, or extracellular amyloid accumulation.[26,52,65–69] However, the nomenclature used to

indicate such different microglia cell states (as opposed to cell types) is confusing and rapidly evolving.[70] Nevertheless, the fact that these cell states are influenced by putative risk genes associated with AD makes their study relevant. For instance, the induction of DAM,[52,53,68,69] also called 'microglia neurodegenerative phenotype' (MGnD) is strongly dependent on TREM2 and APOE function,[52,68] and mutations associated with TREM2 in AD stall the microglia in a homeostatic state, suggesting that the 'disease associated microglial' response is protective.[52,71,72] A more crucial problem is that little is known about the biological repercussions and the impact on the disease of these different microglial cell states, and it is unclear to what extent the transcriptomic changes translate in functional alterations. For instance, it is uncertain whether transcriptome correlate with the proteomic signatures of the cells. Another problem is that while the field has moved away from the dichotomic "M1" and "M2", the new single-cell studies introduced "homeostatic" and "activated" (or DAM-like) nomenclature and have again the tendency to describe in a binary way microglia cell states.[70]

Two major problems complicate further the functional study of microglia in neurodegenerative disorders. One is technical: it is very difficult to culture disease-relevant microglia *in vitro* as they rapidly adopt phenotypes that are very different from those observed *in vivo*. Work is ongoing to improve culture conditions to better mimic physiologically relevant conditions,[73] but the task is not easy. Both transcriptional and proteomic expression of hundreds of proteins are different between *in vitro* and *in vivo* microglia, with major switches in protein synthesis, glucose metabolism, and sensing receptors.[74] The second problem is the large difference in gene expression between mouse and human microglia.[26] Especially when polygenic risk and the potential of gene interactions are considered, it becomes difficult to build models that do not use human cells.

Microglia from human iPS cells can be cultured *in vitro* (with the limitations discussed above), but they can also be transplanted into mouse brains,[24,26,75–77] where they replace the endogenous mouse microglia to a large extent. Intriguingly, the 3D brain environment makes these *in vitro*-generated microglia adapt their gene expression profiles towards those of microglia isolated in human neurosurgery.[26,78] While mouse disease-associated microglia signatures are captured in the human microglia response to amyloid plaques, the human amyloid reactive microglia response also encompasses other cell states described as "HLA response microglia", and two or more "Cytokine

response microglia".[26,78] It appears that the human microglia response to amyloid plaques is more complex than the mouse microglia response. Using the profiles of transplanted human microglia, it is now possible to interpret cell state signatures captured from microglial nuclei isolated from post-mortem AD brains.[79–81] For further discussion of the role of microglia (and their close neighbors, the vascular macrophages) in AD there are several excellent reviews.[65,67,82–84]

Microglia have received relatively less attention in PD, but both animal studies,[85] and human studies show a correlation between reactive microglia and α-synuclein or Lewy body pathology and suggest a role for microglia or monocytes in PD.[34,43,86–90] One example is the role of LRRK2, mutations of which are disease-causing in dominantly inherited PD but which is also associated as a risk gene with sporadic PD.[7,91,92] While LRRK2 is broadly expressed, including in microglia, a non-coding variant associated with PD was identified that affects LRRK2 expression *specifically* in microglia, meaning that part of the risk in PD is conveyed by these cells.[93] Furthermore, the number of microglial cells is increased in PD, and apparently also in other Lewy body diseases,[34,89] and the microglial cell states in disease shifted from a resting state (marked by high P2RY12 expression) towards an activated state (characterized by high expression of HSP90AA1, GPNMB).[33,34] Based on this, several studies have even suggested the microglial protein P2RY12 as a drug target for PD.[86,94] However, as in AD, further work is now required to mechanistically place microglia in the cellular cascade of PD.

Astrocytes

Astrocytes control ion balance, produce neurotrophic factors, form tripartite synapses with neurons, and play pivotal roles in neurovascular integrity.[95–97] The diversity of mouse astrocyte cell populations based on single-cell RNA-sequencing reveals distinct astrocyte sub-populations in different brain regions.[98–100] Astrocytes are also implicated in disease; Alexander's disease is a primary astrocytic deficiency characterized by Rosenthal fibers in astrocytes.[101] In aging-related Tau astrogliosis and some forms of frontotemporal dementia, astrocytes develop Tau pathology.[102] Progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain diseases are tauopathies expressing the four repeat Tau splice variant and are all characterized by tufted astrocytes and astrocytic plaques.[103] It is becoming increasingly evident that infection or injury

profoundly affects astrocytes and causes them to adopt a 'reactive' pathological cell state that is defined by morphological, functional, and transcriptional changes.[96,104]

The most obvious genetic link between astrocytes and AD are APOE gene alleles, with APOE4 increasing the risk of developing AD, and APOE2 protecting (APOE3 is neutral).[8,10,12,105,106] Astrocytes are the main APOE-expressing cell type. Microglia and other cells can produce APOE, but in mice and humans, they only do so after being exposed to amyloid plaques (Figure-2). [52,78,80,107]Importantly the APOE produced by astroglia is required to generate amyloid plaques,[108] and is therefore upstream of the activation process of microglia, that subsequently start to produce APOE as well.[52,78,109,110] Thus, APOE in astrocytes is upstream of amyloid plaque formation and part of an essential feed-forward loop between astroglia and microglia.

The debate on the role of astroglia in pathology is simplified to the question of whether astrocyte responses are beneficial or detrimental. The primary role of astrocytes is to maintain brain homeostasis. Therefore, their contributions to the disease process are likely 'loss-of-function' phenomena, especially in the initial cellular phase of the disease. For instance, an early event induced by amyloid pathology in mouse models and in humans is decreased calcium signaling in astrocytes. Restoring this calcium signaling normalizes early neuronal hyperactivity in AD mouse models.[111] In contrast, at later disease stages, when amyloid plaques further accumulate, reactive astrocytes produce increased calcium oscillations, possibly reflecting an inflammatory state.[112]

In AD, astrocytes form an intriguing ring-like structure surrounding microglia that interact with amyloid plaques.[113,114] Unlike microglia, astrocytes remain largely within their spatial domains upon activation, displaying thickened cellular processes (hypertrophy) demonstrated by dye-filling.[115] Several transcriptome studies have recently started to map the complex series of cell states adapted by astrocytes in AD.[116–118] In mice, astroglia and microglia interact closely, as was demonstrated using spatial transcriptomics to identify a gene co-regulatory network induced by amyloid plaques and involving the two cell types.[119]

While extrapolating findings in mice to the human brain is not always straightforward, some overlap appears.[55] For instance, several of the plaque-induced genes were validated using in situ sequencing

of the human AD brain.[119] In addition, profiling human AD-derived astrocytes reveal the disappearance of subclusters of astrocytes implicated in lipid and oxidative metabolism (*FABP5, HILPDA, and SOD2).* Another human study agrees well with mouse data, revealing 8 astrocyte-cell-states, two of which displayed the most prominent changes in AD, characterized by shifts in ribosomal, mitochondrial, heat-shock, and immune response genes.[80,120,121] Finally, two studies stressed the presence of a population of astrocytes that express high levels of GFAP, and a disease-associated astrocyte (DAA) signature in mice.[116,122] Similar signatures were identified in the snRNA datasets from humans,[117,118] and important reactive astrocytic sub-populations that could be localized using spatial transcriptomics are altered in AD. Notably, differentially regulated genes in astrocytes are involved in neuroprotection proteostasis, phagocytosis, and protein clearance. In addition, a higher number of differentially expressed genes were observed in patients with higher Aβ load relative to Tau-only cases.[117,118]

In PD, enriched astrocytic cell-state markers have been identified as CD44/S100A6, VIM/LHX2 and CYP4F12, while astrocytic populations marked by GJB6/OXTR and CYP4F12 were depleted.[33,34] Astrocytes in PD upregulate the unfolded protein response, heat-shock proteins and response to metal ions.[34,35] α-synuclein filamentous inclusions were seen in astrocytes,[123] suggesting that astrocytes can take up α-synuclein produced in neurons.[124–127] Apparently, this induces the unfolded protein response,[123] mitochondrial impairment,[126] upregulation of major histocompatibility complex genes, and secretion of pro-inflammatory cytokines.[125,127] This is likely functionally important, as exemplified by the causal link between astrocytic unfolded protein response and neurodegeneration in a mouse prion model.[128] When α-synuclein is directly and selectively expressed in astrocytes using the GFAP promoter in mice, there is rapid and progressive motor impairment and degeneration of SN dopaminergic neurons, indicating that α-synuclein accumulation in astrocytes is sufficient to recapitulate core features of human PD.[129]

Many of the genes mutated in familial PD have canonical roles in cellular function and are expressed in astrocytes to similar extents as in neurons.[97,130] Consequently, many major risk genes and causative genes like GBA1 or LRRK2, PINK1, parkin, DJ1 and others act in astrocytes.[97,131] Reactive astrocytes derived from patient iPS cells or primary astrocytes from mice with LRRK2 disease variants are dysfunctional. They show morphological defects, mitochondrial impairment,

lysosomal alterations, dysfunctional chaperone-mediated autophagy, and macroautophagy, increased α-synuclein levels, and increased cytokine release.[132–134] Dopaminergic (DA) neurons degenerate when co-cultured with these astrocytes.[132] Like AD, the loss of astrocytic support and the ultimate conversion of these cells into a reactive state appear to be critical aspects of the disease.

The connections between astroglia and microglia are potential drug targets. Microglial-secreted IL-1α, TNF, and C1q can convert astrocytes into a reactive state, capable of inducing dopaminergic neuron death.[135] A glucagon-like peptide-1 receptor agonist could block the activation of astrocytes by microglia and this partially rescued DA neuronal loss, motor phenotypes, and life-span of mice expressing α-synuclein.[136] Another potential therapeutic inroad blocks GABA signaling in astrocytes to recruit silent DA neurons in the SN to increase their dopamine output.[137]

While astrocytes react to AD and PD pathology in various ways, additional work is now needed to reveal a coherent picture. Many studies are based on relatively low sample numbers, and transcriptomics is limited, not revealing protein, lipid or metabolic changes. Additional morphological and, importantly, functional studies will be needed to fully understand these cells' contribution to ND.

Oligodendroglia

Oligodendrocytes are remarkable cells that wrap myelin sheaths around axons to facilitate saltatory action potential propagation. They are also involved in extracellular potassium buffering and, together with other glial cell types,[138] they provide trophic and metabolic support to neurons, e.g., by shuttling lactate via MCT1 and 2 transporters.[139,140] Oligodendrocyte function is critical for axonal maintenance,[141] exemplified by multiple sclerosis, an autoimmune reaction towards oligodendrocytes that causes a debilitating and progressive neurodegeneration.[142] Also, in AD and PD, oligodendrocytes are important, supported by genetics, pathological observations, and functional studies in animal models.[143–145] Furthermore, an important factor in the context of disease is that the capacity of oligodendrocyte precursor cells (OPCs) to renew the oligodendrocyte pool, reduces with age. This clearly places the oligodendrocyte-neuron interface at particular risk in age-related diseases like NDs.

Historic observations by Alois Alzheimer described the presence of myelin debris and lipid deposits,[146,147] in AD brains. A strong GWAS signal in AD is assigned to the 'BIN1 locus', a gene that is also expressed in oligodendrocytes (but also in microglia and neurons[22,148,149]). BIN 1 has been associated with increased Tau load.[150] Furthermore, Tau pathology in AD correlates inversely with myelination: Tau pathology appears first in cortical regions where myelination happens late in development, and spreads only later to areas with higher levels of myelination.[151,152] Similarly, neurons vulnerable in PD are generally poorly myelinated,[153] and white matter myelin microstructure changes seen in MRI scans of PD patients are predictive of clinical PD subtypes.[154] Fibrillar α-synuclein inclusions are frequently found in oligodendrocytes of PD patient brains and correlate with disease severity. In multiple system atrophy (MSA), an atypical parkinsonian syndrome, α-synuclein inclusions in oligodendrocytes even constitute a neuropathological hallmark.[155] While these observations are correlative, studies in AD patient samples and transgenic amyloid and Tau mouse models show that myelin loss is part of AD progression.[156–161] Aβ oligomers *in vitro* are sufficient to inhibit myelin formation and induce oligodendrocyte cell death via ROS.[162] The consequent demyelination could then predispose neurons to develop Tau pathology.[163] In PD, α-synuclein fibrils are taken up by many cell types, including OPCs and oligodendrocytes *in vitro* and shuttle to neurons via gap junctions.[126,164–166] Hence, OPCs and oligodendrocytes play functions in disease initiation, propagation, and neuronal protection in PD and in AD.

Single-cell technologies have now started to uncover significant regional differences in oligodendrocyte cell physiology.[167] Multiple sclerosis-specific oligodendrocyte alterations have been reported at the single-cell level.[168,169] In AD, single nuclei RNA sequencing of entorhinal and prefrontal cortex samples revealed differential expression of key genes and pathways, including the discovery of reduced expression of the major AD risk gene *APOE* in OPC.[80,81] The significance of this remains unclear and contrasts with the upregulation of APOE in microglia in the disease and the crucial role of APOE in astrocytes for the initiation of amyloid plaques. Recent spatial transcriptomics from the cells around Aβ plaques in AD mouse models revealed the existence of a gene co-expression network enriched for myelin processing in oligodendrocytes.[119] A shared disease-associated oligodendrocyte signature has been identified in multiple CNS-related diseases, including AD (Figure 2).[34,170] In PD, recent evidence suggests that oligodendrocytes are reduced in

the SN of patients and characterized by increased S100B expression.[34] Two additional recent studies reinforce the link of oligodendrocytes in PD, where several PD GWAS linked genes were overrepresented in oligodendrocytes of human and mouse brains,[31,36] which was however not found in others.[23,43] In addition, deregulated genes in bulk RNA sequencing of SN samples from PD patient brains are significantly enriched in genes expressed in the mouse oligodendrocyte lineage.[36] Collectively, the data indicates important, but further to be explored, roles for myelin and oligodendrocyte support in the context of neuronal survival, including transcriptional and functional state changes that ultimately result in the loss of axonal function and ND.

Vascular and glymphatic cells

Breakdown of the blood-brain- and blood CSF barriers and defects in the glymphatic system are recurrently observed in ND. These lead to reduced clearance of pathogenic proteins and reduced perfusion that, in turn, can cause hypoxia and neuronal dysfunction, extravasation of cytokines and neurotoxins, and leukocyte infiltration into the brain, ultimately triggering or altering local immune responses (reviewed in[171–173]). Multiple elements of the vascular niche are involved, most notably altered tight junctions between endothelial cells,[174] pericyte degeneration,[175] and disruption of the vascular extracellular matrix (ECM).[176] Single-cell studies now shed new light on these processes.

Vascular cells are present in similar numbers to glial cells but are typically less represented in single-cell datasets,[177,178] likely due to sample preparation. One solution is VINE-seq (Vessel Isolation and Nuclei Extraction for Sequencing) to obtain a high resolution of single-cell signatures of the brain vasculature (Figure-2).[30] Applied to AD postmortem brain samples, endothelial cells in the hippocampus showed increased expression of inflammation-related genes, including interferon-gamma signaling. These genes were also found upregulated in endothelial nuclei from individuals with AD carrying the APOE4 allele,[30] as well as in endothelial cells isolated from the entorhinal cortex of pathologically confirmed AD donors.[80] This was correlated with greater susceptibility of the hippocampus vasculature in aging and in AD. Nuclei from cells that expressed genes linked to ECM organization (termed M-pericytes) were selectively less abundant in AD, while the proportion of the remaining pericytes did not differ.[30] Functional studies will need to test the role of M-pericytes in blood-brain barrier breakdown in AD and potentially in other diseases displaying vascular ECM disruption.

Interestingly, APOE4 knock-in accelerates blood-brain barrier breakdown in 5xFAD mice independently of amyloid pathology. This occurs via activation of the cyclophilin A-matrix metalloproteinase-9 blood-brain barrier-degrading pathway in pericytes.[179]Heterogeneous differential gene expression responses were observed in the different vascular cell types. Mural cells (pericytes and smooth muscle cells) showed a profile implicating deregulated vasoconstriction and compromised blood flow, consistent with reduced perfusion observed by MRI in AD patients.[180] Interestingly, this transcriptional profile is reminiscent of 2 rare hereditary small-vessel diseases that lead to vascular dementia, CADASIL and CARASIL, suggesting a common molecular basis for impaired blood flow and a potential link with cognitive decline.[181]

Meningeal lymphatic drainage removes Aβ from the brain, and its importance is evident when the dorsal lymphatic vasculature in 5xFAD mouse models was photo-ablated.[182] These animals displayed increased meningeal Aβ burden, increased dystrophic neurites, aberrant activation of myeloid cells (IBA1+) and accelerated cognitive decline.[182] This indicates that reduced meningeal lymphatic drainage is sufficient to cause a transition from homeostatic to activated microglia, an aberrant induction of vascular repair, and increased leukocyte transmigration mechanisms.[182] Future studies translating these findings to humans are important,[183] especially given the vastly different brain sizes and, thus different scales of required lymphatic drainage.

A considerable fraction of putative risk genes in PD and AD are highly expressed in mouse meningeal lymphatic endothelial cells.[182] APOE, which is typically associated with expression in microglia and astrocytes, is also robustly expressed in human smooth muscle cells and meningeal fibroblasts. Other genes putatively linked to GWAS signals were found in all other cell types of the vessel. This was underappreciated until recently due to the under-representation of vascular and lymphatic vessel cells in single-cell or single-nuclei analysis. For example, the recent studies that mapped AD risk SNPs in a microglia-specific enhancer and an oligodendrocyte-specific regulatory element of the PICALM gene used datasets that did not comprise vascular cells.[22,23] This underscores the need to be cautious when interpreting high-resolution omics data, where sample preparation can bias the results towards particular cell types.

Selective neuronal vulnerability and resilience

Dysfunction of specific neuronal populations and circuits causes abnormalities that define the disease. For example, hippocampal and cortical defects cause memory loss and dementia. In PD, the motor symptoms arise from a nigrostriatal dopamine deficiency. While it is unclear how specific these neuronal deficits are, the vulnerability of these neurons is built into the very definition of the NDs.[184] However, in most cases neuronal dysfunction or death often does not directly correlate with the protein aggregates characteristic of these diseases with the exception of Tau. For example, Lewy Bodies in PD do not necessarily cause cell death,[185] and there is evidence from model organisms suggesting that aggregate formation of Tau, α-synuclein, TDP-43, Ataxin, and others can, in some cases, even be protective. Another confounder is that human studies are performed on postmortem brains, with patients already in an advanced stage of their disease. This complicates the identification of the cells in which aggregation and/or dysfunction was initially triggered and obviates a deeper understanding of mechanisms leading to synaptic loss, which is a critical early feature of AD and PD.[186,187] At the end stage of the disease, cellular dysfunction tends to be widespread and complex, and many secondary events and processes blur the picture. Consequently, the question of neuronal vulnerability remains of central interest in ND research.[188,189] What are the initial triggers before pathology is visible, and why can some neurons cope where others seem to fail?

Selective neuronal vulnerability in neurodegeneration is often reduced to the questions when and where toxic aggregates form. In AD, pathological Tau accumulation occurs in excitatory neurons (e.g., cholinergic basal forebrain neurons, entorhinal cortex, subiculum, hippocampus).[190–194] Conversely, many inhibitory neurons, granule neurons in the dentate gyrus, and layer VI neurons of the entorhinal cortex are spared.[195] In PD, neuronal loss and neuronal dysfunction are present far beyond the midbrain dopaminergic neurons[196] (reviewed in[41,185]) as exemplified by olfactory impairment, sleep disturbances or constipation years prior to the onset of the pathognomonic motor features. These non-motor problems are mostly non-responsive to treatments that restore dopaminergic tonus in the brain,[6,197] indicating that cells other than DA neurons are involved.[41] Hence, aggregates and other pathological hallmarks (e.g., granulovacuolar degeneration (GVD) in AD) are important markers[198]. Still, they do not reveal the full complexity of all affected neuronal (and non-neuronal) cell types and are merely descriptive and correlative phenomena. Neuronal

features have been associated with vulnerability, including large axonal arbors, high synapse numbers, low calcium-binding capability, or pacemaker activity,[41,185] but again, no causality has been demonstrated. New methods are needed to score the contributions of specific neurons or brain areas that are functionally impaired before any degeneration is visible.

The advent of single nucleus sequencing, soma sequencing, and spatial technologies have begun to unveil the complexity of neuronal cell types and the effects of disease processes. Neuron atrophy is widespread in AD, but unbiased single nuclei RNA sequencing of human prefrontal cortex and entorhinal cortex of AD patient samples show lower numbers of specific excitatory neurons that are positive for RORB, CTC-340A15.2 and CTC-535M15.2 (Figure-3).[122] Likewise, excitatory neurons are affected in the anterior cingulate cortex in patients with Parkinson's dementia or dementia with Lewy bodies.[89] One study on AD brain samples found a loss of GAD1 and PVALB inhibitory neurons,[199] which could explain the neuronal hyperexcitability and epilepsy seen in early AD, but this needs confirmation.[200–202] One issue is that analyses in postmortem brain samples probe the remaining surviving neurons but not those that died. An interesting approach is to use FAC sorting to isolate cells with cytoplasmic Tau aggregates and those without. This strategy identified a signature of 63 pre-synaptic and trans-synaptic signaling genes in cells that accumulate Tau compared to those that do not.[203]

In PD, there is incomplete knowledge of the neuronal cell types underlying most of the non-motoric problems, and also, not all DA neurons are equally affected. Classical neuropathological methods have established that ventral tegmental area (VTA) DA neurons and dorsal SN par compacta (SNc) neurons are relatively spared, while their ventral SNc counterparts are vulnerable.[41,185] Consistently, a recent single-nucleus study comprising >22k SN DA neurons found ten subtypes, of which the ventral-most, marked by *SOX6/AGTR1,* was preferentially lost in PD and characterized by the higher expression of GWAS hits (Figure-3).[33] Another PD single-cell study found RIT2 as a marker for the vulnerability of DA-related neurons,[35,204] and other recent work uncovered a new PD-associated DA cell state, marked by high expression of *CADPS2*,[34] that is also deregulated in cellular models of PD.[205] However, this awaits confirmation as only a small number of cells was analyzed.[34] Finally, an earlier single-cell qPCR study found a subset of ALDH1A1 positive DA neurons within the SNc and VTA to be particularly vulnerable to 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP), a neurotoxin that causes DA death and induces parkinsonism.[206] While these studies have great promise to identify molecular signatures of vulnerable (or resilient) neurons, at present, most lack analysis of the complexity of neuronal subtype diversity in the human brain, or they focus on specific 'classical' brain areas identified in pathological studies, e.g., SN DA neurons in PD.

Ideally, single-cell analyses of diseased patient brains are combined with single-cell trajectories in animal models to monitor the development of neuronal dysfunction at the earliest stages. This will necessitate cross-species comparisons.[207] In one study, >200 unique neuron types across entire fruit fly brains were assayed to uncover genes and pathways associated with vulnerability and resilience to pathogenic Tau and α-synuclein, which were then used to confirm and predict vulnerable and resilient cell-types in human brain snRNA-seq datasets.[188] These and other examples (e.g.,[189,208–210]) reveal the power of single-cell approaches to identify pathways of vulnerability and resilience in specific neurons. However, there are caveats. These include that single nucleus RNA sequencing does not capture the entire transcriptome of the neuron (e.g., cytoplasm, axons, and dendrites). Another issue is the size and complexity of the human brain, which -at present- precludes a genuinely unbiased approach. Making links between observations from model systems, where broader analyses and perturbations are possible,[100,211,212] to the human brain are increasingly valuable.[207,213]

Future directions

Single-cell technologies have already proven their value for the generation of new hypotheses, but there are clear limitations: (1) There is often large inter- and intra-study variability, decreasing statistical power to detect deregulated pathways. Studies are also often underpowered, as the number of cases and controls that can be studied with these expensive methods is limited. (2) Often, only mRNA is analyzed as a proxy for describing cellular processes, but RNA abundance does not always predict protein or lipid function.[214] (3) The vast majority of measurements are carried out in dissociated cells, which omits the critical spatial component of a highly structured organ such as the brain. (4) Study designs are typically cross-sectional and thus do not allow for establishing causality. An important concern is that human single-cell and single-nuclei data are collected from individuals with various genetic backgrounds, collected postmortem, towards the late stages of ND.

This undoubtedly introduces variance that complicates data integration and interpretation. Tools to streamline data integration across labs and conditions are urgently needed.[215]

The availability of single-cell brain datasets is currently mostly limited to mRNA measurements, with an increasing number of studies reporting chromatin accessibility and, more recently, other epigenetic modifications.[23,216–218] With the advent of commercially available or open-source methods to measure two or more of these parameters from the same cell,[219,220] multimodal data integration will be facilitated, and an unambiguous link between the modalities can be established where otherwise computational integration algorithms were necessary.[221,222] While these readouts are invaluable in assessing disease-related cell states and for interpreting GWAS risk SNPs in a cell-type-specific manner, the different modalities can be discordant.[223] Moreover, it remains challenging to infer protein levels from mRNA data, and protein co-expression modules associated with the disease are not necessarily observed in corresponding RNA-based network.[224] Thus, it is desirable to push proteomics to the single cell level, maybe by pooling similar cells defined by morphology or transcriptional state or by function from different brain areas, both in health and disease conditions. Several different approaches are already available, amongst them dual mRNA and protein measurements of dissociated cells through antibody pools that are conjugated to unique oligonucleotide barcodes.[225–227] With increasing sensitivities of mass cytometers, high throughput single-cell proteomics of dissociated cells is already on the horizon.[228] Proteins localized to axons and dendrites, can be biotinylated to detect them selectively in subsequent bulk mass spectrometry. This approach has been taken to disentangle the subcellular proteomes of midbrain dopaminergic neurons,[229] providing a valuable resource. To complete our understanding of NDs with cellular resolution, also single-cell or cell-type specific metabolomics and lipidomics are necessary.[230–232] Importantly, mounting evidence indicates disease-modifying mechanisms related to lipid metabolism in NDs.[233–239] Finally, to decipher disease mechanisms and to understand selective vulnerability, in particular the unique structures, electrophysiological properties and brain-wide connectivity across different neuron types will - in addition - need to be integrated with the molecular definitions of cell types across different time points.[213]

Several spatially-resolved transcriptomics technologies have become commercially available or published by academic labs, each trading-off spatial resolution, detection efficiency, and the

number of targets that can be interrogated simultaneously (reviewed in[240]). These spatial methods either rely on the physical isolation of individual cells, e.g., NICHE-seq[241] and proximID[242], imaging, e.g., *in situ* sequencing,[243] MERFISH[244] and SeqFISH+,[245] or sequencing, e.g., Spatial Transcriptomics,[246] and Slide-seq.[247] New advancements in spatial sequencing, such as MERFISH, CosMx, Stereo-seq, and sci-Space technologies can capture the subcellular resolution of the specific mRNAs in axons or in dendrites,[208,248,249] in some cases with enlarged capture areas in tissue chips up to several cm^2.[208] Altogether, spatial omics methods will be key to our understanding of how brain cells interact and influence each other. Here again, technological advances are supplemented with powerful computational tools to model how gene expression within a given cell is influenced by interacting cells, e.g., CellPhoneDB,[250] CellChatDB,[251] or NicheNet.[252]

Capturing early defects in NDs is critical - since treatments at this stage would likely have the largest impact. We discussed above already the limitations of postmortem brain material. Nevertheless, a comparison of transcriptomes obtained from brains in an early *vs.* late stage of the disease have revealed early cholesterol metabolism deregulation in astrocytes in AD and early disturbed synaptic homeostasis changes in PD dopaminergic neurons.[35,253] However, to uncover the earliest cell-type specific molecular mechanisms of neurodegeneration, we need disease models that can capture these initial steps. Disease models are also important for establishing causality.

IPS cells provide exciting possibilities to dive deep into the human biology of neurodegeneration. The flexibility of this system makes it a well-suited model for studying human disease. Technically, reprogramming cells into iPS cells erases their somatic epigenetic pattern, including marks of aging and disease; this is often a disadvantage. For example, 4-month-old iPS cell-derived cerebral organoids showed similarity to 13 weeks post-conception human embryonic brain.[254] Given the role of aging as a primary risk factor for NDs, an attractive alternative is to use transcription-factor-based direct conversion of already differentiated cells, typically fibroblasts. Such directly converted "induced neurons" showed more mature marker expression and functionality.[255] Interestingly, converted induced neurons from AD donors reproduced transcriptomic signatures of AD, including the downregulation of genes related to synapse organization and function and the upregulation of stress- and protein-folding-related genes. This study also suggests that neuron-autonomous mechanisms may confer vulnerability to AD pathology. Caveats and limitations have to be

acknowledged for both cellular models. Up to 72% of skin fibroblast-derived iPSCs contain ultraviolet light (UV)-related DNA damage, while 26.9% of blood-derived iPSCs harbour mutations in the BCOR oncogene.[256] However, the direct conversion of fibroblasts to neurons suffers also from limitations. There is a technical problem in obtaining sufficient fibroblast biopsies and having a good case-control setup, including the control of genetic heterogeneity. It is also not yet clear to what extent the epigenetic signatures in the fibroblasts used to generate iN reflect the epigenetic signatures of aged neuronal subtypes and other cells in the brain. Studies using iPSC models derived from sporadic AD cases[257] or from familial AD cases[258] concluded that neurons derived from iPSC, still capture AD-relevant signatures. Hence, more work is needed to be able to properly evaluate iPSC vs. iNs, and investigators should take this carefully into account before setting-up experiments.

Since omics efforts generate numerous hypotheses at a fast pace, we need scalable ways to test them in highly multiplexed analysis systems such as Perturb-seq or CROP-seq screens.[259–262] Another important aspect is that cells in culture lack the *in vivo* 'brain context'. Grafting experiments creating human-mouse chimera, while not high throughput, may be a powerful complement. Such chimeric human-mouse models have been created for several cell types, including neurons and microglia[25,77,263–265] or oligodendrocytes.[266,267] A final approach to be able to use primary human brain cells are organotypic brain slice cultures from brain pieces removed during neurosurgery,[268] but obviously this material is limited.

Moving forward, concerted efforts across countries and laboratories are required. Initiatives to reduce the administrative burden associated with human data access and sharing, to enable the integration of datasets from different studies, and to facilitate the re-use of code and tools, are critical. Similarly, the exchange of patient materials, i.e., iPS cells with interesting genetic backgrounds, needs facilitation instead of increasing regulation and bureaucracy.

Several well-characterized iPSC from patients and their respective isogenic controls are becoming available (ALS),[269] Primary Tauopathies,[270] and iPSC Neurodegenerative Disease Initiative (iNDI)). The Alzheimer's Disease Data Initiative (ADDI) provides an "AD workbench" with associated data science tools that supports the interoperability of datasets from multiple data

platforms. Many coordinated research initiatives such as the Parkinson's Progression Markers Initiative (PPMI), the Accelerating Medicines Partnership Alzheimer's Disease (AMP-AD), the Chan Zuckerberg Neurodegeneration Challenge Network (NDNC), the Aligning Science Across Parkinson (ASAP) initiative, AnswerALS, NIH iNDi project, the UK Dementia Research Institute's Multi-'omics Atlas Project (MAP) and iPSC project (IPMAR), and others, generate comprehensive sets of correlated clinical data and research data, including omics data, that can be accessed for research purposes.

Conclusion

While the single-cell biology revolution is ongoing, the therapeutically important question is to decide how early a given cell type is involved and whether their reaction contributes to the progression of disease severity. The challenge ahead will lie in understanding how different cells influence each other during the disease process as a key step towards the prioritization of cell types and druggable pathways that are *upstream* of neuronal dysfunction and death, where processes might still be reversible. The danger with multi-omics approaches is that they are, in essence, a complex form of descriptive science and that this might drag the field even further into correlative and speculative proposals. The most important challenge across neurodegeneration research is to find ways to establish causality. Besides enabling targeted therapeutic interventions, these insights will also reveal novel biomarkers for the early diagnosis of disease. With this increasing wealth of molecular information, clinical disease categories will have to be refined and sometimes redefined, as the molecular causes rather than the clinical picture must be used to guide the choice of disease-modifying treatment. Ultimately, individuals will then likely receive a combination therapy to restore a protective cellular context and to modify an upstream early disease trigger, thereby delaying or possibly preventing disabling symptoms.

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Declaration of interests

B.D.S. has been a consultant for Eisai and AbbVie over the last three years. B.D.S is also a scientific founder of Augustine Therapeutics and a scientific founder and stockholder of Muna Therapeutics. P.V. is the scientific founder of Jay Therapeutics. The other authors declare no competing interests.

Figure Legends

Figure 1: Hypothetical temporal changes in disease transition in neurodegenerative diseases. Schematic representations of the disease's progression from the biochemical (blue line) to the cellular (green line) and clinical phases (red line) are indicated. The most notable changes occurring in every phase are indicated in the grey box below. MCI-mild cognitive impairment.

Figure 2: Cellular disease-associated markers and disease-associated processes during the disease. Glial cells' response in the cellular phase is characterized by the expression of distinct cellular markers (or cellular states). Markers represented in red (Alzheimer's disease) and green (Parkinson's disease) of every cell type and their associated cellular pathways inferred from the single-cell studies. OPC, oligodendrocyte precursor cell. Up/down indicates cell-types proportional changes as reported in.[33] OPC-oligodendrocyte progenitor cell; EC-endothelial cells; SMC-smooth muscle cells.

Figure 3: The cellular phase of neurodegenerative diseases. Schematic representation of the interaction of different phases in the disease's progression from the biochemical to the clinical phases. In the cellular phase, pathological protein aggregation within vulnerable neurons (e.g., Tau, α-syn, TDP-43) or accumulation of amyloid plaques evoke a damaging response from glial cells. Vulnerable neuronal populations are marked by the expression of distinct markers represented in red (Alzheimer's disease) and green (Parkinson's disease). References for Alzheimer's disease[122,199] and Parkinson's disease.[33–35]

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