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Confronting ALS: understanding multicellular contribution to neurodegeneration : computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

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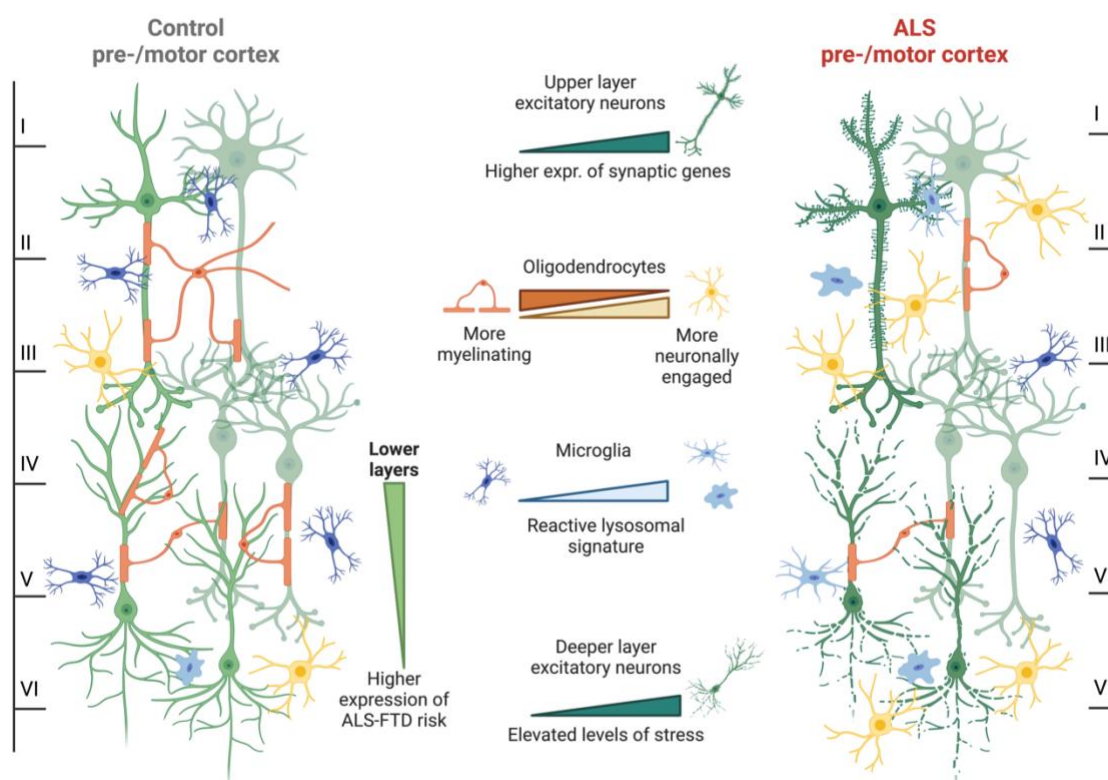
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Chapter 2:

Single-nucleus sequencing reveals enriched expression of genetic risk factors in Extratelencephalic Neurons sensitive to degeneration in ALS

In this chapter we describe findings from RNA sequencing analysis and molecular characterization of motor cortices from sporadic ALS patients and age matched controls in an effort to expand our knowledge on disease pathogenesis at a single-nucleus levels and different cell type resolution.



Graphical abstract and working model. Our study highlights cell type specific changes in premotor/motor cortex of sporadic ALS patients. Specifically, we identify upregulation of synaptic molecules in excitatory neurons of upper cortical layers, interestingly correlating to hyperexcitability phenotypes seen in patients. Moreover, excitatory neurons of the deeper layers of the cortex, that project to the spinal cord and are most affected by the disease, show higher levels of cellular stresses than other neuronal types. Correspondently, oligodendrocytes transition from a highly myelinating state to a more neuronally engaged state, probably to counteract stressed phenotypes seen in excitatory neurons. At the same time, microglia show a reactive state with specific upregulation of endolysosomal pathways.

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Single-nucleus sequencing reveals enriched expression of genetic risk factors in Extratelencephalic Neurons sensitive to degeneration in ALS

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Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder characterised by a progressive loss of motor function. The eponymous spinal sclerosis observed at autopsy is the result of the degeneration of extratelencephalic neurons, Betz cells (ETNs, Cortico-Spinal Motor Neuron). It remains unclear why this neuronal subtype is selectively affected. To understand the unique molecular properties that sensitise these cells to ALS, we performed RNA sequencing of 79,169 single nuclei from cortices of patients and controls. In unaffected individuals, we found that expression of ALS risk genes was significantly enriched in *THY1*⁺-ETNs and not in other cell types. In patients, these genetic risk factors, as well as genes involved in protein homeostasis and stress responses, were significantly induced in a wide collection of ETNs, but not in neurons with more superficial identities. Examination of oligodendroglial and microglial nuclei revealed patient-specific changes that were at least in part a response to alterations in neurons: downregulation of myelinating genes in oligodendrocytes and upregulation of a reactive state connected to dysfunctional endo-lysosomal pathways. Our findings suggest that the selective vulnerability of extratelencephalic neurons is partly connected to their intrinsic molecular properties sensitising them to genetic and mechanistic mechanisms of degeneration.

Amyotrophic Lateral Sclerosis (ALS) is a neuromuscular disease with survival typically limited to 2-5 years from onset, the most common motor neuron disease in aging individuals and the neurodegenerative disease with one of the earliest onsets in the mid-to-late 50s¹. Although specific genetic causes have been identified, most cases are sporadic (~90%), have no family history and unknown etiology², rendering modelling of non-genetic forms of the

disease difficult³. Variants in genes associated with ALS can contribute to a related disorder, Frontotemporal Dementia (FTD), leading to the view of ALS and FTD as different clinical manifestations of shared molecular causes. Bulk RNA-sequencing of ALS post-mortem brains have identified differences^{4,5} and similarities between sporadic and familial⁶ cases and highlighted shared profiles independent of disease onset^{7,8}. While they have provided valuable insight, these studies have had limited resolution on the cell-type specificity of the disease.

The most striking feature in ALS/FTD is the formation of protein aggregates of TAR DNA-binding protein-43 (TDP-43) in over 95% of cases of ALS and ~50% of FTD cases, mostly in neurons⁹, providing at least one shared mechanism. While the pattern of degeneration is similar, it is still unknown how familial mutations and sporadic onset might converge on the formation of these aggregates and how it specifically affects classes of extratelencephalic Cortico-Spinal Motor Neurons, i.e. Betz^{10,11} and von Economo cells^{12,13}. Moreover, strong evidence demonstrated that cells other than neurons are key mediators of disease progression and it remains unclear how these might contribute to the disease¹⁴⁻¹⁷.

Methods to study heterogeneity at a single-cell level have rapidly advanced and their application to human post-mortem brain tissue is beginning to emerge, especially for neurodegenerative diseases¹⁸⁻²⁷. However, a comprehensive view of the changes across cell types in ALS has not been performed. In this study, we applied single-nuclei RNA sequencing and *in vitro* human induced Pluripotent Stem Cells modelling to investigate specific changes in cortical cell types in sporadic ALS. Our profiling identified the intrinsically higher expression of ALS/FTD risk factors in specific classes of extratelencephalic excitatory neurons. In ALS patients, these neurons selectively express higher levels of genes connected to unfolded protein responses and RNA metabolism. We also found that, excitatory neurons vulnerability is accompanied by a decrease in myelination-related transcripts in oligodendroglial cells and an upregulation of reactive, pro-inflammatory state in microglial cells connected to senescence. We provide a preliminary, insightful view of disruptions triggered in human motor cortices in ALS and implicate aging-associated mechanisms like altered proteostasis, inflammation and senescence to specific cell type in the disease.

Results

Profiling of ALS cortex by single-nucleus RNA-sequencing

To better understand factors that contribute to the specific degeneration of classes of excitatory neurons, we used snRNAseq to profile motor/pre-motor cortex grey matter from sporadic (sALS) patients and age-matched controls with no neurological disease using Drop-seq technology²⁸ (Fig. 1a, Extended Data Table 1, Extended Data Fig. 1a-c). After screening for RNA quality, 79,169 barcoded droplets from 8 individuals were analysed ($n=5$ sALS, $n=3$ Control), with a mean of 1269 genes and 2026 unique molecular identifiers (UMIs) (Extended

Data Fig. 1d). We used Seurat²⁹, single-cell analysis package, to cluster and annotate groups according to canonical markers of brain cell types³⁰: excitatory and inhibitory neurons, oligodendrocytes, oligodendrocyte progenitor cells (OPCs), microglia, astrocytes, and endothelial cells (Extended Data Figure 1e,f). The observed cell type distribution corresponded to previous studies³¹ and enabled robust categorization for downstream analysis. Cellular distribution was homogeneous between sexes and individuals, except for a modestly lower number of astrocytes in ALS samples (Extended Data Fig. 1g-i).

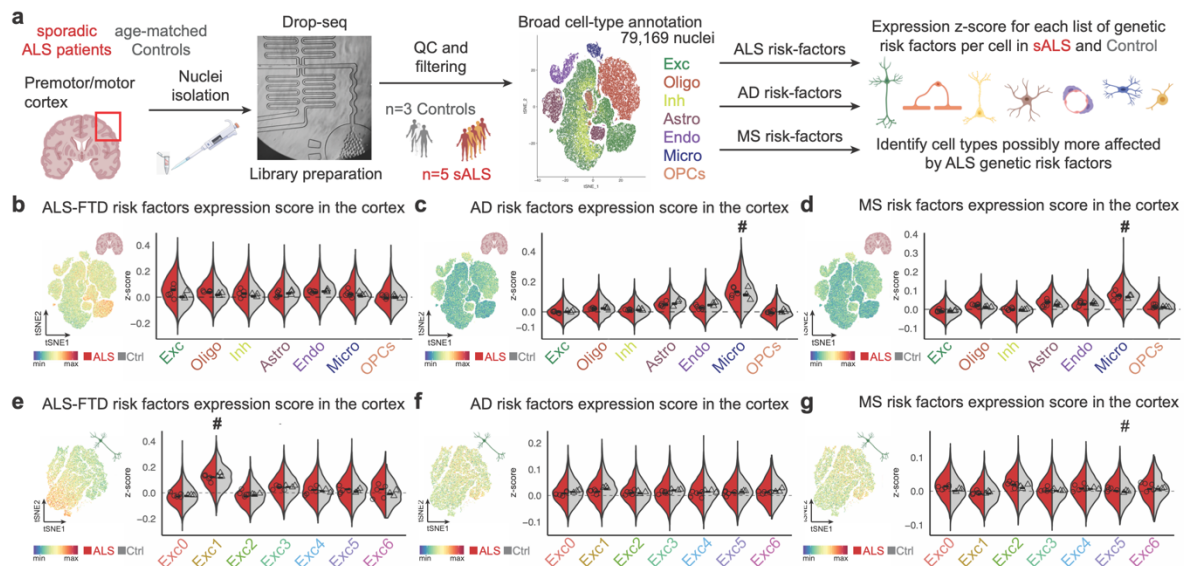


Figure 1 Cellular susceptibility to ALS-FTD in the human cortex. **a**, Diagram of workflow for isolation of nuclei from cortices of ALS patients and age-matched controls followed by single-nucleus RNA sequencing and assessment of expression of gene modules associated with neurodegenerative diseases. **b-d**, t-SNE projections and Violin plots of z-scores for expression of genes associated with the ALS-FTD (b), AD (c) and MS (d) in the different cell types identified (bars denote median for each side of the violin – symbols: average score per individual). **e-g**, t-SNE projections and Violin plots of z-scores for expression of genes associated with the ALS-FTD (e), AD (f) and MS (g) in the different subtypes of excitatory neurons (bars denote median for each side of the violin – symbols: average score per individual).

Elevated expression of ALS/FTD risk genes in a specific class of excitatory neurons

To potentially identify cell types underlying ALS pathophysiology, we examined the expression of known familial genes for ALS/FTD and variants identified as risk factors from genome-wide association studies (GWAS). These genes were expressed to a highly variable degree between cell types many of them were ubiquitously expressed as already known in the field² (Extended Data Fig. 2a). We then computed a “module score” for this set of genes³²; this metric generates a standardised z-score for the expression of each gene and sums it up as a total score for the gene set, here a positive score suggests higher expression of this gene set compared to the average expression of the module across the dataset. We also computed parallel module scores for lists compiled from latest GWAS for neurological disorders that affect the cortex but not specifically Betz cells: AD^{33,34} and MS³⁵ (Fig. 1a, Extended Data Table 2). No clear preferential expression for ALS/FTD gene list was identified (Fig. 1b), as it might

have been anticipated by the scattered and ubiquitous pattern of expression. On the other hand, AD and MS modules showed enrichment for their respective lists in microglia, as expected based on the strong immune signatures of that characterise these diseases and the involvement of immune cells in neurodegeneration³³⁻³⁷ and shown by other reports³⁰ (Fig. 1c,d). These results confirm knowledge in the field, underlying the strength of this analysis, and confirm our results in an unbiased, single-cell resolution.

Considering the selective loss of excitatory neurons in ALS, we further analysed these cells. We found 32,810 nuclei from excitatory neurons with unbiased clustering identifying seven subgroups (Exc0-6) that expressed known markers of different cortical layers, equally distributed in our cohort (Extended Data Fig. 2b-f). Analysis of the ALS/FTD module in these cells showed a positive score in *THY1*-expressing subgroup Exc1 (Normalised Enrichment Score=1.834) (Fig. 1e, Extended Data Fig. 2g,h) and no significant enrichment for AD and MS modules (Fig. 1f,g). We decided to further dissect the identity of these cells and investigate if they could be ETNs (group containing Betz cells).

We identified three subgroups expressing markers of subcerebral projection neurons: Exc1, Exc5 and Exc6 (Fig 2a). Exc5 and Exc6 expressed canonical markers *FEZF2*, *BCL11B* and *CRYM*³⁸; Exc1 expressed *THY1*, enriched in human layer V¹⁸ and used as a reporter for CSMNs³⁹, and high levels of neurofilament chains, markers of ET neurons *in vivo*⁴⁰ (Fig. 2b). Recent reports dissected the transcriptomic identity of layer V extratelencephalic neurons in the human Motor Cortex⁴¹. We detected expression of their markers in these groups with Exc1 expressing *SERPINE2* and *POU3F1*, specific of ETNs⁴¹, and *NEFH* and *STMN2*, broad markers of MN^{40,42} (Fig 2c). Because of the anatomical location of our samples and the presence of ETNs across motor-related areas⁴³, we plotted markers specific to layer V neurons of regions adjacent to the Motor Cortex like von Economo cells⁴⁴, affected in FTD⁴⁵, and other Long-Range Subcerebral Projecting Neurons (LR-SCPNs)⁴⁶ and confirmed that all three groups expressed these markers (Fig. 2d,e). To further characterised the spatial expression of these markers we leveraged a publicly available single-cell, spatial dataset of the human dorsal cortex⁴⁷. We confirmed that markers of layer V neurons, such as *THY1*, *STMN2* and *SNCG*, are expressed in Exc1 (Fig. 2f) and that these markers are also expressed in layer V (L5) of the spatial dataset (Fig. 2g,h and Extended Data Fig. 3a,b). This evidence suggests that Exc1, Exc5 and Exc6 express markers of extratelencephalic neurons of cortical areas affected by ALS/FTD.

To further confirm that *THY1*^{high}-neurons expressed higher levels of ALS/FTD genes, we ran module score analysis in two datasets that identified *THY1*^{high} cortical neurons^{18,48}. In these studies, *THY1*-neurons expressed ETNs markers, layer V, von Economo and LR-SCPNs markers (Extended Data Fig. 3c-i) and, expressed higher levels of the ALS/FTD module score (Extended Data Fig. 3l-m). Analysis of the spatial transcriptomic dataset⁴⁷,

confirmed that the top 10 ALS/FTD-associated genes most highly expressed in Exc1 (Extended Data Fig. 2g) are highly expressed in deeper layers of the cortex, specifically in layer V (Fig. 2i,j and Extended Data Fig. 3n). Studies in human^{13,49} and mouse⁵⁰ showed that deep layer neurons have a higher propensity to form TDP-43 aggregates, hallmark of ALS/FTD. Here we provide a possible link to their specific vulnerability.

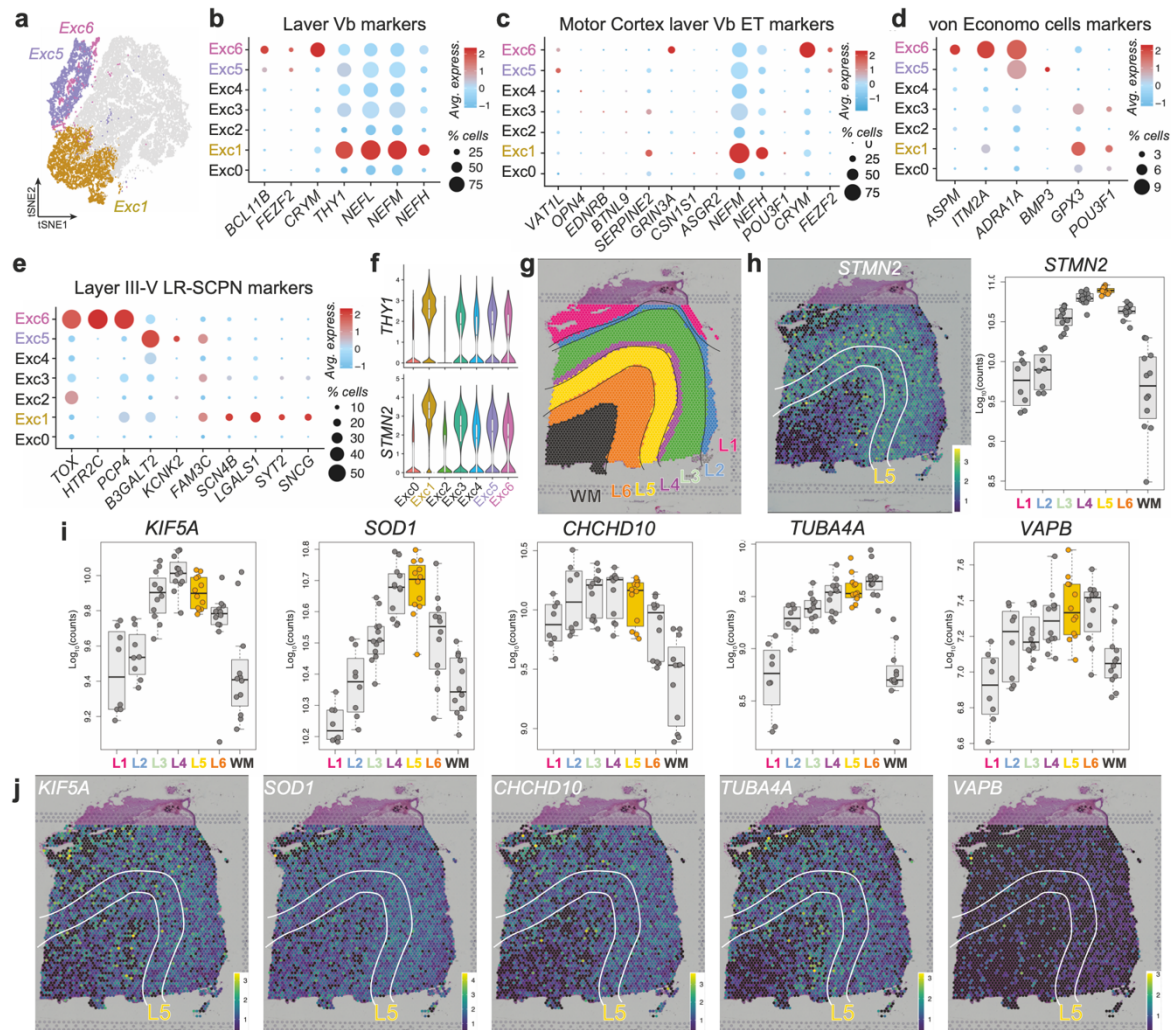


Figure 2 ALS-FTD susceptible neurons are L5-ET Neurons. **a**, t-SNE projection of presumptive layer V neurons. **b**, Dotplot representing expression of Layer V markers. **c**, Dotplot for markers of LVb Extratelencephalic neurons of human Motor Cortex. **d**, Dotplot representing expression of von Economo cells markers. **e**, Dotplot representing expression LR-SCPN markers. **f**, Representative Violin for markers of layer V Extratelencephalic neurons of human Motor Cortex. **g**, Visual depiction of layers identification by Maynard et al. 2021 (publically available). **h**, Spotplot depicting expression of layer Vb Motor Cortex marker, STMN2, identified as enriched in THY1-Exc1, with corresponding boxplot quantification. **i-j**, Boxplots and corresponding spotplots for the expression of top 5 ALS/FTD associated genes expressed in Exc1.

Cellular burden on excitatory neurons is higher in deeper layers

We next examined how the enriched expression of ALS/FTD genes relates to changes that occur in excitatory neurons in response to ALS. We conducted differential gene expression (DGE) analysis between neurons from patients and controls, across all excitatory cells and within each subgroup (Fig. 3a). We then selected genes significantly upregulated in

patients globally (DGEall) and within each subgroup (DGE0-6), calculated module-scores for each set and investigated whether certain neuronal subtypes might have similar responses to ALS (Extended Data Table 3). This analysis showed a correlation between scores in groups expressing deep layer markers and the global changes identified in patients (Fig. 3b), suggesting that pathology in lower cortical layers are driving the observed alterations. For instance, groups expressing ETNs markers (Exc1, Exc5, Exc6) shared many upregulated genes with each other and with the global signature (Fig. 3c), whereas genes upregulated in upper layers of the cortex, a region relatively spared of pathology, shared less similarities (Fig. 3d). Intriguingly, this class of genes is, like genetic risk factors, constitutively expressed at higher levels in Exc1-ETNs (Fig. 3b), advocating for a proposed interplay between genetics and molecular pathways that sensitises ETNs to ALS⁵¹.

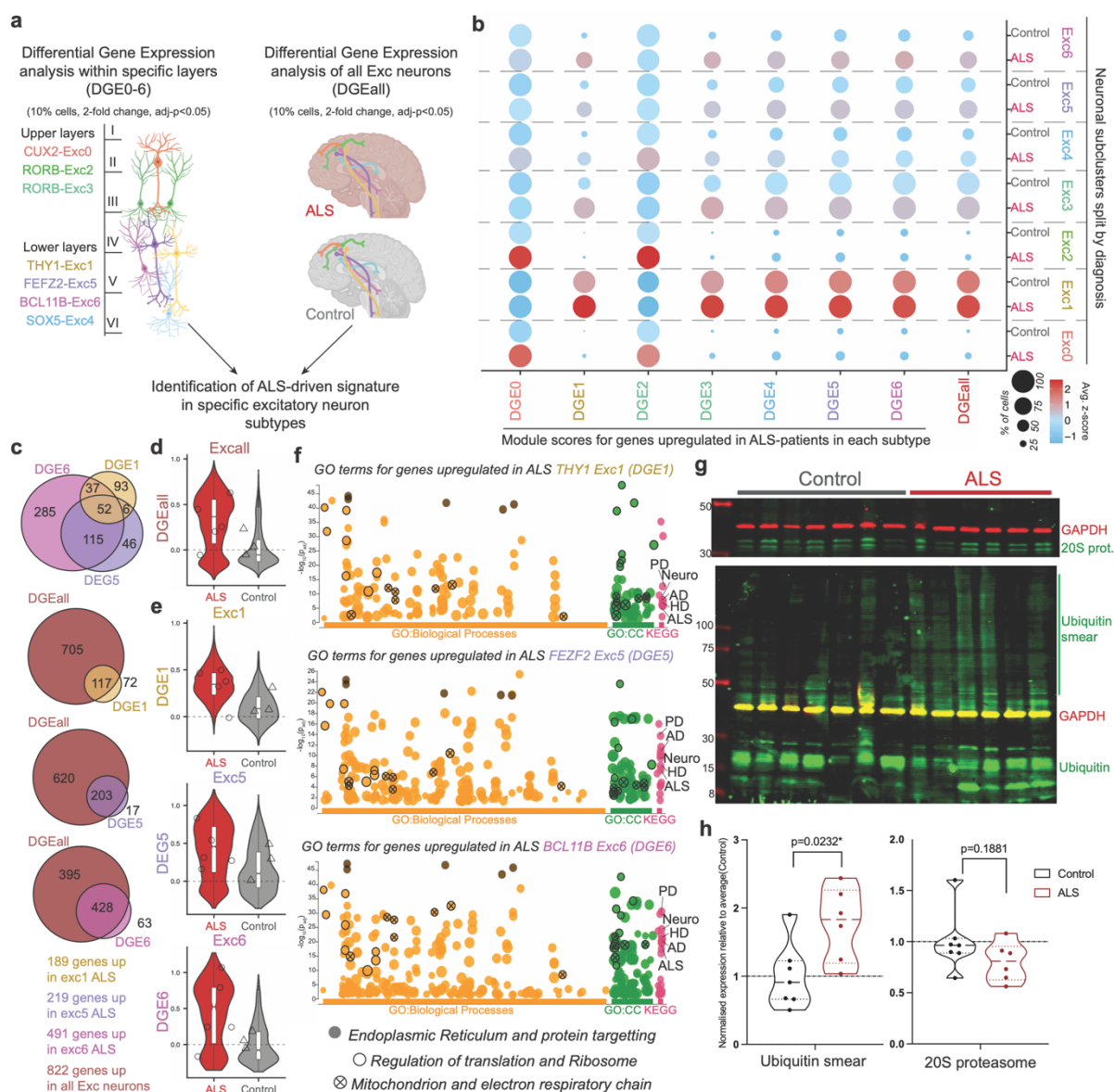


Figure 3 ALS excitatory neurons present increased expression of stress-related pathways. **a**, Schematic of Differential Gene Expression Analysis. **b**, Dotplot representing scores for genes upregulated in each subgroup of Exc neurons (DGE0-6) and globally upregulated in all Exc (DGEall). **c**, Comparison of genes globally upregulated in ALS (DGEall) with genes upregulated in classes of L5-ETNs (genes expressed by >10% of cells, >2-FC, adjusted p-value<0.05). **d**, Violin plots of z-scores for genes globally upregulated in all excitatory cells (DGEall) in all excitatory neurons (geometric boxplots represent median and interquartile ranges – symbols: average score per individual). **e**, Violin plots of z-scores for genes upregulated in classes of L5-ETNs (DGE1, DGE5, DGE6) in the three groups (geometric boxplots represent median and interquartile ranges – symbols: average score per individual). **f**, Gene Ontology analysis for genes upregulated in L5-ETNs classes (DGE1,5,6), highlighted terms are shared between the three (CC=Cellular Components). **g-h**, Western Blot quantification of ubiquitin accumulation and 20S proteasome subunit from Motor Cortices of ALS patients and age-matched controls.

Subsequent Gene Ontology (GO) analysis showed that DEGs in CUX2-cells were associated with synaptic biology (Extended Data Figure 4a,b), which could be due to changes in synaptic activity of degenerating neurons in deeper cortical layers. In contrast, DEGs identified in classes of ETNs were connected to cellular stresses previously associated with ALS^{1,2}, even from studies with thousands of patients⁵² (Fig. 3e). Interactome analysis confirmed the coordinated alterations in the expression of genes that function in translational machinery, mitochondria, protein folding, and degradation pathways connected to the proteasome and proteostasis and many were shared with transcriptional changes identified in patients' excitatory cells as a whole (Extended Data Fig.4c,d-5). Interestingly, these pathways were specifically upregulated in neurons of deeper cortical layers rather than upper layer (Extended Data Fig. 4e,f). Comparison with other studies underlined similarities of these pathways with genes upregulated in excitatory neurons from MS patients¹⁸ but not neurons from AD patients²⁰ (Extended Data Fig. 4g,h), suggesting that similar processes might be at the base of neurodegeneration but these changes are not universal.

Presently, *in vitro* modelling of sporadic ALS requires high numbers of lines and high-throughput methods and needs further standardization⁵³⁻⁵⁵, we therefore decide to implement a system that would allow to probe disruptions of proteostasis in human neurons and test if any of these changes parallel any of the disruptions seen in ALS patients and interpret what proportion of the complex transcriptomic signature may be associated with proteostatic stress specifically in neuronal cells. In order to do so, we implemented transient proteasome inhibition as a model to induce TDP-43 nuclear loss as seen in patients' Betz cells⁴⁹, phase separation⁵⁶, stress granules⁵⁷ and other ALS-related dysfunction in human neurons^{5,58} (Extended Data Fig. 6a). To recapitulate proteostatic stress we applied a proteasome inhibitor to human Pluripotent Stem Cells (hPSC)-derived neurons^{58,59} and induced nuclear loss of TDP-43 (Extended Data Fig. 6b,c). Bulk RNA-sequencing analysis showed widespread changes after treatment, with a significant overlap of upregulated genes between stressed hPSC-neurons and sALS-neurons, specifically proteasome subunits and heat-shock response-associated chaperonins and GO analysis of shared genes confirmed the upregulation of proteasomal and chaperone complexes (Extended Data Fig. 6d-g). Moreover, genes upregulated in both conditions show a significant overlap with transcripts misregulated after downregulation of TDP-43 in neurons⁵⁸

(Extended Data Fig. 6h). This confirms that some changes identified in sALS neurons are connected to neuronally intrinsic proteostatic alternations and at least in part connected to alterations in TDP-43.

To confirm hindered proteostasis in ALS cortex, we selected a second cohort of sALS patients and controls. We extracted protein, confirmed increased insoluble TDP-43 in patients (Extended Data Fig. 6i-j) and showed that, despite the presence of core proteasomal subunits, pathology is accompanied by the accumulation of highly ubiquitinated proteins, hallmark of impaired proteostasis (Fig. 3f,g). These findings suggest that proteasome inhibition orchestrate alterations like those observed in ETNs from ALS patients, underscoring the connection between neuronal stress and loss of proteostatic homeostasis.

Oligodendroglial cells respond to neuronal stress with a neuronally-engaged state

To reach deep into the cord ETNs are dependent on robust axonal integrity⁶⁰ and because others detected changes in myelination in ALS motor cortex¹⁶ and in FTD frontal cortex²⁷, we analysed nuclei from myelinating cells. 19,151 nuclei from oligodendroglia were clustered in five groups: one of OPCs – Oliglia3, and four of oligodendrocytes – Oliglia0,1,2,4 (Fig. 4a-c, Extended Data Fig. 7a-b). We noted a significant depletion of ALS-nuclei in Oliglia0 whereas Oliglia1 and Oliglia4 were enriched in patients (Fig. 4d). GO analysis for genes enriched in each group revealed that Control-enriched Oliglia0 was characterised by terms connected to oligodendrocyte development and myelination and expressed higher levels of myelinating genes, e.g. *CNP*, *OPALIN*, *MAG* (Fig. 4e, Extended Data Fig. 7c-e). Conversely, ALS-enriched Oliglia1 showed terms for neurite morphogenesis, synaptic organization and higher expression of postsynaptic genes *DLG1*, *DLG2*, *GRID2* (Fig. 4f, Extended Data Fig. 7f-h). Intriguingly, expression of neuronal RNAs has been specifically found in classes of oligodendrocytes in primate motor cortex⁴¹.

Global differential gene expression analysis supports a shift from a myelinating to a neuronally-engaged state with upregulation of genes involved in synapse modulation and decrease of master-regulators of myelination, as confirmed by GO analysis (Fig. 4g-i, Extended Data Fig. 7j,k). Loss of myelination is exemplified by the expression of G-protein coupled receptors (GPCRs) that mark developmental milestones: *GPR56*, expressed in OPCs⁶¹, and *GPR37*, expressed in myelinating cells⁶², were lowly expressed in ALS-enriched subgroups and globally downregulated (Extended Data Fig. 7i). Impaired myelination is consistent with previous studies identifying demyelination in sALS patients¹⁶.

To further explore these changes, we compared them with published reports that identified shifts in oligodendrocytes (Extended Data Table 4)¹⁹. Comparison of Jäkel et al.¹⁹ with our study revealed that Control-enriched Oliglia0 most closely resembled highly

myelinating, *OPALIN*⁺ cells from Jäkel (Extended Data Fig. 8a,b), while ALS-enriched Oliglia1 and Oliglia4 aligned to not-actively myelinating Jäkel1 (Extended Data Fig. 8c,d), with many shared genes (Extended Data Fig. 8e-h). To confirm this shift, we ran validations on protein extracts from patients and controls and showed that oligodendrocyte-specific, myelin-associated proteins CNP and MBP are downregulated in motor cortices from patients (Fig. 4j-k). The data so far shows how activation of stress pathways in deep layer neurons is accompanied by a shift in oligodendrocytes from active myelination to oligo-to-neuron contact (Extended Data Fig. 8i).

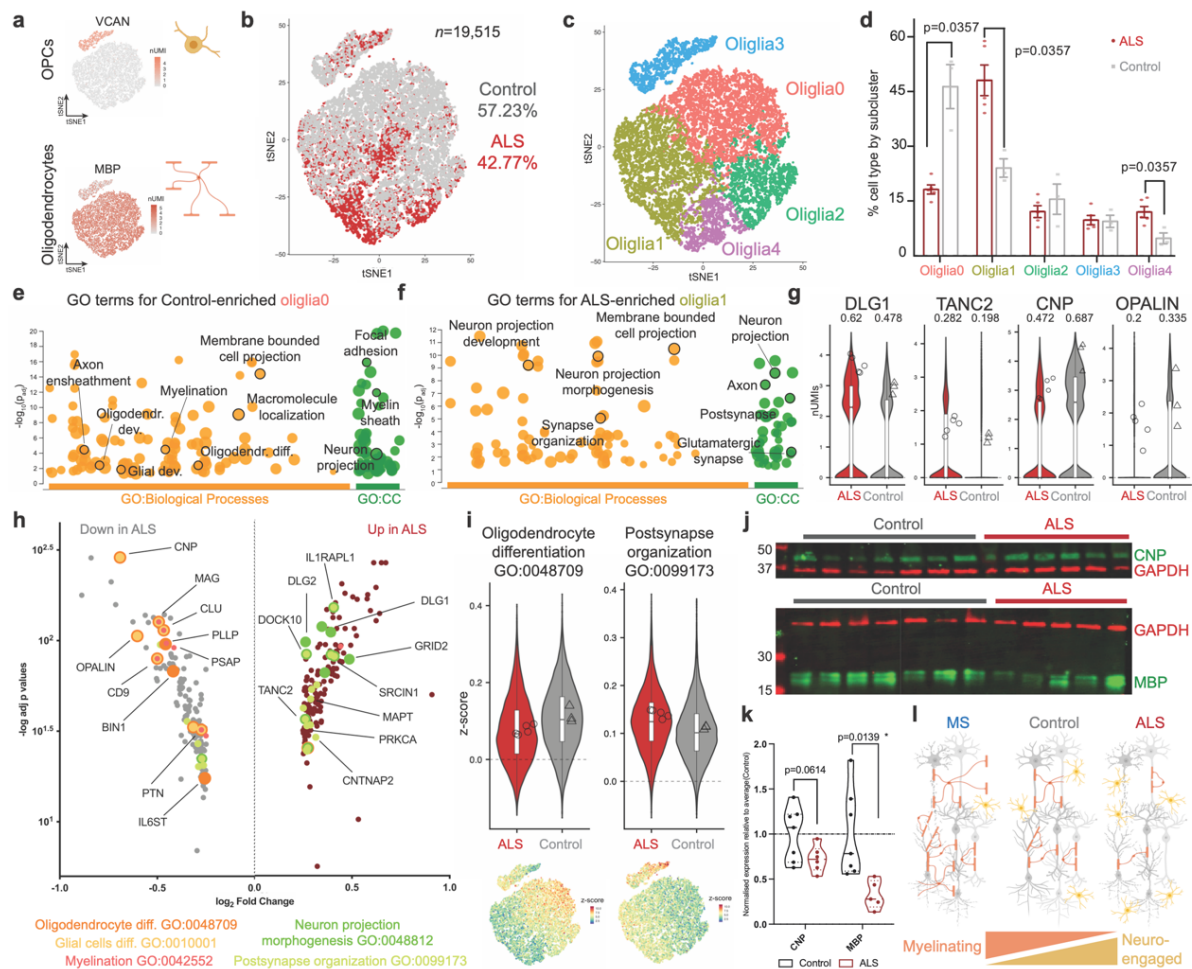


Figure 4 In ALS, oligodendroglial cells decrease their myelinating machinery in favour of a neuro-engaged state. **a**, t-SNE projection of OPCs and oligodendrocytes markers. **b**, t-SNE projection of oligodendroglia (ALS n=8,372 nuclei, Control n=11,168 nuclei). **c**, t-SNE projection of subclusters within oligodendroglia. **d**, Distribution of subclusters by diagnosis. **e**, Gene Ontology analysis for genes characteristic of Control-enriched *oliglia0*, highlighted terms involved in myelination (CC=Cellular Components). **f**, Gene Ontology analysis for genes characteristic of ALS-enriched *oliglia1*, highlighted terms involved in neuro-engaged functions. **g**, Violin plots of representative genes for neuro-supportive functions (left) and myelination (right) (geometric boxplots for median and interquartile ranges – symbols: log2(AverageExpression) per individual) (fraction of cell expressing). **h**, Volcano plot of differentially expressed genes in oligodendroglia. Highlighted genes identified in GO terms related to myelination (orange) and neuro-engaged functions (green). **i**, Violin plots representing z-score for selected GO terms and related t-SNE projection (median and interquartile ranges – symbols: average score per individual). **j-k**, Western Blot quantification of CNPase and MBP from Motor Cortices of ALS patients and age-matched controls. **l**, Diagram illustrates shift of oligodendrocytes states.

Microglial activation is characterised by an ALS-specific endo-lysosomal response

Mouse models⁶³, patient samples⁶ and function of ALS-related genes in myeloid cells⁶⁴⁻⁶⁶ have demonstrated the importance of microglia as modifiers of disease so we interrogated changes in this cell type. In the 1,452 nuclei from microglia (Fig. 5a, Extended Data Fig. 9a), we identified 159 genes upregulated in patients and, remarkably, many were associated with endocytosis and exocytosis, previously implicated in ALS^{65,66} (Fig. 5b). Several of these genes were also associated with microglial activation (*CTSD*) and neurodegenerative disorders (*APOE*) (Fig. 5c,d). Interestingly, genes associated with AL/FTD were upregulated: *TREM2*, *OPTN*, *SQSTM1/p62*, *GRN* (Fig. 5e). GO analysis for upregulated genes confirmed a pro-inflammatory state highlighting activation of endo-lysosomal pathways, secretion and immune cell degranulation previously associated with myeloid cells in ALS^{65,66} (Fig. 5f,g). Further subclustering identified three groups: homeostatic Micro0, “Disease Associated Microglia”-like Micro1, and cycling Micro2 (Extended Data Fig. 9b,c). Notably, genes that characterised Micro1 were also upregulated in sALS (Extended Data Fig. 9d,e), in conjunction with a downregulation of homeostatic genes and upregulation of reactive pathways (Extended Data Fig. 9f-i).

To identify modulators of this signature, we used the Connectivity Map (CMap) pipeline⁶⁷, which contains gene expression data of 9 human cell lines treated with thousands of perturbations allowing association between a given transcriptomic signature and a specific alteration. This analysis revealed that genes dysregulated in microglia positively correlated with regulators of cell cycle and senescence, *KLF6* and *CDKN1A/p21*, suggesting an exhaustion of microglial proliferation. On the other hand, we found a negative correlation with a type I-interferon-associated responses (*IFNB1*), which is targeted in treatments for neurological diseases to reduce inflammation⁶⁷ (Extended Data Fig. 10a). Given the stress signature identified in neurons, we wondered whether these transcriptomic changes might be driven by neuronal apoptosis. We differentiated microglia-like cells (iMGLs)⁶⁸ and neurons (piNs)⁵⁹ from hPSCs, triggered neuronal apoptosis and then introduced apoptotic neurons to iMGLs *in vitro* (Extended Data Fig. 10b-c). Quantitative assessment of selected transcripts by RT-qPCR confirmed that dead neurons lead to significant downregulation of homeostatic genes (Extended Data Fig. 10d), upregulation of genes involved in the endo-lysosomal trafficking (specifically *CTSD*, *ITGAX*, *LGALS3*, *SQSTM1/p62*) and downregulation of markers of actively cycling cells (Extended Data Fig. 10e-f), suggesting that changes identified in microglia from patients are, at least in part, a response to neuronal apoptosis.

We next asked if these changes were a general response to neuronal disease or restricted to ALS. By comparing our results with published snRNA-seq studies in AD²⁰ and MS⁶⁹, we identified dysregulation of lipid metabolism (*APOE*, *APOC1*, *SPP1*) as a common

feature in microglia, and genes associated with DAMs were shared between ALS and MS (*CTSD*, *GPNMB*, *CPM*, *LPL*) and ALS and AD (e.g. *TREM2*) (Fig. 5h). Genes specifically upregulated in ALS were related to vesicle trafficking, myeloid cell degranulation and the lysosome (e.g., *SQSTM1/p62*, *LGALS3*, *GRN*, *ASAHI*, *LRRK2*). This evidence suggests the induction of a shared microglial reactive state in neurodegenerative diseases, yet in ALS neuronal death activates changes connected to dysfunctional endo-lysosomal pathways.

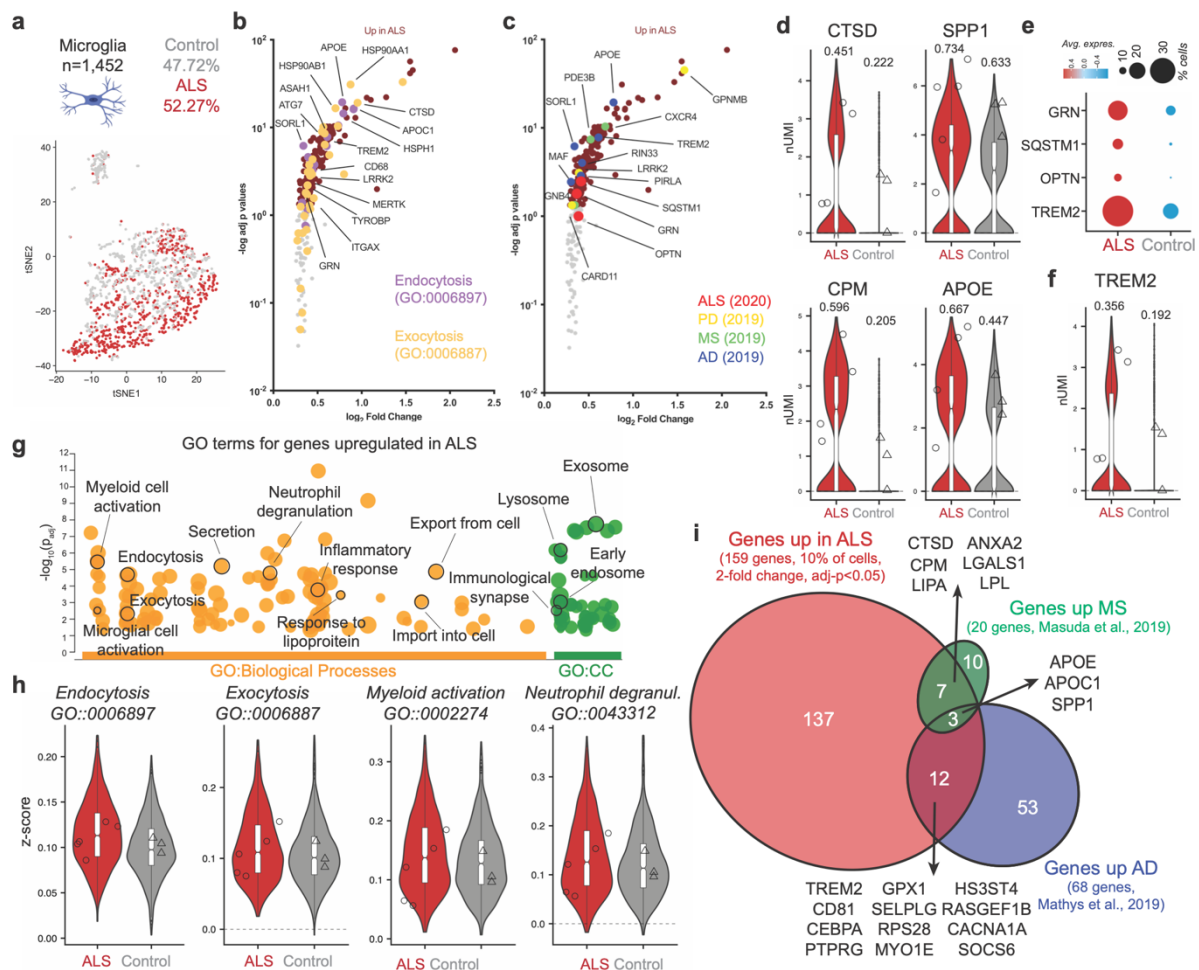


Figure 5 Disease-Associated Microglia signature in ALS. **a**, t-SNE projection of microglia (ALS n=759 nuclei, Control n=693 nuclei). **b,c**, Volcano plot of genes upregulated in microglia from ALS. Genes identified in GO terms for endocytosis and exocytosis (**b**), genes associated to neurodegenerative diseases in (**c**). **d**, Violin plots of representative genes upregulated in ALS patients associated with reactive microglia (geometric boxplots represent median and interquartile ranges – symbols: log₂(AverageExpression) per individual) (fraction of cell expressing). **e**, Dotplot representing expression of genes associated with ALS/FTD pathogenesis. **f**, Violin plots of representative ALS/FTD gene upregulated in ALS (geometric boxplots represent median and interquartile ranges – symbols: log₂(AverageExpression) per individual) (fraction of cell expressing). **g**, Gene Ontology analysis for genes upregulated in ALS microglia, highlighted terms involved in myeloid cells biology and/or pathogenesis of ALS. **h**, Violin plots representing z-score for selected, statistically significant GO terms from f (geometric boxplots represent median and interquartile ranges – symbols: average score per individual). **i**, Comparison of genes upregulated in microglia from ALS with genes upregulated in microglia in other neurodegenerative diseases.

Discussion

A key question in the study of neurodegeneration is why certain cell types are more susceptible in different diseases⁷⁰. In this study, we identified the enrichment for ALS/FTD associated genes in a class of ETNs which provides a connection between this neuronal type and its propensity to accumulate TDP-43 aggregates^{11,13,50} leading to their gradual loss in ALS/FTD¹⁰. This enrichment is not recapitulated for risk factors connected to AD and MS, related to immune processes and are more enriched in microglia³⁰. One study suggested that ALS-associated variants connected to autophagy and protein clearing are most highly expressed in glutamatergic neurons⁵¹ and these findings were later corroborated adding the importance of neuronal morphology and ribonucleotide metabolism⁵², here we provide a more detailed dissection of which subtypes of cells that might be.

Additionally, we identified a broadly shared transcriptomic signature of cellular stress pathways in classes of deep layer excitatory neurons. These alterations in RNA translation, proteostasis and mitochondrial function have previously been involved in models of ALS^{1,2}. Our study not only recapitulates these changes, it also highlights their cell-type specificity and links them to the identification of rare mutations in regulators of these pathways in familial forms of ALS⁷¹. These molecular mechanisms are confirmed to be connected to proteasomal function by our neuronal human *in vitro* model, underlying the importance of protein homeostasis in neurons and its connection to ALS. The nuclear nature and the low-coverage of this kind of sequencing but also the small sample size in our study does not allow a confident, further dissection of the specifically neuronal changes in RNA biology identified in *in vitro* models and patient samples^{58,72}. It remains intriguing to speculate how RNA metabolism and proteostasis might be mis-regulated in extratelencephalic neurons and specifically Betz cells, mouse models where these pathways are specifically altered in CSMNs might help shed a light on their interplay in this specific neuronal type.

We suggest two mechanisms by which ETNs are rendered more susceptible to ALS: the intrinsically higher expression of risk factors which is coupled with processes of neurodegeneration happening broadly in classes of ETNs that might exacerbate and contribute to vulnerability of these cells in a combinatorial effect. Recent snRNA-sequencing studies have unravelled susceptibility of specific neuronal subtypes in other diseases: mid-layer RORB⁺ neurons accumulate tau aggregates and are depleted in AD^{22,73}; upper layer CUX2-neurons are more affected by meningeal inflammation in MS¹⁸; ventral dopaminergic neurons in Parkinson's Disease²³ and ET neurons affected in ALS/FTD as described by our study and spinal cord motor neurons as suggested in recent reports^{74,75}. Impairment of proteostatic mechanisms seems to be a common theme in degenerating neurons regardless of the disease, however, only in ALS these changes are specifically connected to upregulation of transcripts connected to RNA metabolism, trend that appears to go in opposite direction in

AD⁷³. Further investigation into the transcriptomic signatures and integrative analyses of these studies might mark the beginning of a new era in the understanding of selective neuronal vulnerability to degeneration in different diseases.

Emerging studies have shown that glial cells are important modifiers in ALS/FTD. For instance, defects in oligodendrocyte maturation and myelination are present in *SOD1-G93A* mice and removing toxic SOD1 from this lineage improves survival¹⁶. In our study, we show that changes in processes involved in oligodendrocyte differentiation and myelination may contribute to neuronal degeneration and/or be a coordinated response to the disease and appear to contrast those described in MS¹⁹. Moreover, we revealed perturbations in key myelin-regulators, such as *OPALIN*, *CNP*, and *MAG*, across multiple oligodendrocyte clusters but in these cells only, as opposed to AD where myelination-related changes were present across multiple cell types^{20,24,25}. Given the similarities in the stress signature identified in neurons in this study with changes in MS lesions but not in AD patients, it is puzzling how changes in myelination might be a consequence or the cause of neuronal degeneration.

Intriguingly, recent work has shown the expression of neuronal transcripts in oligodendrocytes of human motor cortex⁴¹ and regional distribution of different types of oligodendrocyte in the nervous system might explain differential responses to disease⁷⁶. The upregulation of synaptic transcripts in oligodendrocytes of ALS patients might represent phagocytic activity of this cell type in neurodegenerative contexts⁷⁷ or the need for synaptic proteins in the formation of myelin sheath⁷⁸. These speculations are interesting if coupled with the upregulation of synaptic machinery in upper layer CUX2-neurons and the documented loss of postsynaptic molecules in ET neurons in ALS⁷⁹. Moreover, a recent snRNAseq study of FTD cortices identified changes in myelinating cells in response to neuronal loss and specifically underlined the importance of cell-to-cell communication in neurodegeneration²⁷. Finally, recent GWAS studies trying to associate specific cell types to ALS risk factors have pointed at excitatory neurons but also myelinating cells and inhibitory neurons as more sensitive to genetic risks for the disease⁵². These observations suggest a coordinated response of neurons in the Cortico-Spinal motor circuit in an attempt to compensate for loss of neuronal inputs to the cord. Further investigations could focus on shifting oligodendroglial states in disease models and determine changes in disease progression in the scope to complement efforts aimed to controlling neuronal activity⁸⁰.

Finally, we found distinct transcriptional perturbations in ALS-associated microglia, particularly in endo-lysosomal pathways. We and others have implicated ALS/FTD-associated gene *C9orf72* in endosomal trafficking and secretion in myeloid cells^{65,66} and the upregulation of lysosomal constituents, e.g. *CTSD*, was identified in this study and by others in patients⁸¹. Coupled with the upregulation of ALS/FTD-associated genes *SQSTM1/p62*, *OPTN*, *TREM2* and *GRN*, this suggests a mechanistic convergence on vesicle trafficking and pro-

inflammatory pathways that may initiate and/or exacerbate the homeostatic-to-DAM transition in ALS/FTD. We also delineated interferon-response-related changes, as identified by others in *C9orf72*-ALS⁸², providing a parallel between sporadic and familial ALS. Overall, differentially expressed transcripts had partial overlap with those in microglia surrounding amyloid plaques in AD^{20,21} and microglia associated with demyelinating lesions in MS⁶⁹, suggesting that drugs specifically modulating myeloid cells in other neurodegenerative diseases may provide a basis for new therapeutic approaches for ALS/FTD and warrants further study. Recent reports have shown how Disease Associated Microglia and their activity might actually be beneficial in disease contexts⁸³, studies specifically manipulating microglial states might elucidate the “friend or foe” role of these cells in ALS.

In summary, we show that classes of ETNs require the expression of a collection of genetic risk factors for ALS/FTD with pivotal roles in proteostasis, either because of their peripheral metabolic needs or aging. This intrinsically higher expression of disease-associated genes might be at the bottom of a “first over the line” mechanism leading to disruption of homeostasis in groups of deep-layer excitatory neurons. These alterations trigger a cascade of responses: superficial neurons upregulate synaptic genes to supplement for lost inputs to the cord; oligodendroglia shift from a myelinating to a neuronally-engaged state; microglia activate a pro-inflammatory signature in response to neuronal apoptosis. Our study offers a view in which neurocentric disease vulnerability sparks responses in other neuronal subtypes and glial cells, but it also shows that clear enrichment of ALS/FTD-related genes in ETNs is not necessarily the main genetic driver and it is coupled with processes engaging disease related genes in different cells, i.e. microglia. This view is a first insight into the disruptions of cortical biology in ALS and provides a connection between age-related changes in cellular components and mechanisms associated with ALS⁸⁴. Future investigations should consider multicellular disruptions in ALS/FTD, where the survival of the neuron is unmistakably pivotal, but targeting other cells to reduce inflammation, promote myelination, and bolster neuronal circuitry may re-establish a neuroprotective environment.

Limitations of this study

One limitation of this study is the small size of the cohort. ALS is a very heterogeneous disease⁷, and a smaller cohort size cannot fully recapitulate the diversity identified in patients. However, only recently biobanks have been able to collect enough samples to generate reports with dozens and dozens of individuals^{7,8}, we hope that the increase in samples availability and affordability of single-cell technology will allow a more comprehensive view of transcriptomic changes in ALS at single-cell level. Moreover, a bigger cohort size would allow a more stringent analysis of differentially expressed genes by “pseudo-bulking” single-nuclei dataset

at the individual level within a cell type before nominating DEGs. We also recognise that our study would benefit from additional validation at RNA and/or protein level, we hope that in the future this kind of studies would accompany bioinformatics analyses with more validations. These validations would also elucidate some of the findings in this study. For example, are oligodendrocytes in ALS patients really expressing higher levels of neuronal genes or is this an artifact coming from contaminations⁸⁵? As mentioned in the text, oligodendrocytes in the motor cortex have been shown to express synaptic transcripts⁴¹, immunofluorescent staining would further prove this point at the single cell level. Nonetheless, we believe that this study provides original and novel insights the involvement of different cell types in ALS and a different view in the motor cortex of ALS patients.

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Methods

Human donor tissue. Frozen post-mortem human cortical samples from cases of sporadic ALS patients and age-matched controls were obtained from the Target ALS Neuropathology Core that drew upon the repositories of five institutions. Specimens from the medial, lateral, or unspecified motor cortex were grouped together. Additional post-mortem human samples of the posterior frontal cortex consistent with the motor cortex from ALS patients and controls were obtained at MGH using a Partners IRB approved protocol and stored at -80°C.

Isolation of nuclei. RNA quality of brain samples was assessed by running bulk nuclear RNA on an Agilent TapeStation for RIN scores. Extraction of nuclei from frozen samples was performed as previously described⁸⁶. Briefly, tissue was dissected and minced with a razor blade on ice and then placed in 4 ml ice-cold extraction buffer (Wash buffer (82 mM Na₂SO₄, 30 mM K₂SO₄, 5 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH) containing 1% Triton X-100 and

5% Kollidon VA64). Tissue was homogenized with repeated pipetting, followed by passing the homogenized suspension twice through a 26 ½ gauge needle on a 3 ml syringe (pre-chilled), once through a 20 mm mesh filter, and once through a 5 mm filter using vacuum. The nuclei were then diluted in 50 ml ice-cold wash buffer, split across four 50 ml tubes, and centrifuged at 500xg for 10 minutes at 4°C. The supernatant was discarded, the nuclei pellet was resuspended in 1 ml cold wash buffer.

10X loading and library preparation. Nuclei were DAPI-stained with Hoechst, loaded onto a hemocytometer, and counted using brightfield and fluorescence microscopy. The solution was diluted to ~176 nuclei/ul before proceeding with Drop-seq as described in ref.¹⁵²⁸. cDNA amplification was performed using around 6000 beads per reaction with 16 PCR cycles. The integrity of both the cDNA and tagmented libraries were assessed for quality control on the Agilent Bioanalyzer as in ref.⁸⁷. Libraries were sequenced on a Nova-seq S2, with a 60 bp genomic read. Reads were aligned to the human genome assembly (hg19). Digital Gene Expression files were generated with the Zamboni Drop-seq analysis pipeline, designed by the McCarroll group^{86,88}.

Filtering of expression matrices and clustering of single nuclei. A single matrix for all samples was built by filtering any barcode with less than 400 genes and resulting in a matrix of 27,600 genes across 119,510 barcodes. This combined UMI matrix was used for downstream analysis using Seurat (v3.0.2)²⁹. A Seurat object was created from this matrix by setting up a first filter of min.cells=20 per genes. After that, barcodes were further filtered by number of genes detected nFeature_RNA>600 and nFeature_RNA<6000. Distribution of genes and UMIs were used as parameters for filtering barcodes. The matrix was then processed via the Seurat pipeline: log-normalized by a factor of 10,000, followed by regressing out UMI counts (nCount_RNA), scaled for gene expression.

After quality filtering, 79,830 barcodes and 27,600 genes were used to compute SNN graphs and *t*-SNE projections using the first 10 statistically significant Principal Components. As previously described^{89,90}, SNN-graphed *t*-SNE projection was used to determine minimum number of clusters obtain at resolution=0.2 (FindClusters). Broad cellular identities were assigned to groups on the basis of differentially expressed genes as calculated by Wilcoxon rank sum test in FindAllMarkers(min.pct=0.25, logfc.threshold=0.25). One subcluster with specifically high ratio of UMIs/genes was filtered out resulting in 79,169 barcodes grouped in 7 major cell types of the CNS: excitatory neurons, oligodendrocytes, inhibitory neurons, astrocytes, endothelial cells, microglia, oligodendrocyte progenitor cells (OPCs). Markers for specific cell types were identified in previously published human scRNAseq studies^{30,31}.

Analysis of cellular subtypes were conducted by subsetting each group. Isolated barcodes were re-normalised and scaled and relevant PCs were used for re-clustering as a separate analysis. This newly scaled matrix was used for Differential Gene Expression analysis with the MAST algorithm⁹¹ in Seurat R package as previously reported^{19,21,23,25,26} with parameters FindAllMarkers(min.pct=0.10, logfc.threshold=0.25) and subclustering for identification of subgroups. Gene scores for different cellular subclusters were computed in each re-normalised, re-scaled sub-matrix using the AddModuleScore function in Seurat v3.0.2.

Gene Ontology, Interactome and Gene Set Enrichment Analyses. For GO terms analysis, we selected statistically significant up-regulated or down-regulated genes identified in each subcluster as described before (adj p-values<0.05, LFC=2). These lists were fed in the gProfiler pipeline⁹² with settings: use only annotated genes, g:SCS threshold of 0.05, GO cellular components and GO biological processes (26th of May 2020 – 9th of December 2021), only statistically significant pathways are highlighted. For oligodendrocytes cells (Extended Data Fig.8) statistically significant up-regulated genes identified in each subcluster as described before (adj p-values<0.05, LFC=2) were used for synaptic specific Gene Ontology analysis using SynGO⁹³ (12th of June 2020). Interactome map was built using STRING⁹⁴ protein-protein interaction networks, all statistically significant upregulated genes were used, 810 were identified as interacting partners using “experiments” as interaction sources and a medium confidence threshold (0.400), only interacting partners are shown in Extended Data Figure 6. Gene Set Enrichment Analysis was performed using GSEA software designed by UC San Diego and the Broad Institute (v4.0.3)⁹⁵. Briefly, gene expression matrices were generated in which for each subcluster each individual was a metacell, lists for disease-associated risk genes were compiled using available datasets (PubMed – ALS/FTD – Supplementary Table 2) or recently published GWAS for AD^{33,34} and MS³⁵.

Generation of Microglia-like Cells. Microglial-like cells were differentiated as described⁶⁸ with minor modifications^{37,89}. Briefly, hPSCs were cultured in E8 medium (Stemcell technologies) on Matrigel (Corning), dissociated with Accutase (Stemcell technologies), centrifuged at 300xg for 5 minutes, resuspended in E8 medium with 10µM Y-27632 ROCK Inhibitor, 2M cells are transferred to a low-attachment T25 flask in 4ml of medium and left in suspension for 24 hours. The first 10 days of

differentiation are carried out in iHPC medium: IMDM (50%, Stemcell technologies), F12 (50%, Stemcell technologies), ITS-G 2% v/v (ThermoFisher), L-ascorbic acid 2-Phosphate (64 μ g/ml, Sigma), monothioglycerol (400 mM, Sigma), PVA (10 mg/ml; Sigma), Glutamax (1X, Stemcell technologies), chemically-defined lipid concentrate (1X, Stemcell technologies), non-essential amino acids (NEAA, Stemcell technologies). After 24h (day0), cells are collected and differentiation is started in iHPC medium supplemented with FGF2 (Peprotech, 50 ng/ml), BMP4 (Peprotech, 50 ng/ml), Activin-A (Peprotech, 12.5 ng/ml), Y-27632 ROCK Inhibitor (1 μ M) and LiCl (2mM) and transferred in hypoxic incubator (20% O₂, 5% CO₂, 37°C). On day 2, medium is changed to iHPC medium plus FGF2 (Peprotech, 50 ng/ml) and VEGF (Peprotech, 50 ng/ml) and returned to hypoxic conditions. On day4, cells are resuspended in iHPC medium supplemented with FGF2 (Peprotech, 50 ng/ml), VEGF (Peprotech, 50 ng/ml), TPO (Peprotech, 50 ng/ml), SCF (Peprotech, 10 ng/ml), IL-6 (Peprotech, 50 ng/ml), and IL-3 (Peprotech, 10 ng/ml) and placed into a normoxic incubator (20% O₂, 5% CO₂, 37°C). Expansion of haematopoietic progenitors is continued by supplementing the flasks with 1ml of iHPC medium with small molecules every two days. On day10, cells are collected and filtered through a 40mm filter. The single cell suspension is counted and plated at 500,00 cells/well of a 6 well plate coated with Matrigel (Corning) in Microglia differentiation medium: DMEM/F12 (Stemcell technologies), ITS-G 2%v/v (Thermo Fisher Scientific), B27 (2%v/v, Stemcell technologies), N2 (0.5%v/v, Stemcell technologies), monothioglycerol (200 mM, Sigma), Glutamax (1X, Stemcell technologies), NEAA (1X, Stemcell technologies), supplemented with M-CSF (25 ng/ml, Peprotech), IL-34 (100 ng/ml, Peprotech), and TGFb-1 (50 ng/ml, Peprotech). Induced Microglia-like cells (iMGLs) are kept in this medium for 20 days with change three times a week. On day 30, cells are collected and plated on poly-D-lysine/laminin coated dishes in Microglia differentiation medium supplemented with CD200 (100 ng/ml, Novoprotein) and CX3CL1 (100 ng/ml, Peprotech), M-CSF (25 ng/ml, Peprotech), IL-34 (100 ng/ml, Peprotech), and TGFb-1 (50 ng/ml, Peprotech) until day 40.

Feeding of apoptotic neurons to Microglia-like Cells. For feeding assays, neurons were generated from human iPSCs using an NGN2 overexpression system as described previously^{59,96,97}. Day30 hiPSC-neurons “piNs” were treated with 2 μ M H₂O₂ for 24 hours to induce apoptosis. Apoptotic neurons were gently collected from the plate and the medium containing the apoptotic bodies was transferred into wells containing day40 iMGLs. After 24 hours, iMGLs subjected to apoptotic neurons and controls were collected for RNA extraction.

RNA extraction and RT-qPCR analysis. RNA was extracted with the miRNeasy Mini Kit (Qiagen, 217004). cDNA was produced with iScript kit (BioRad) using 50 ng of RNA. RT-qPCR reactions were performed in triplicates using 20 ng of cDNA with SYBR Green (BioRad) and were run on a CFX96 Touch™ PCR Machine for 39 cycles at: 95°C for 15s, 60°C for 30s, 55°C for 30s.

Generation of hiPSC-derived neurons for bulk RNA sequencing. Human embryonic stem cells were cultured in mTESR (Stemcell technologies) on matrigel (Corning). Neurons were generated from HuES-3-Hb9:GFP based on the motor neuron differentiation protocol previously described^{58,98}. Upon completion of the differentiation protocol, cells were sorted via flow-cytometry based on GFP signal intensity to yield GFP-positive neurons that were plated on PDL/laminin-coated plates (Sigma, Life technologies). Neurons were maintained in Neurobasal medium (Life Technologies) supplemented with N2 (Stemcell technologies), B27 (Life technologies), glutamax (Life technologies), non-essential amino acids (Life technologies), and neurotrophic factors (BDNF, GDNF, CNTF), and were grown for 28 days before the application of the proteasome inhibitors MG132 for 48 hrs.

RNA was extracted using RNeasy Plus kit (Qiagen), libraries were prepared using the Illumina TruSeq RNA kit v2 according to the manufacturer's directions, and sequenced at the Broad Institute core with samples randomly assigned between two flow chambers. The total population RNA-seq FASTQ data was aligned against ENSEMBL human reference genome (build GRCh37/hg19) using STAR (v.2.4.0). Cufflinks (v.2.2.1) was used to derive normalized gene expression in fragments per kilo base per million (FPKM). The read counts were obtained from the aligned BAM-files in R using Rsubread. Differential gene expression was analyzed from the read counts in DESeq2 using a Wald's test for the treatment dosage and controlling for the sequencing flow cell.

Western blot analysis. As previously described tissue was minced, lysed in RIPA buffer with protease inhibitors (Roche) and sonicated⁹⁹. After centrifugation, the supernatant was collected as soluble fraction and the insoluble pellet was resuspended in 8M urea buffer (Bio-Rad, 1632103). After protein quantification by BCA assay (ThermoFisher), ten micrograms of proteins were preheated in Laemmli's buffer (BioRad), loaded in 4-20% mini-PROTEAN® TGX™ precast protein gels (BioRad) and gels were transferred to a PDVF membrane. Membranes were blocked in Odyssey Blocking Buffer (Li-Cor) and incubated overnight at 4°C with primary antibodies. After washing with TBS-T, membranes were incubated with IRDye® secondary antibodies (Li-Cor) for one hour and imaged with Odyssey® CLx imaging system (Li-Cor). List of primary antibodies can be found in Appendix.

Proteasome activity assay. Neurons were sorted in 96-wells plates and, after two weeks of maturation, treated for 24 hours. Cells were washed with 1xPBS, exposed to ProteasomeGlo® (Promega, G8660) and incubated for 30 minutes at RT. Fluorescence was measured using a Cytation™3 reader (BioTek).

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Author contributions

The study was designed by F.L., D.M. and directed and coordinated by K.E. and S.A.M with input from B.S. and I.K. Manuscript writing by F.L. and D.M., with support from O.P. and A.B. F.L. performed bioinformatics analysis with the help of S.D.G., D.M. and D.M. D.M. and I.C. supported obtaining post-mortem samples and carried out nuclei isolation and RNA-sequencing with M.G. and L.B.; M.T., O.P., A.B., A.C. and B.J.J. performed bioinformatics analyses of bulk RNA-sequencing and helped with

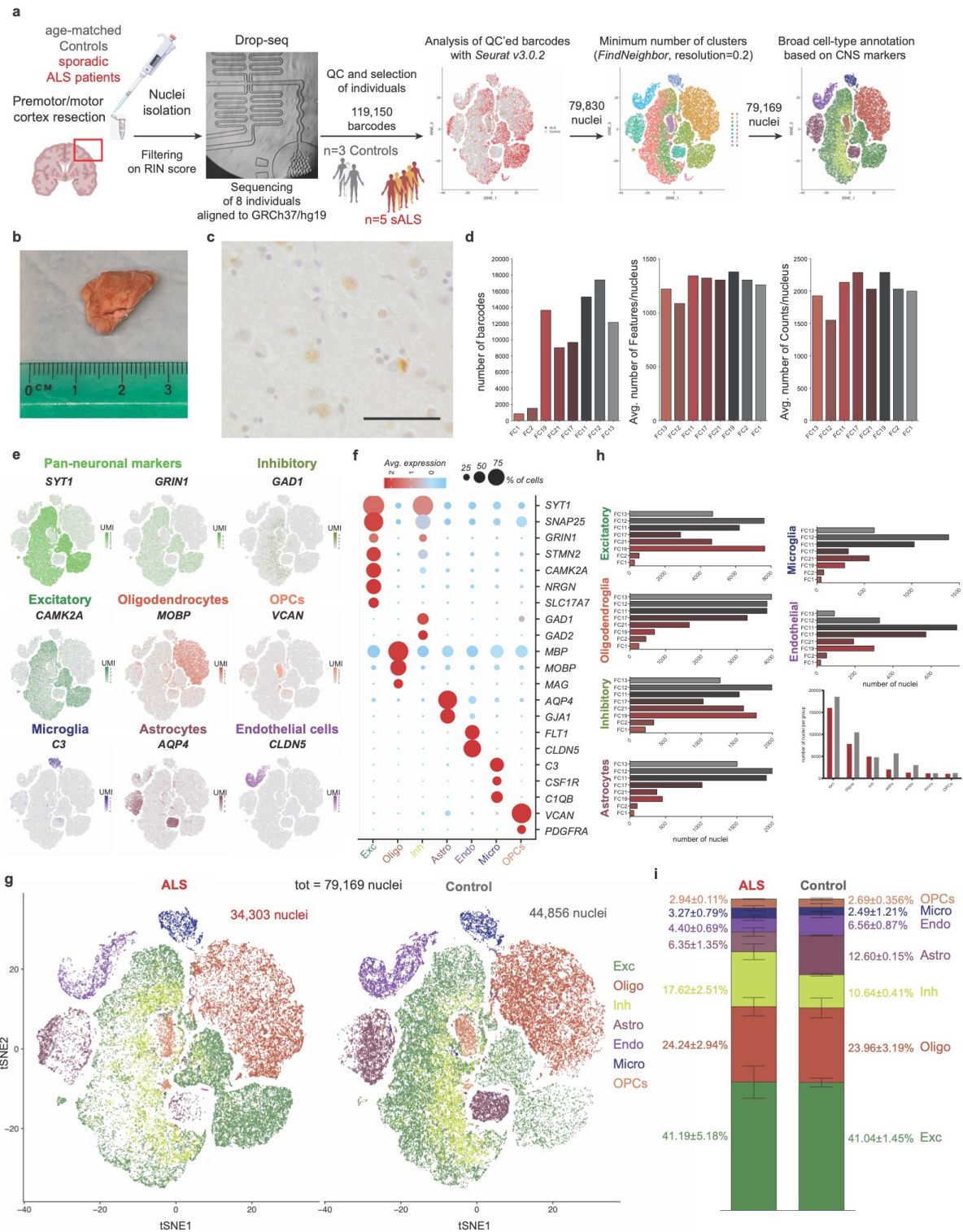
protein and RNA validation with cellular models; J.M.M. performed analysis of published datasets; M.T. and B.S. contributed to microglial biology section; K.E. acquired primary funding.

Competing interests

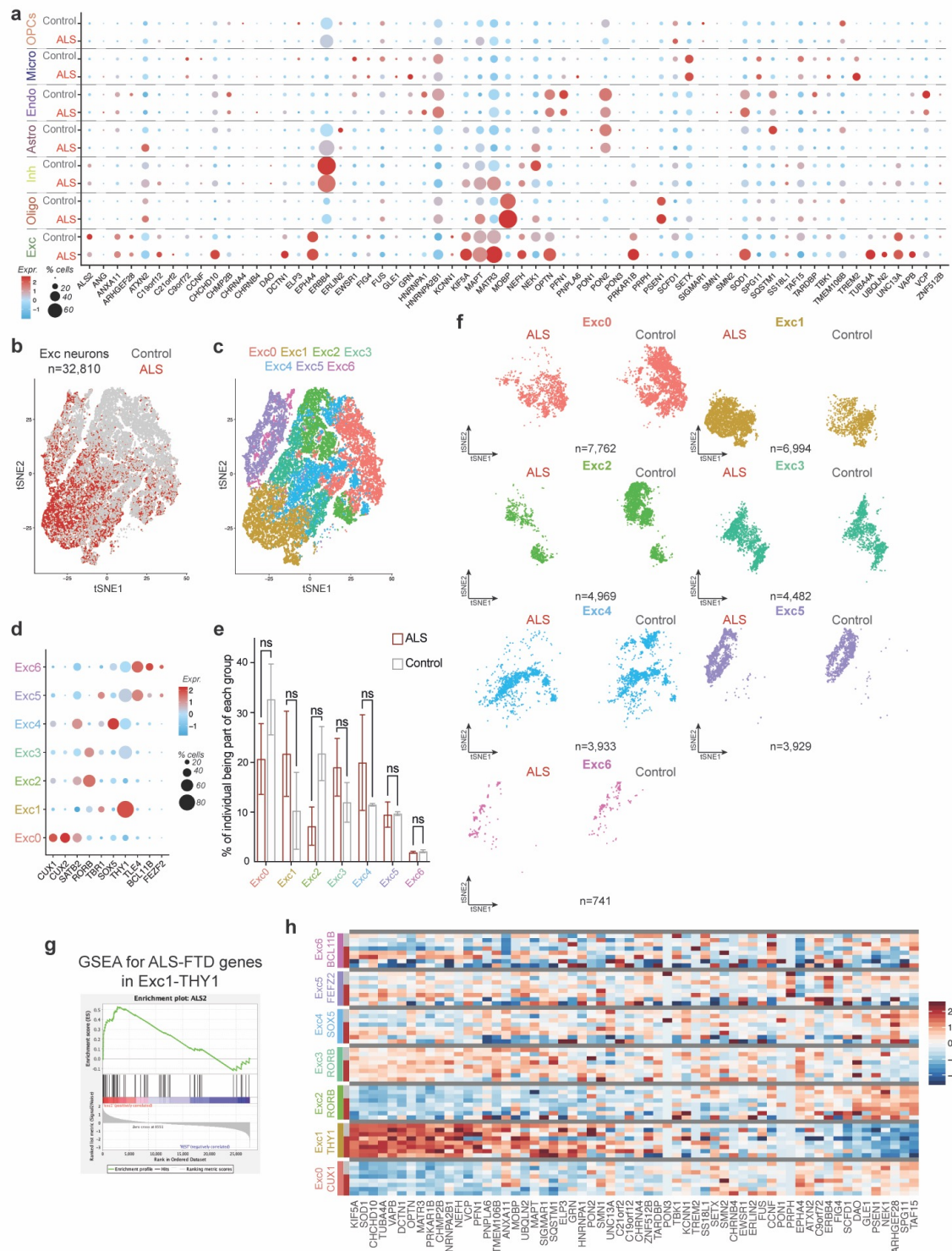
I.K. is an employee at UCB Pharma and holds stock options. K.E. is a cofounder of Q-State Biosciences, Quralis, Enclear Therapies and is group vice-president at BioMarin Pharmaceutical. K.E. and F.L. are authors on a pending patent "Single-nuclei characterization of amyotrophic lateral sclerosis frontal cortex" (US 2022,17535070).

Additional information

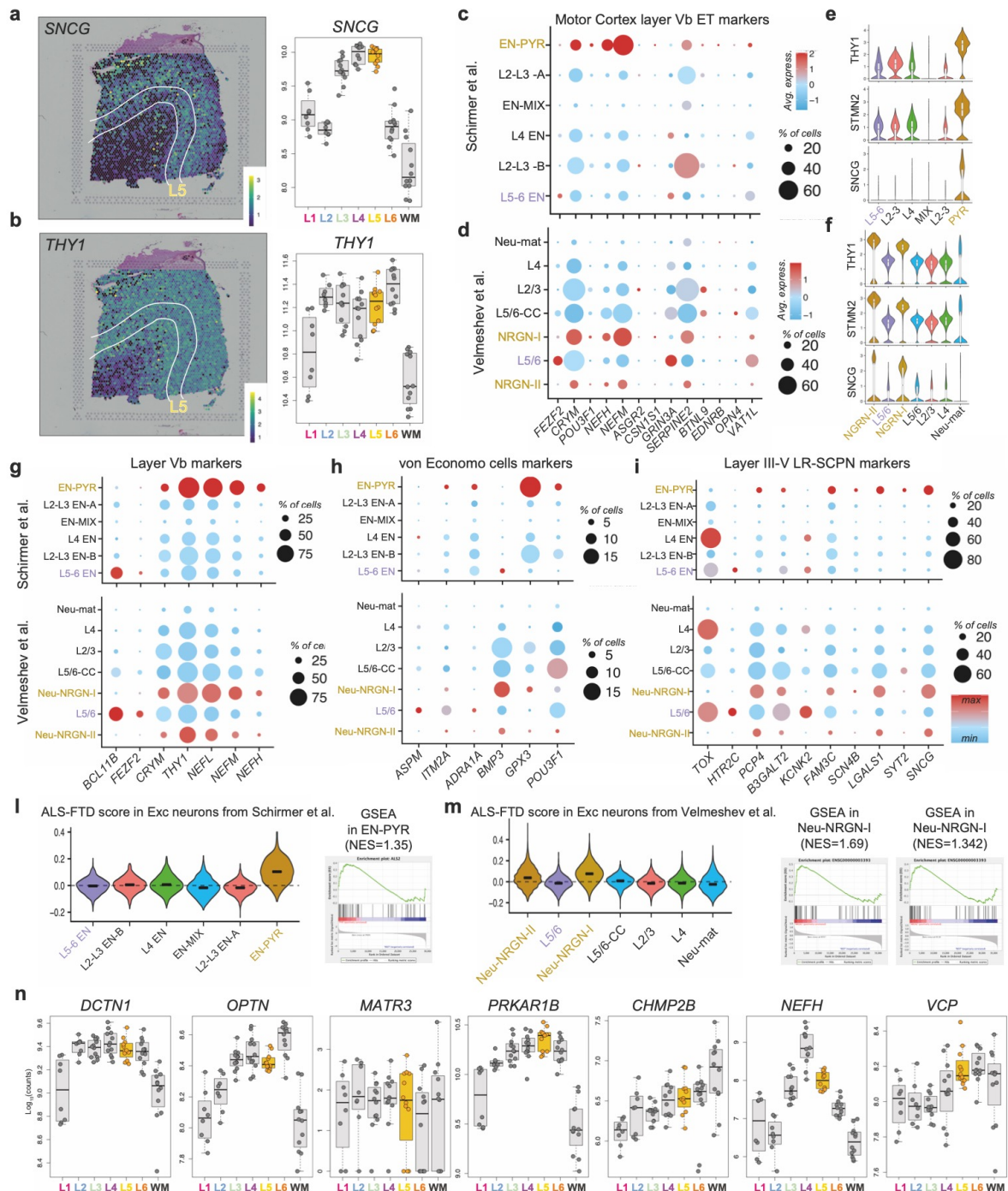
We worked to ensure diversity in experimental samples through the selection of genomic datasets. One or more of the authors self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.



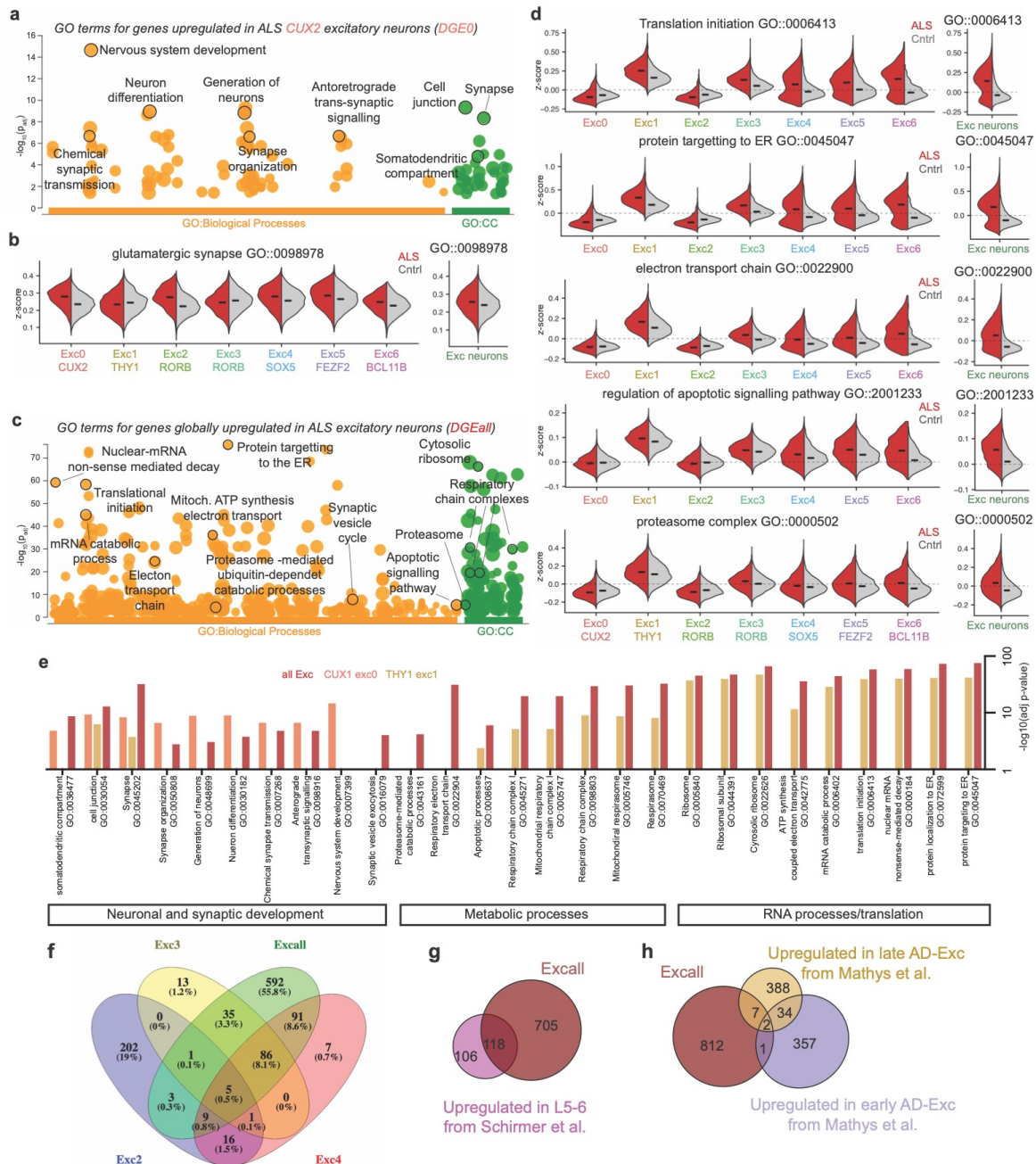
Extended Data Fig. 1 | Technical parameters of snRNAseq and cell-type distribution across individuals. **a.** Schematic diagram of workflow for isolation of nuclei from cortices of ALS patients and age-matched controls followed by single-cell RNA sequencing by DropSeq, library generation and Quality Controls for analysis with Seurat 3.0.2 **b.** Frozen tissue from one of the individuals. **c.** Staining for TDP-43 in one of the patient sample, note neurons with skein-like inclusions and faint nuclear staining (scale bar 25μm). **d.** Quality controls post-filtering (FC – Frontal Cortex): number of total nuclei detected (barcodes), average number of genes per nucleus (nFeatures), and average number of UMIs (Unique Molecular Identifiers) per nucleus (nCounts). **e.** t-SNE projections of the whole cohort with expression of broad cell type markers. **f.** Dotplot representing percentage of cells expressing additional cell type specific markers. **g.** t-SNE distribution of whole cohort with annotated cell types split by diagnosis (ALS patients $n=5$, age-matched Controls $n=3$, $n = 79,169$ total nuclei). **h.** t-SNE distribution of whole cohort with annotated cell types split by diagnosis (ALS patients $n=5$, age-matched Controls $n=3$, $n = 79,169$ total nuclei). **i.** Fraction of each cell types identified in whole cohort split by diagnosis.



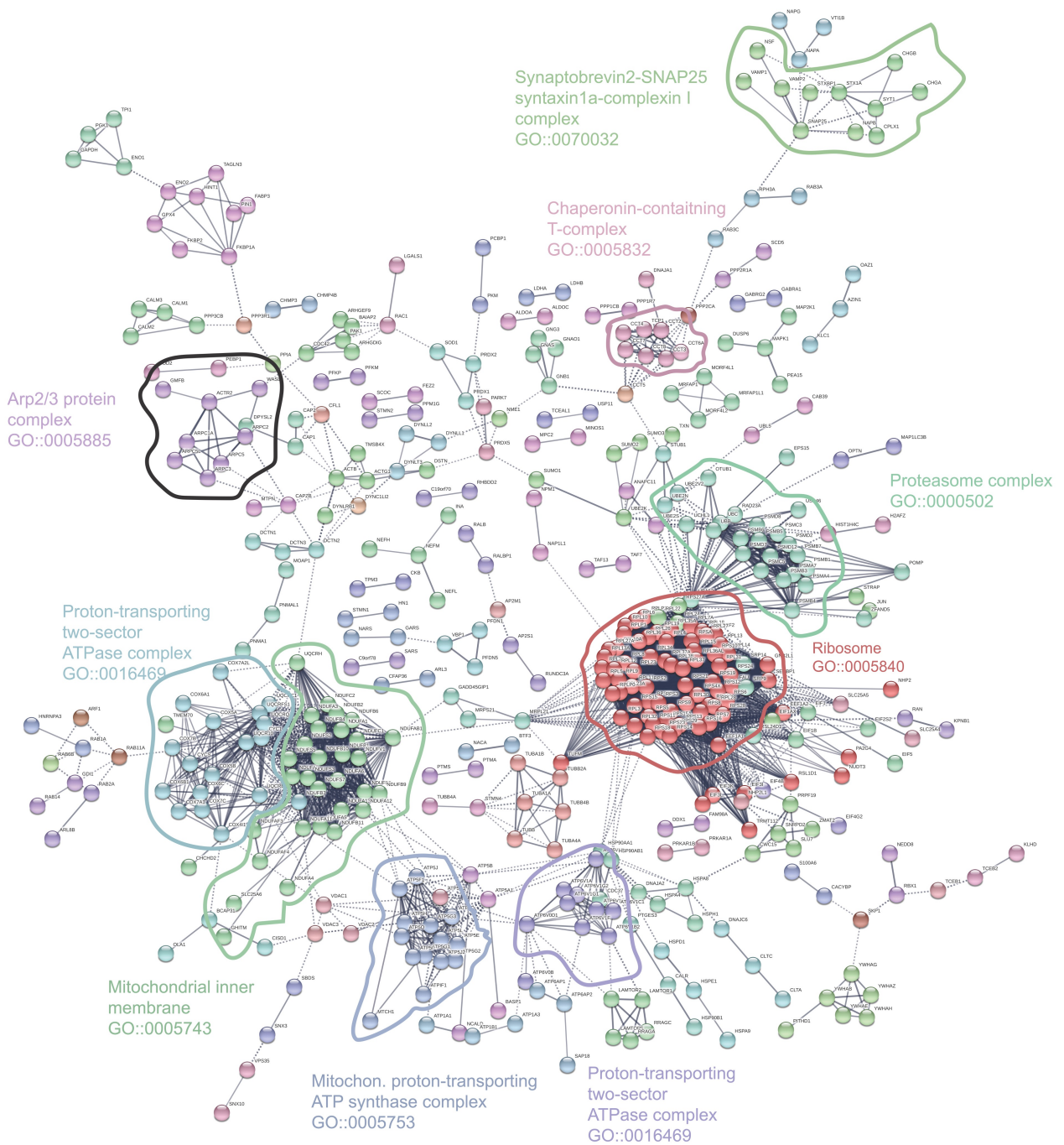
Extended Data Fig. 2 | Expression of ALS-FTD associated genes in different cellular subtypes and excitatory neurons subtypes. **a.** Dotplot representing expression of gene associated with the ALS-FTD spectrum in each cell type identified in the whole cortex split by diagnosis. **b.** t-SNE projection of excitatory neurons clusters (ALS $n=15,227$ nuclei, Control $n=17,583$ nuclei). **c.** t-SNE projection of subclusters identified in excitatory neurons represents different, biologically relevant neuronal layers (*FindNeighbor*(res=0.2)). **d.** Dotplot representing percentage of cells expressing broad markers for different cortical layers. **e.** Distribution of excitatory neurons in subclusters by diagnosis. **f.** t-SNE projection of excitatory neurons by clusters and by diagnosis. **g.** Get Set Enrichment Analysis for the ALS-FTD associated genes in Exc1 cortical neurons. **h.** Heatmap representing expression of gene associated with the ALS-FTD spectrum in each excitatory neurons identified split by diagnosis.



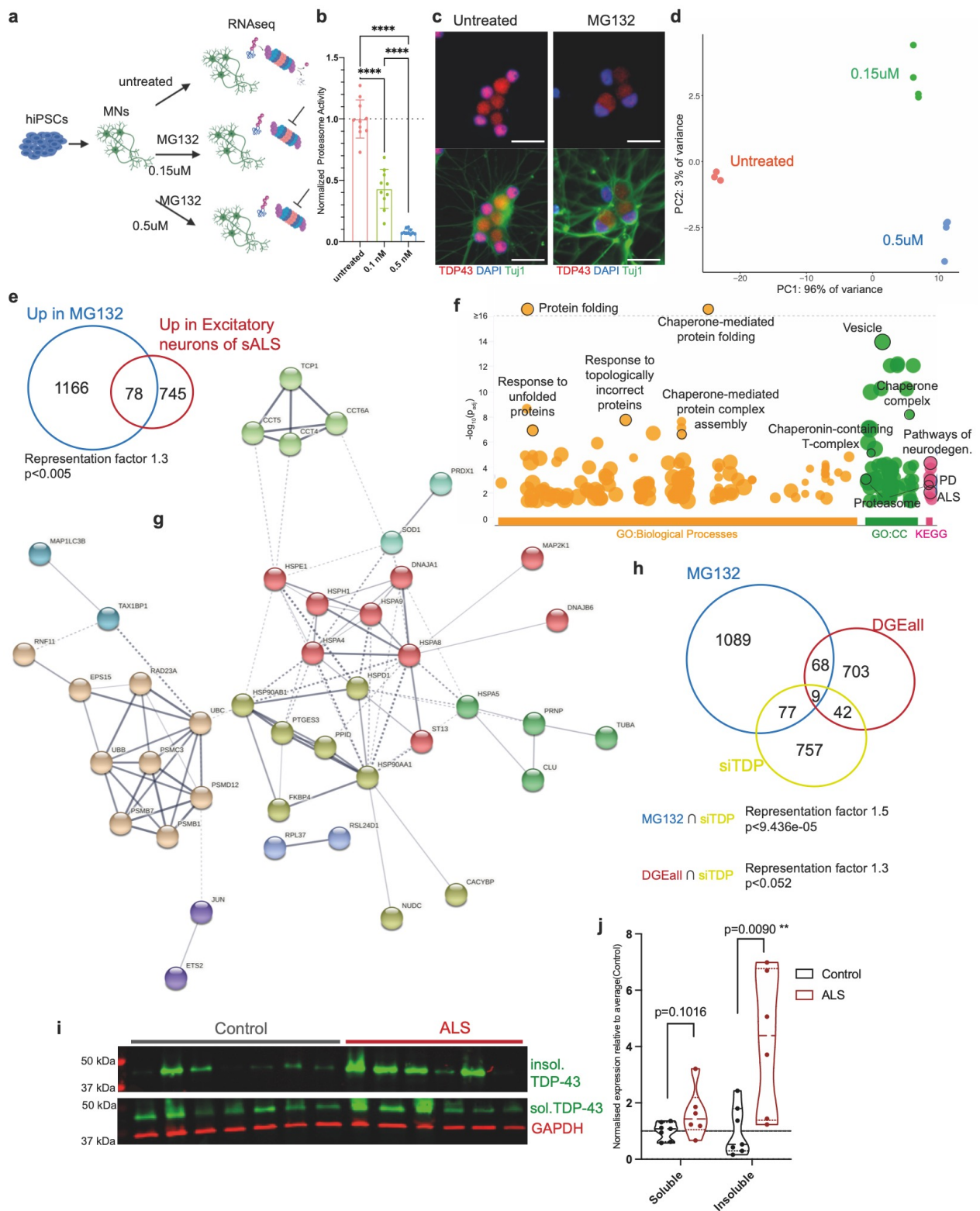
Extended Data Fig. 3 | L5-ETNs/CSMN-like neurons express higher levels of ALS-FTD related genes. **a,b.** Spotplot and corresponding boxplot from Maynard et al. for the expression of layer Vb Motor Cortex marker, *SNCG* and *THY1*, identified as enriched in Exc1. **c,d.** Dotplot and representative Violin plots for markers of L5 ExtraTelencephalic neurons of human Motor Cortex in Schirmer et al. **e,f.** Dotplot and representative Violin plots for markers of L5 ExtraTelencephalic neurons of human Motor Cortex in Velmeshev et al. **g-i.** Dotplot representing expression of Layer V markers (d), von Economo markers (e), LR-SCPN markers (f) in Schirmer et al. and Velmeshev et al. **i.** Violin plots and corresponding Gene Set Enrichment Analysis of z-scores for expression of ALS-FTD-associated genes in THY1-neurons identified by Schirmer et al. (bars denote median). **m.** Violin plots and corresponding Gene Set Enrichment Analysis of z-scores for expression of ALS-FTD-associated genes in THY1-neurons identified by Velmeshev et al. (bars denote median). **n.** Boxplot from Maynard et al. for the expression of top 10 ALS/FTD associated genes identified in Exc1.



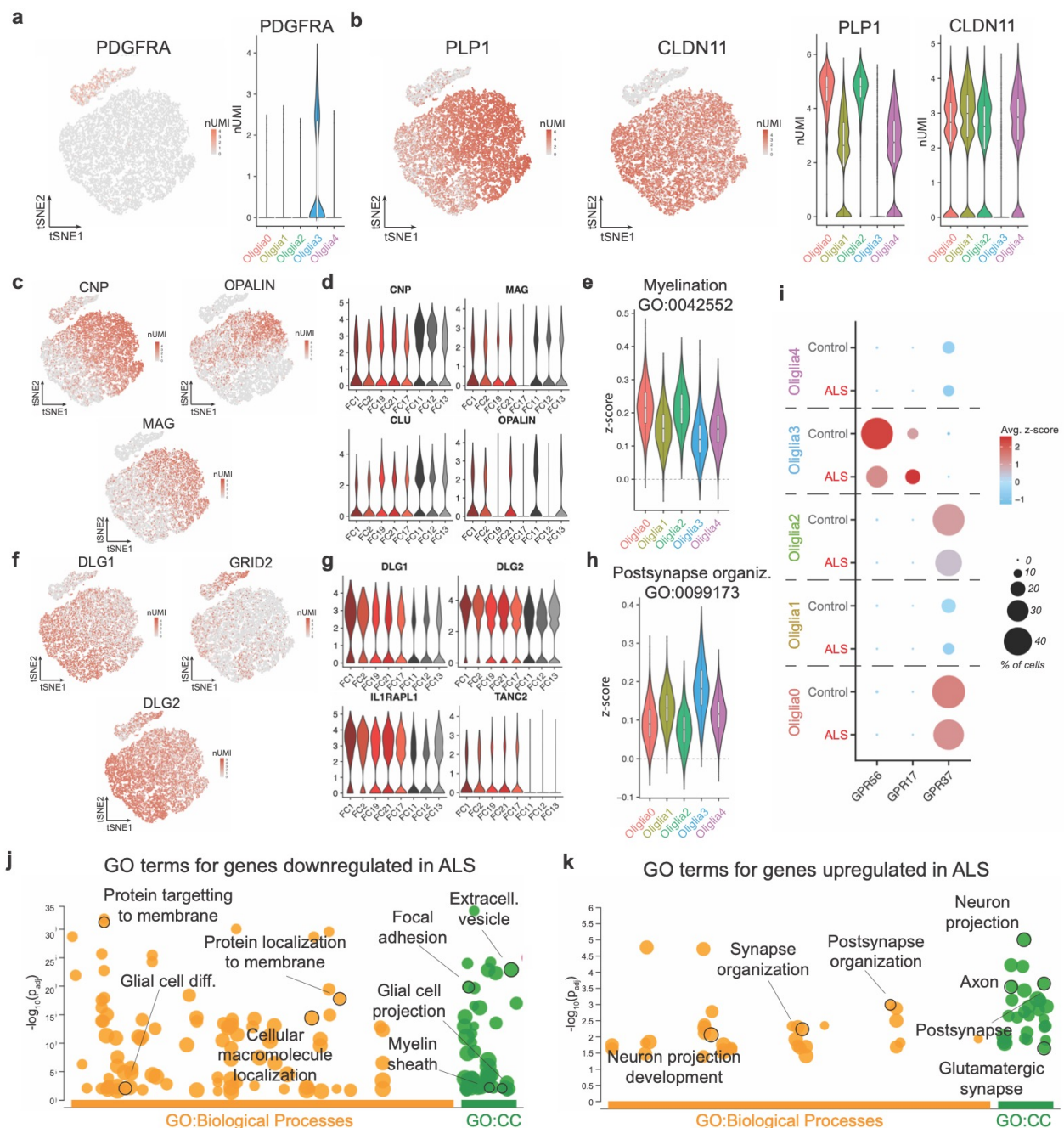
Extended Data Fig. 4 | Classes of L5-ETNs express higher levels of stress pathways. **a.** Gene Ontology analysis for genes globally upregulated in ALS excitatory neurons (DGEall). **b.** Violin plots representing z-scores for selected, statistically significant GO terms upregulated in lower layers in each subgroup (left) and globally (right). **c.** Gene Ontology analysis of terms for genes upregulated in CUX2-Exc0 group (DGE0), highlighted terms involved in synaptic biology (CC=Cellular Components). **d.** Violin plot representing z-scores for selected, statistically significant GO terms upregulated in upper layers in each subgroup (left) and globally (right). **e.** Representation of $-\log_{10}(\text{adjusted p-values})$ of selected GO terms from previous figures for CUX2-Exc0 and THY1-Exc1 groups and globally. **f.** Venn Diagram depicting shared upregulated genes between other excitatory neurons and global signature. **g-h.** Venn Diagram depicting shared upregulated genes between treated MS excitatory neurons from Schirmer et al. and AD excitatory neurons from Mathys et al. with changes found in this study.



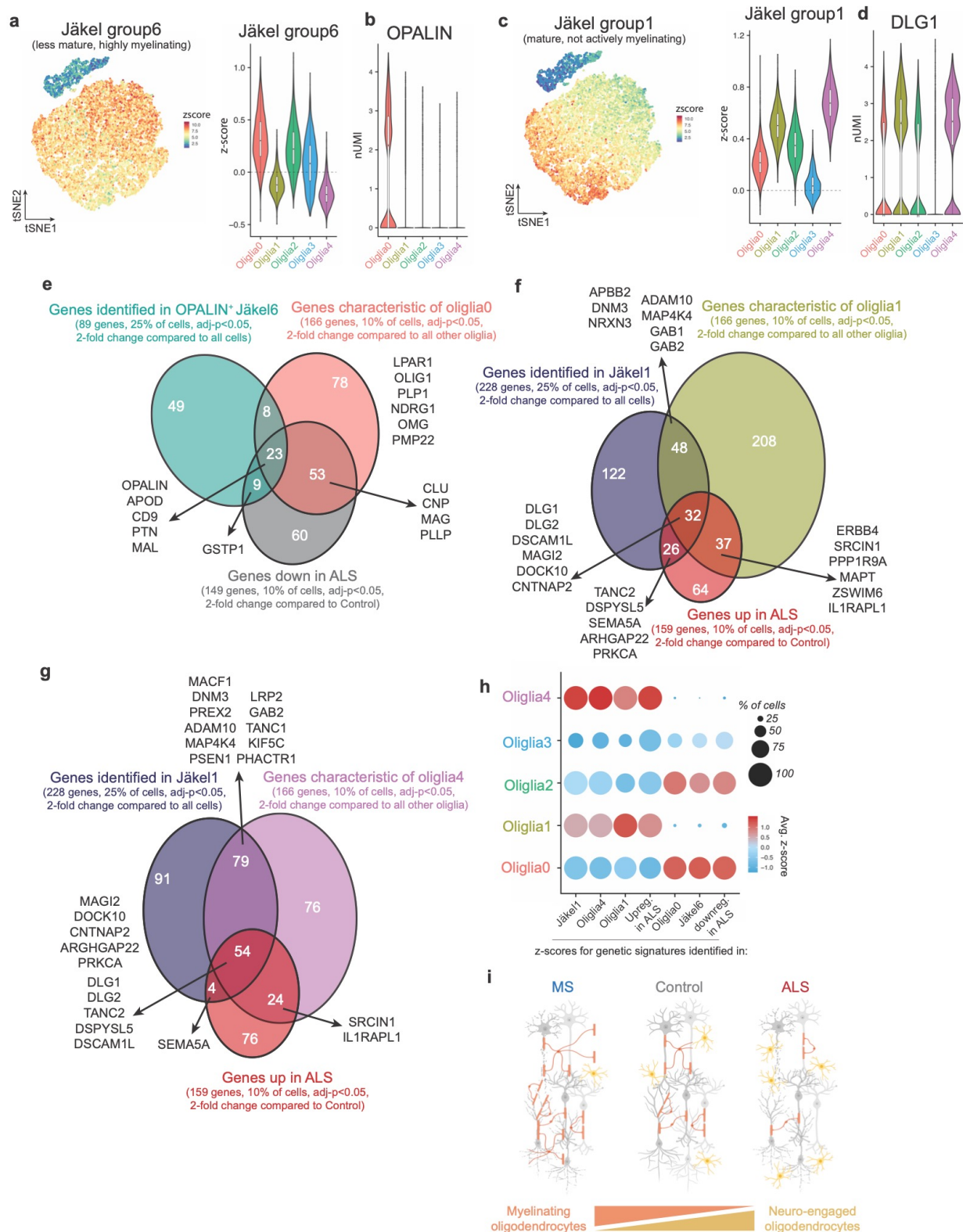
Extended Data Fig. 5 | Global protein-protein interaction network for genes upregulated in ALS excitatory neurons. Color-coding derived from MLC clustering (4) to identified closely related groups of proteins.



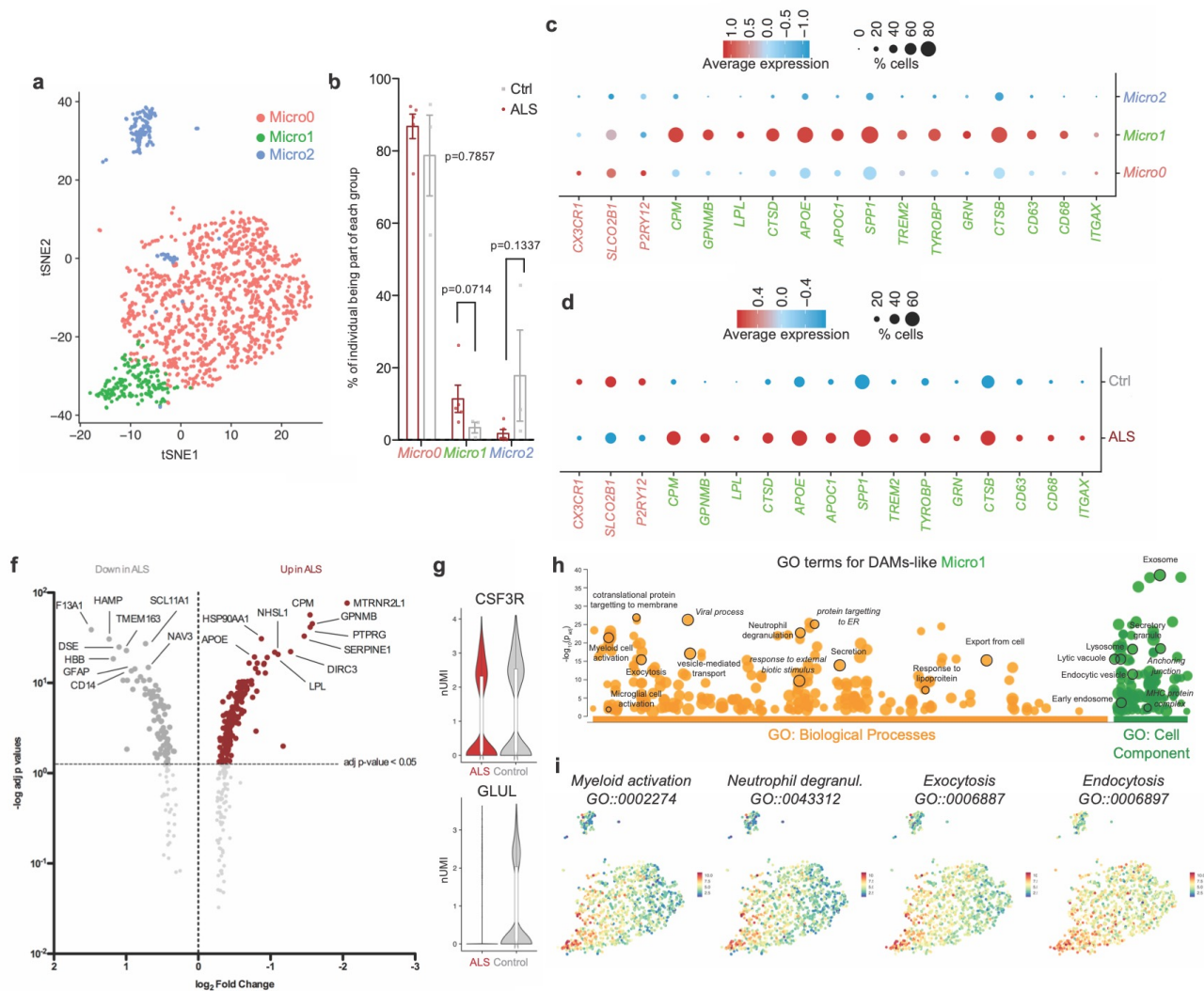
Extended Data Fig. 6 | Proteostatic stress in hPSC-derived neurons resembles changes in excitatory neurons from brain of ALS patients. **a.** Diagram of neuronal differentiation from Pluripotent Stem Cells and treatment with proteasome inhibitors for bulk RNA-sequencing. **b.** Quantification of proteasome inhibition. **c.** Immunofluorescence of TDP-43 localisation after treatment. **d.** Principle Component Analysis plot showing strong effect of treatments compared to untreated controls. **e.** Venn Diagram depicting shared upregulated genes between treated hPSC-derived neurons and excitatory neurons from ALS patients. **f.** Gene Ontology analysis for shared genes in (e), highlighted terms involved in protein folding and neurodegenerative diseases (CC=Cellular Components). **g.** Protein-protein interaction network of shared genes from (d). **h.** Venn Diagram depicting shared upregulated genes in treated hPSC-derived neurons (MG132), excitatory neurons from ALS patients (DGEall) and genes misregulated in human neurons after TDP-43 siRNA from Klim et al. **i-j.** Western Blot quantification of soluble and insoluble TDP-43 from MotorCortex of ALS patients and age-matched controls.



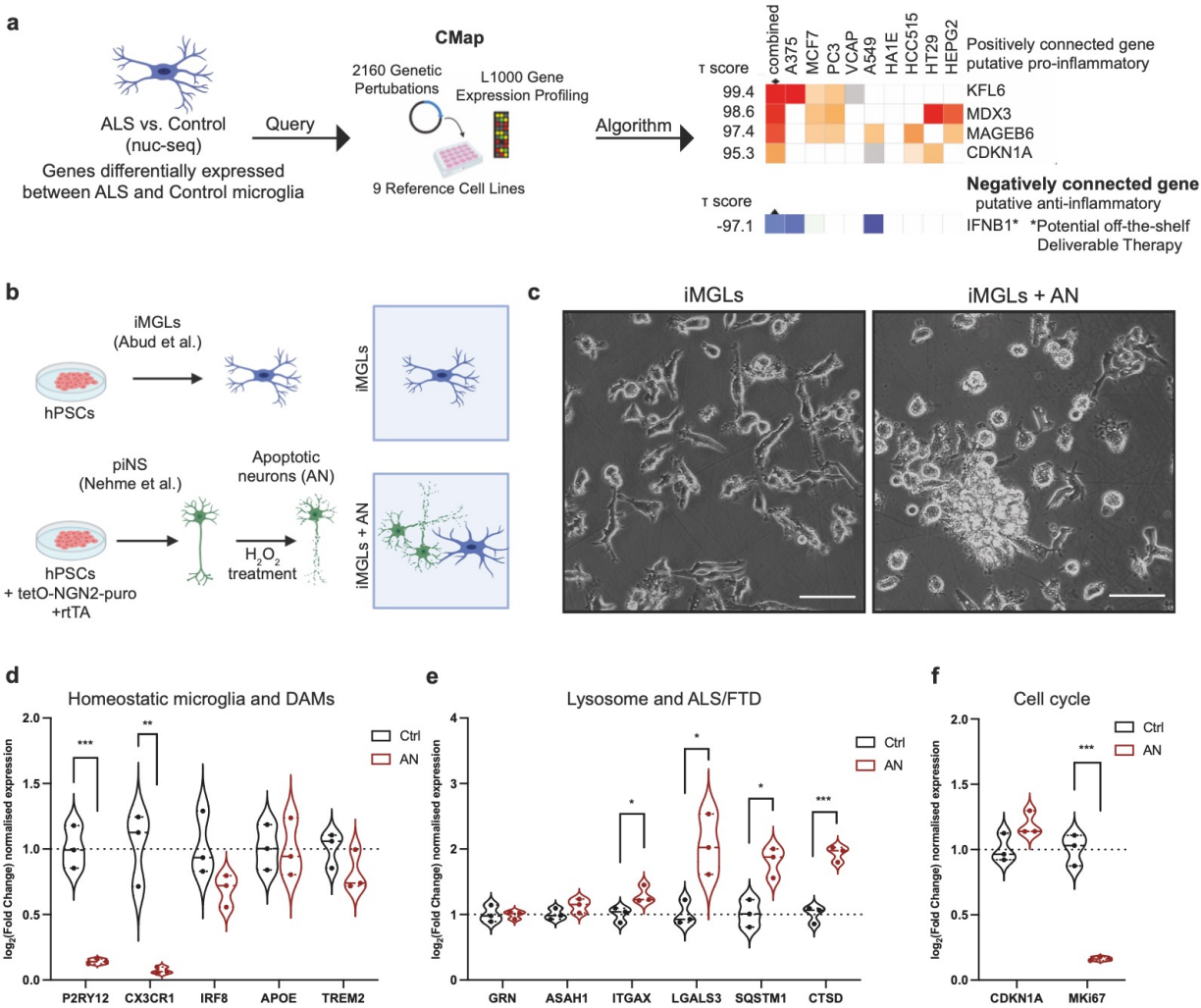
Extended Data Fig. 7 | Oligodendrocytes polarize between myelinating and neuro-engaged states. a. t-SNE projection and Violin plot of markers of Oligodendrocyte Progenitor Cells (OPCs). **b.** t-SNE projection and Violin plots of markers of mature oligodendrocytes. **c-d.** t-SNE projection and Violin plots of markers of actively myelinating oligodendrocytes. **e.** Violin plots representing z-score for selected GO terms by cluster. **f-g.** t-SNE projection and Violin plots of markers of neuro-engaged oligodendrocytes. **h.** Violin plots representing z-score for selected GO terms by cluster. **i.** Dotplot representing genes characteristic of maturation and development of OPCs in myelinating oligodendrocytes in each subcluster split by diagnosis. **j.** GO analysis for genes downregulated in ALS oligodendrocytes, highlighted terms involved in myelination (CC=Cellular Component). **k.** GO analysis for genes upregulated in ALS oligodendrocytes, highlighted terms involved in neuro-supportive functions (CC=Cellular Component).



Extended Data Fig. 8 | Comparison of ALS-driven changes with other studies with similar signatures disrupted in the disease. a. *t*-SNE projection and violin plot representing z-score for genes characteristic of highly myelinating, OPALIN⁺ oligodendrocytes in Jäkel et al. **b.** Violin plot showing OPALIN expression in our dataset. **c.** *t*-SNE projection and violin plot representing z-score for genes of mature, not-actively myelinating oligodendrocytes in Jäkel et al. **d.** Violin plot showing DLG1 expression in our dataset. **e.** Comparison of genes downregulated in oligodendroglia from ALS patients with genes characteristic of highly myelinating, OPALIN⁺ subtypes identified by this study (oligia0) and by Jäkel et al (Jäkel6), highlighted genes are shared with GO terms shown in figures. **f,g.** Comparison of genes upregulated in oligodendroglia from ALS patients with genes characteristic of mature, lowly myelinating groups in this study (oligia1 and 4) and by Jäkel et al (Jäkel1), highlighted genes are shared with GO terms shown in figures. **h.** Dotplot representing z-scores for the genetic signatures identified in the actively myelinating cells, the mature lowly myelinating cells and DEGs identified in this study. **i.** Diagram illustrates proposed shift of oligodendrocyte states.



Extended Data Fig. 9 | Shared features between ALS-driven changes and reactive subcluster of microglia. **a.** t-SNE projection of subclusters identified within microglia (Micro0 = Homeo = homeostatic, Micro1 = DAMs = Disease-associated microglia, Micro2 = Cycling cells). **b.** Distribution of microglia within clusters by diagnosis. **c.** Distribution of microglia within subclusters by individual. **d.** Dotplot representing genes identified as characteristic of Homeostatic microglia and DAMs by subcluster. **e.** Dotplot representing genes identified as characteristic of Homeostatic microglia and DAMs by diagnosis. **f.** Volcano plot of statistically significant differentially expressed genes between Control and ALS microglia (top ten upregulated and top ten downregulated genes highlighted). **g.** Violin plots of representative DEGs downregulated in ALS patients of genes associated with homeostatic microglia. **h.** Gene Ontology analysis of terms associated with genes characteristic of DAMs microglia, highlighted terms playing important role in microglial biology and/or pathogenesis of the disease. **i.** t-SNE projections representing z-score for selected, statistically significant GO terms.



Extended Data Fig. 10 | Apoptotic neurons upregulate lysosomal genes in microglia. **a.** Schematic of workflow and results from the Connectivity Map project for the genes upregulated in ALS microglia. Heatmap shows what cellular signature is most closely related to the query. **b.** Diagram of microglia and neuronal differentiation from Pluripotent Stem Cells, induction of apoptosis neurons and feeding to iMGLs. **c.** Brightfield images of untreated day 40 iMGLs and day 40 iMGLs fed apoptotic neurons for 24 hours. **d.** RT-qPCR quantification of selected ALS-FTD-associated and lysosomal genes 24h after feeding iMGLs with apoptotic neurons. **e.** RT-qPCR quantification of homeostatic and DAMs genes after feeding. **f.** RT-qPCR quantification of cell cycle-associated genes after feeding.