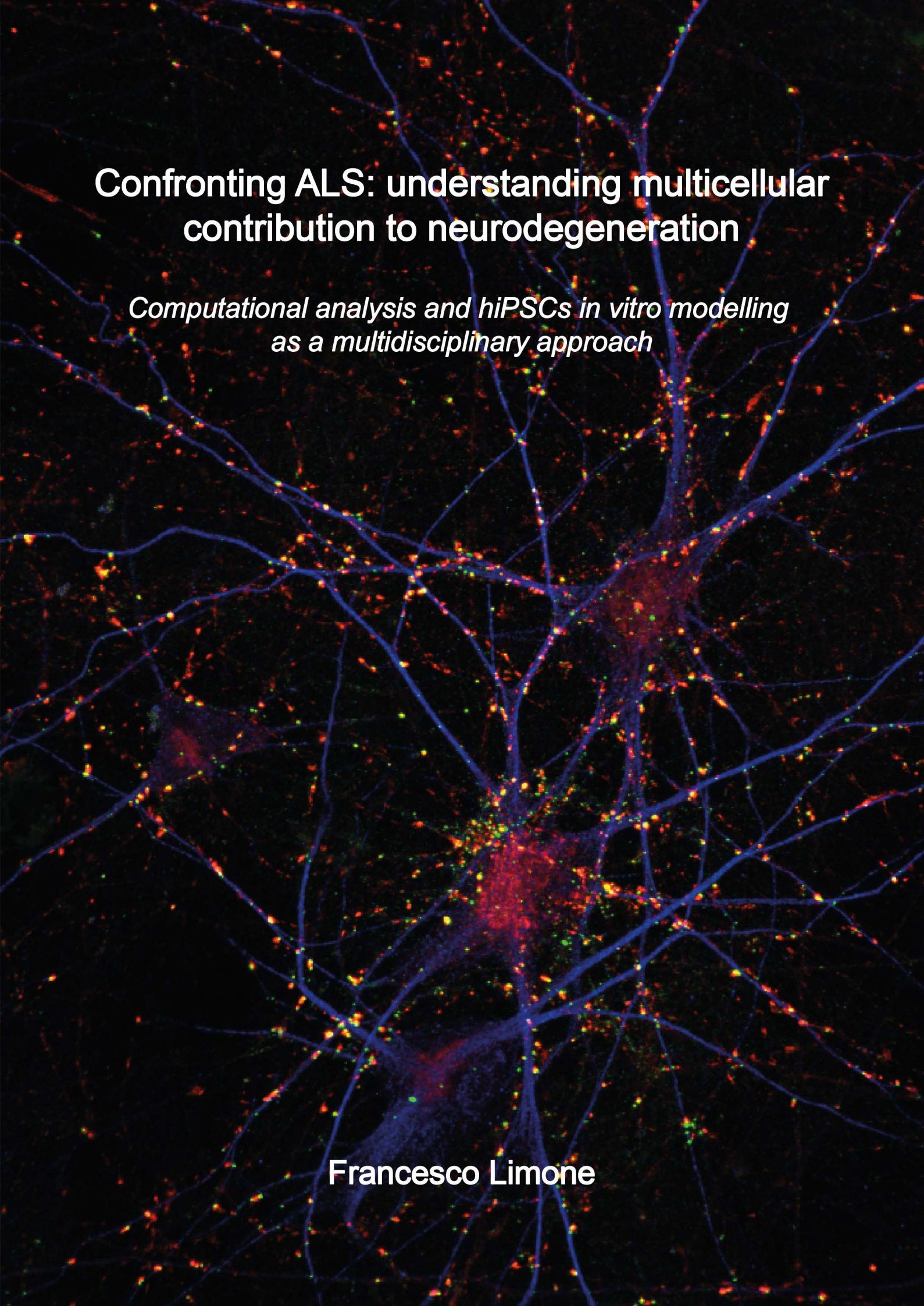
Confronting ALS: understanding multicellular contribution to neurodegeneration : computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

Limone, F.



Confronting ALS: understanding multicellular contribution to neurodegeneration

Computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

Francesco Limone

Confronting ALS: understanding multicellular contribution to neurodegeneration

Computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

Francesco Limone

Confronting ALS: understanding multicellular contribution to neurodegeneration

Computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

PhD thesis by Francesco Limone

Leiden University Medical Center, Harvard University

On cover: Synaptic staining of liMoNes, Irune Guerra San Juan – Sara Bolzon @sarabolzon.art

Confronting ALS: understanding multicellular contribution to neurodegeneration

Computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op dinsdag 26 september 2023
klokke 15:00 uur

door

Francesco Limone
geboren te Latina, Italië
in 1992

Promotors:

Prof. Dr. N. Geijsen

Prof. Dr. K. C. Eggan (Harvard University)

Promotiecommissie:

Prof. Dr. M. E. Drukker

Prof. Dr. R. C. Hoeben

Prof. Dr. C. L. Mummery

Prof. Dr. R. J. Pasterkamp (UMC Utrecht)

Prof. Dr. J. J.G.M. Verschuuren

Index

Chapter 1: Introduction

1. Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder that affects cortical and spinal motor neurons and is characterised by loss of motor function and muscle control leading to death[1]. First described by the founder of modern neurology Jean-Martin Charcot and his team of neuroscientists at La Salpêtrière hospital in Paris in 1869[2-6], ALS is the most common motor neuron disease with adult onset and the most frequent neurodegenerative disorder with insurgence at midlife, in the mid-to-late 50s[7]. The scarring of descending corticospinal tracts (sclerosis) is the result of Cortico-Spinal Motor Neuron (CSMNs/Betz cells/upper MN) degeneration and the gradual loss of the connection between the cortex and lower, spinal motor neurons (MNs). Loss of control of inputs in the motor circuit results in defects in regulation of electrical activity in MNs and disruption of synaptic contact with the muscle that results in muscular atrophy (amyotrophy)[8]. To date, it remains unclear why these neuronal subtypes are selectively affected by the disease.

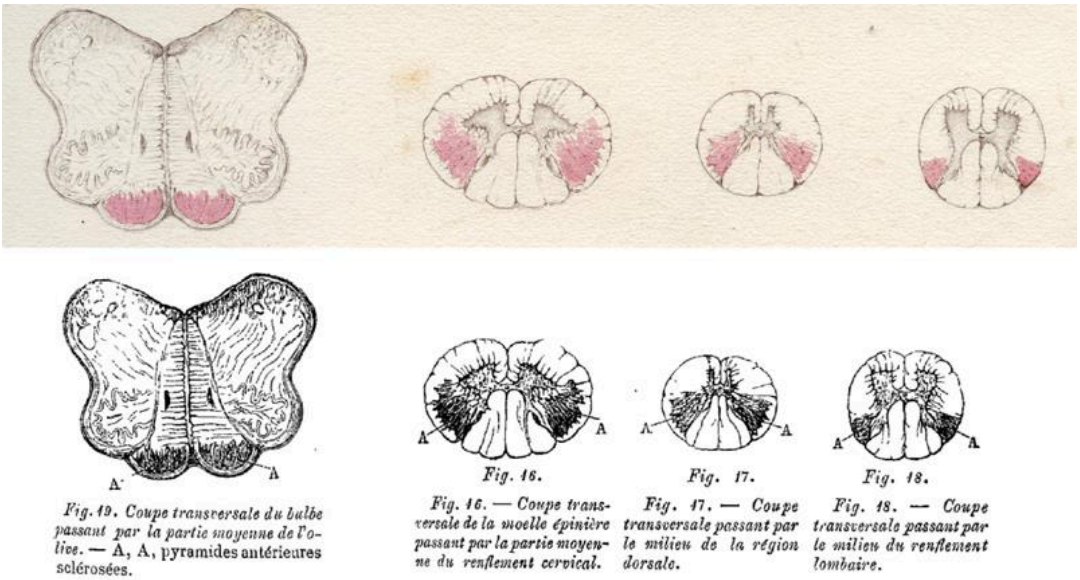


Figure 1 First description of ALS from Joffroy's thesis[5]*. Top: positioning of medullar pyramids of the corticospinal tracts. Bottom: cross sections of the medulla supposedly drawn by Charcot himself depicting sclerotic areas (AA) through medulla oblongata (bulbe), cervical, thoracic and lumbar spine (moelle cervicale, dorsale et lombaire). Adapted from Duyckaerts et al.[6], Charcot's originals often used in his famous leçons du mardi at La Salpêtrière culminated in "Anatomie pathologique de la moelle épinière", property of "Musée de l'Assistance Publique-Hôpitaux de Paris" at the Neuropathology Department of Hôpital Pitié-La Salpêtrière.*

ALS is an unforgivably fast, progressive disease with survival typically limited to 2-5 years from diagnosis. Complicating this scenario, diagnosis is extremely hard requiring an extensive clinical examination by a skilled neurologist in conjunction with electromyography and several tests to exclude other diseases of motor neurons that might resemble ALS[9]. Current treatment strategies mostly focus on palliative care, symptoms management and respiratory support. The only two approved medications, Riluzole and Edaravone, which act by modulating synaptic firing of the motor system and reduce oxidative species, only prolong life by a few months[10,11] and many of the promising drug candidates found experimentally failed to pass preclinical stages[12]. The current state of clinical knowledge on the disease implies that the only efficient way to counteract symptoms is early diagnosis and timely intervention to manage rather than prevent degeneration, prompting the field to identify new, more rapid and efficient ways to diagnose and treat ALS[13].

2. ALS genetic causes

Although genetic studies have immensely advanced our knowledge of ALS, only ~10% of cases are inherited and classified as familial (fALS), whereas 90-95% of diagnoses are sporadic in origin (sALS), occurring without family history and often no known genetic cause[8]. Several studies demonstrated that roughly half of fALS cases are connected to a handful of genes[14,15]: *SOD1*, *TARDBP* (*TDP-43*), *FUS* and *C9ORF72* being the most common ones and rare variants in other genes implicated as well[16], mostly autosomal, inherited as dominant traits and frequently with high penetrance.

The first gene to be discovered in families affected by the disease is SOD1. Cu/Zn superoxide dismutase 1 (*SOD1*), first identified in 1993, is now recognised to be connected to ~20% of familial ALS cases[17]. SOD1 is a ubiquitously expressed and involved in reducing oxidative stress species. Mutations in this gene create aberrant, mutant protein aggregates in MN and cause disease through toxicity rather than loss of function of the wild-type protein[18,19]. Since then, many other genes have been identified as cause of fALS[1,8,16], three of them are worth discussing in more details since they explain most of the heritability. The most common inherited cause of ALS in European populations is an hexanucleotide repeat expansion in intronic region of *C9ORF72*[20,21]. This gene normally harbours a short set of repeats but in affected individuals the expansion can encompass hundreds to thousands. C9ORF72 has been implicated in vesicle trafficking, autophagy, immune function and RNA metabolism and its connection to ALS entails both a loss of function of normal *C9ORF72* gene product and the production of aberrant RNAs and peptides from the expansion itself that create both RNA foci and protein aggregates. Mutations in *TARDBP/TDP-43*[22,23] and *FUS*[24,25] each account for

roughly 5% of familial cases. Interestingly, both proteins are ubiquitously expressed and have a pivotal role in RNA biology, shuttling between the nucleus and the cytoplasm controlling RNA stability, splicing and transport[14,26]. Other genes have been associated with rare forms of fALS including for example: *VCP*, *OPTN*, *TBK1*, *SQSTM1*/*p62*, *UBQLN2*, *DCTN1*, *PFN1*, *MATR3*, *CHCHD10*, *TUBA4A*[1,8,14-16,27]. These genes are involved in mechanisms listed above like vesicle trafficking, autophagy, immune functions, RNA metabolism but also protein homeostasis, axonal transport and cytoskeletal dynamics.

The seemingly loose connection between all these pathways and the often-ubiquitous expression of these genes renders the understanding of molecular mechanisms underlying ALS very challenging. Additionally, even though sporadic cases are defined as being present without familial history, 3-5% of them can still be explained by genetic mutations also found in fALS. Complicating this scenario, some of these variants have intermediate penetrance[28] with rare genetic variation being disproportionally frequent in sALS, with many loci that act as modifiers of the disease containing genes involved in even disparate molecular and cellular functions, such as *MOBP*, *NEK1*, *SARM1*, *UNC13A*, *SCFD1*, *KIF5A* and others[29-31]. The intermediate penetrance of certain mutations, the cumulative knowledge on disease modifiers and the partial heritability (established at 60% in twins studies[32]) result in many cases being more familial clusters rather than classical mendelian inheritance[33] and has brought about the notion that ALS could be an oligogenic disease[31,34].

3. ALS pathological manifestations

Besides the uncertainties in underlying mechanisms produced by this complex genetic landscape, the core pathological finding in ALS remains motor neuron death. This degeneration is always accompanied by loss of corticospinal tracts resulting in lateral scarring of the spinal cord and spastic control of muscles. As the disease progresses, a common feature identified in most cases, regardless of their familial or sporadic nature, is the accumulation of cytoplasmic protein aggregates, mostly in neurons[35]. These common, skein-like accumulations can be composed of different proteins and are highly ubiquitinated[35]. It was only in 2006 that a major change in the understanding of ALS pathobiology occurred when Virginia Lee and colleagues identified that the major component of these aggregates was TAR DNA/RNA-binding protein 43 (TDP-43)[36].

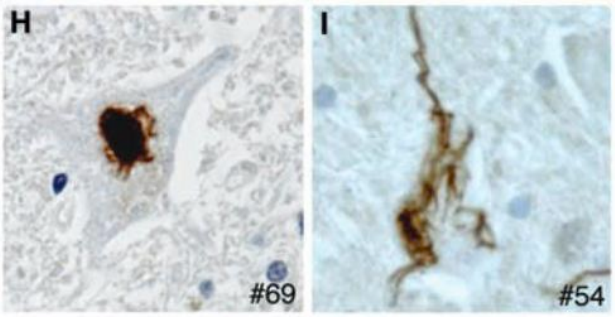


Figure 2. Identification of TDP-43 inclusions in sporadic ALS. *Immunostaining with anti-TDP-43 labelling round nuclear aggregates (H) and nuclear loss with skein-like aggregates (I) in spinal cord motor neurons (original images from Neumann et al, 2006, Science[35]).*

Other pathological features are specific to certain genes, e.g. intranuclear RNA foci[20] in C9orf72-patients with ubiquitinated aggregates rich in SQSTM1/p62[37], in addition to TDP-43 aggregates. Also, mutant proteins of SOD1 and FUS result in aggregation of these proteins even though somewhat pathologically distinct since they do not present TDP-43 accumulation.

Accumulation of TDP-43 was identified even before ALS-causative mutations in the TARDBP gene[22] and it has now been confirmed and replicated by many studies that have found neuronal TDP-43 protein aggregates in most patients[1,8]. These aggregates are often associated with nuclear loss of the protein and studies have proven that this RNA-binding protein is found to be insoluble in over 97% of cases, providing at least one convergent mechanism for molecular disruption in ALS[26].

4. The Motor Neuron Disease spectrum: ALS, ALS/FTD, FTD

Studies that identified TDP-43 accumulation in ALS have also identified these aggregates in brains of patients affected by FrontoTemporal Dementia (FTD)[36]. FTD, also known as frontotemporal lobar degeneration (FTLD), is the second most common form of dementia after Alzheimer's disease (AD) and is characterised by loss of cortical neurons in fronto-temporal cortical regions resulting in decreased cognitive function[38]. Only around 50% of FTD cases present TDP-43 pathology, whereas the rest is characterised by aggregates of either FUS or tau[39]. Similarities in pathological features include loss of cortical neurons[1] which might start in different regions, more frontal in FTD and specifically motor in ALS, but that then spreads to other motor-related areas of the cortex and the brainsteam[40].

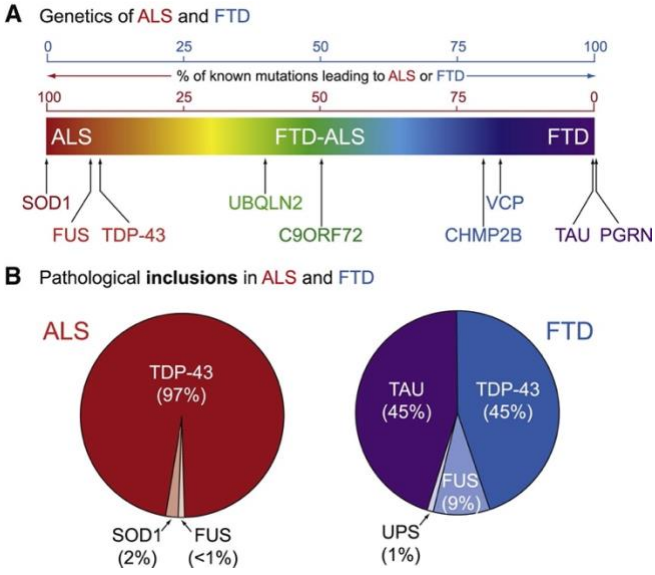


Figure 3. Overlap of pathology and genetics in the ALS and FTD (from Ling et al.[14]). *(A) Major causative genes of ALS (red) and FTD (blue) positioned along the spectrum of manifestations of the two types of degeneration. (B) Break-down of cases presenting inclusions of different proteins in ALS and FTD.*

It is estimated that 15% of FTD patients present symptoms of motor neuron disease and that around 20% of ALS patients develop symptoms typical of dementia, not counting for the fact that as many as 50% of ALS patients might exhibit some degree of cognitive impairment[40]. The overlap in symptoms and pathology is partially explained by overlapping genetics. One of the most common mutations identified in FTD is the same intronic repeat expansion in *C9ORF72* identified in ALS[20,21]. Moreover, a few rare mutations in other genes have been known to cause

symptoms of both ALS and FTD: *TARDBP/TDP-43*, *FUS*, *UBQLN2*, *VCP*, *CHMP2B*, *SQSTM1*, *OPTN*[14,40]. However, some of the genes involved seem to only cause symptoms for one of the two diseases, for example *SOD1* is only found in ALS cases and *MAPT* in FTD[14,40]. The overlap in symptoms and diagnoses and the shared variants in several genes associated with the two diseases support the notion that ALS and FTD represent different manifestations of shared molecular causes and that most patients sit on a motor-neuron-disease-dementia continuum forming a spectrum of ALS-ALS/FTD-FTD[41].

5. Molecular mechanisms and cell types underlying ALS pathogenesis

The complex genetics and the involvement of several molecular pathways is partially the result of incomplete knowledge on the molecular causes of ALS, where degeneration is highly heterogeneous and caused by different genetic and environmental factors[42] triggering pathophysiology in the complex, multicellular milieu of the aging human brain and spinal cord. Nonetheless, many groups have demonstrated how several molecular pathways are disrupted and what cell types might be more susceptible or implicated in these disruptions. Corroborating these findings in human tissue, recent studies have shown that even though neuronal death remains the primary pathological feature, pathogenesis is contributed by other non-neuronal cells, especially astrocytes, microglia and myelinating glia[43]. In this section, we will summarise the knowledge accumulated around different molecular pathways involved in the disease in specific cell types, with a particular focus on studies corroborating findings in primary patient samples, connections to the genetics of familial disease and mentioning pivotal studies in murine models that demonstrated how different cell types other than neurons might play a role in disease initiation, progression and prevention.

The current view on disease mechanisms implicates three main categories of cellular pathways: protein homeostasis (proteostasis), RNA metabolism and cytoskeletal dynamics. However, the ubiquitous expression of ALS/FTD implicated genes, their role in different pathways and the tight interplay of some of these pathways make the mechanisms disrupted by the disease not exclusive and with broader implications. The complex interplay of mechanisms and genetics may result in a plethora of molecular and cellular abnormalities: protein and RNA granules may disrupt both proteostasis through formation of aggregates and RNA metabolism by sequestering RNA-binding proteins, this in turn may result in and/or be aggravated by stress of the endoplasmic reticulum (connected to both regulation of translation and protein folding and production), mitochondrial dysfunctions (highly reliant on quick protein production and local control of RNA metabolism), and altered nuclear-to-cytoplasmic trafficking of both RNA and proteins[8]. Moreover, disruptions of similar mechanisms in different cell types might result in very different phenotypes. For example, disruption in vesicle

trafficking associated with several ALS-FTD causative genes, such as C9orf72 and TBK1, might result in altered neuronal excitability and synaptic function in neurons, whereas in glial cells it has been shown to lead to microglial activation and impaired immune functions[1]. Furthermore, sequential order of dysfunction but also the interplay of these dysfunction in cellular processes might be both beneficial or detrimental to motor neuron viability. This multi-layered model implies an arabesque of genetics and cellular biology resulting in multicellular disruptions in ALS/FTD, where the neurocentric degeneration is accompanied by many other alterations and re-establishing a neuroprotective environment might be as essential as supporting neuronal survival to re-cement the integrity of the neuronal motor circuitry.

5.1 Corticospinal and spinal motor neurons

Motor neuron susceptibility was recognised as a key neuropathological characteristic connected to ALS symptoms since its description by Charcot, with loss of cells in the ventral horn of the spine and sclerosis of descending tracks in lateral columns[4,5,44]. At the beginning of the 1900s, loss of giant, corticospinal Betz cells in cortices of patients was also recognised[45] and connected to loss of muscle tone[5], involving the whole motor circuit in disease pathology. Accumulation of ubiquitinated proteins was then found in spinal motor neurons[35,46], ubiquitin-positive aggregates that were mostly composed of TDP-43 inclusions[36,47] in both ALS and FTD. The primarily neuronal pathology drove researchers to focus on pathways that might be disrupted in neurons.

Because of the central role of TDP-43 in RNA biology and metabolism[26,48], many groups have focused on RNA dysfunctions in neurons. Not only *TDP-43*[22] but other ALS/FTD-related genes are RNA-binding proteins and play pivotal roles in RNA metabolism, such as *FUS*[24], *HNRNPA1*[49], *MATR3*[50], *ATXN2*[51], *TAF15*[52] but also the RNA-related molecular processes connected to *C9orf72*[20,53]. Many of these proteins are found to form cytoplasmic stress granules, transient low-complexity aggregates composed of RNA and proteins that arise after phase separation and live as membraneless organelles in cells[53,54]. These granules can sequester mRNAs[16] preventing normal translation and act as a self-replicating cytoplasmic sinks[1]. When pathological, granule formation can also lead to excessive aggregation of RNA-binding proteins and their depletion from the nucleus accompanied by impaired nucleocytoplasmic transport[55,56] and loss-of-function of these proteins[57]. For TDP-43 specifically, this may result in alterations in its splicing activity[58], dysregulation of alternative splicing events with emergence of aberrant splicing (e.g. cryptic exons)[59-62], as well as impaired transport of neuronal mRNAs along axons[63] and failed autoregulation[58], some of these disrupted mechanisms overlap with FUS-dependent aggregation[57]. Other disrupted mechanisms are connected to the hexanucleotide repeat expansion in *C9orf72* that can be

transcribed in both sense and antisense fashion to produce short RNA transcripts that form RNA foci in neurons[20,64] resulting in the sequestering of RNA-binding proteins[65].

A second obvious sequitur stemming from the identification of highly ubiquitinated aggregates is the role of proteostasis. Initial studies have shown that SOD1 mutations result in stress of the endoplasmic reticulum (ER) and accumulation of misfolded proteins in neurons[66]. The ubiquitin-proteasome system, the unfolded protein response (UPR), autophagy and other pathways involved in protein folding and degradation are a large component of degenerative mechanisms in ALS, especially if we take into account the number of genes connected to fALS/fFTD that are involved in these pathways: *UBQLN2*[67], *SQSTM1*/p62[68], *OPTN*[69], *VCP*[70], *CHMP2B*[71], *VAPB*[72], *TBK1*[28], *FIG4*[73], *GRN*[74], *C9orf72*[20,53]. Moreover, Dipeptide Repeats (DRPs) derived from *C9orf72* hexanucleotide RNA foci can form neuronal aggregates that sequester proteasome subunits compromising neuronal proteostasis[75]. Other organelles associated with dysfunctions of the disease are mitochondria, mostly through the antioxidant role of SOD1 protein[19], the oxidative stress found in spinal cord of patients[76] and mutations in *CHCHD10*, a mitochondrial gene associated with rare familial cases of ALS[77].

As the largest, asymmetric cells in the human body, with axons that can reach more than one metre in length, motor neurons are extremely reliant on axonal transport. Four fALS genes have been connected to defects in cytoskeletal dynamics and axonal transport (*TUBA4A*[78], *DCTN1*[79], *PFN1*[80] and *KIF5A*[81]) and two have been connected sporadic-associated variants (*NEFH*[82] and *PRPH*[83]). Axonal defects also include poor RNA transport by TDP-43 mentioned above[63] but also the involvement of several ALS genes in vesicular transport (*OPTN*[69], *VAPB*[84], *CHMP2B*[71], *VCP*[70]) and specifically synaptic vesicles, *UNC13A*[85]. Interestingly, the most consistent diagnostic tool for ALS in both sporadic and familial cases is electrophysiological studies that identified hyperexcitability in the motor circuit of patients[86,87]. Moreover, one of the few and most promising biomarkers identified for diagnosis is neurofilament[88] and one of the two drugs approved for treatment of ALS, Riluzole, acts by modulating neuronal firing[10], pointing at axonal biology and synaptic activity as an extremely important aspect of the disease.

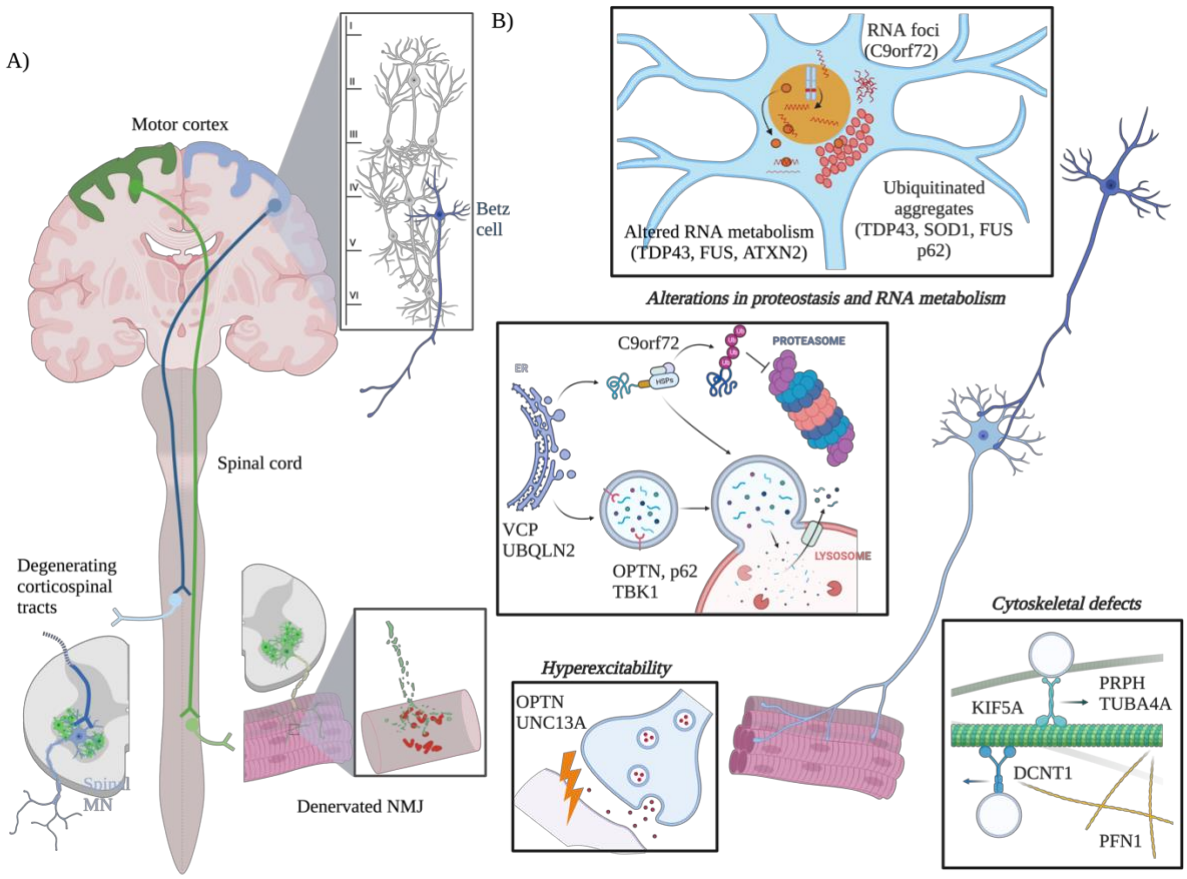


Figure 4 Mechanisms disrupted in neurons by ALS. A) Areas of the nervous system affected by ALS. *Upper motor neurons (Betz cells) in the motor cortex start to be affected with consequent degeneration of corticospinal tracts resulting in lower motor neurons being affected and losing muscle control through Neuromuscular Junction (NMJ).* ***B) Cellular mechanisms implicated in the disease.*** *Summarised schematics of some of the molecular mechanisms discussed in the text. Some of the genes connected to familial forms of the disease are highlighted in their role in biological functions. Partially reconstructed with the help of Irune Guerra San Juan and adapted from Taylor et al. 2016[1].*

5.2 Astrocytes

Astrocytes are glial cells responsible for modifying the chemical microenvironment of neurons participating in synaptic function and forming and modulating the blood-brain barrier. Reactive astrocytes have been described by many studies in both cortices and spinal cords of ALS patients[89,90], leading to speculation that they might be contributors to ALS/FTD. Interestingly, one of the first studies dissecting the molecular mechanisms underlying astrocytes in ALS identified that in patients these cells reduce the expression of astrocyte-specific glutamate transporter GLT-1 (EEAT2/*SCL1A2*)[91]. This mechanism is connected to the hyperexcitability phenotypes seen in patients' motor neurons and thought to be at the base of failure to remove excessive glutamate at synapse, resulting in neurons overfiring and glutamate excito-toxicity[92].

In vivo and *in vitro* studies have confirmed the view that astrocytes play a role in disease initiation and progression. Astrogliosis and reactive astrocytes have bene identified in several mouse models of SOD1, TDP43 and C9orf72[43], and selective deletion of mutant

SOD1^{G85R} from astrocytic lineage delayed disease onset and slowed down progression in animal models[93]. Moreover, knock-out of reactive astrocytes factors in SOD1 mouse models can also result in delayed disease progression, underscoring the important role of astrocytes in neuronal support[94]. *In vitro* studies that co-cultured astrocytes with motor neurons have shown that astrocytes isolated from both familial and sporadic patients[95,96] and from mouse models[97,98] decrease human motor neuron survival through soluble factors and change the electrophysiological properties of the neuronal networks, underscoring the notion that astrocytes might play an active role in inducing and/or promoting neuronal loss in ALS.

5.3 Oligodendrocytes

Oligodendrocytes and Oligodendrocytes Progenitor Cells (OPCs, a.k.a. NG2 glia) are responsible for myelinating axons of the Central Nervous System (CNS) and help maintain strong electrical connectivity in brain and spinal cord circuitries[99]. Oligodendrocytes pathology has been identified in several studies in ALS patients[100] and the relevance of this cell type in the disease is underscored by findings of TDP-43 inclusions in these cells as well[101].

Neurosupportive function of oligodendrocytes in ALS appears to be mediated by MCT1, a lactate transporter hypothesised to be to metabolically support neurons, shown to be downregulated in both mouse models and human primary tissue[102]. Moreover, loss of myelinating cells was observed in SOD1^{G93A} mouse models and, similarly to astrocytes, Cre-mediated removal of mutant SOD1^{G37R} in NG2 glial precursors delayed onset of symptoms and increased survival[103]. The involvement of oligodendrocytes in the disease is corroborated by loss of myelin and myelinating components in both spinal cords and motor cortices of sporadic ALS patients[103], but also by the identification of *MOBP*, oligodendrocytes-specific and basic component of the myelination machinery, as a disease-associated locus and modifier of disease for both ALS[29] and FTD[104] risks.

5.4 Microglia

Microglia are the resident immune cells of the CNS and have many functions including developmental roles, immune surveillance, debris clearance and defence from pathogens. It is now recognised that spinal cord from ALS patients present high microglial density with abnormal morphology[105,106], associated with reactive microglial cells, and microglial activation being identified in motor cortices as well[107].

Mouse models have provided numerous insights into microglial involvement in the disease. Microglial activation is believed to be occur even prior to symptoms occurrence in SOD1 mouse models[108]. Moreover, one of the earliest studies focusing on cell type specific contributions to disease proved that Cre-mediated depletion of SOD1^{G37R} in myeloid cells could mediate disease progression, confirming an active role of microglia in ALS

pathogenesis[109]. Many studies have since focused on the role of these cells in SOD1 models showing that an initial neuroprotective effect transitioned into loss of neurotrophic support and gain of a toxic state[43]. These reports have recently been confirmed by single-cell RNA-sequencing studies (scRNA-seq) that identified reactive microglia in SOD1^{G93A} mouse model[110] and described it as Disease-Associated Microglia (DAM)[110,111]. As with astrocytes, *in vitro* co-cultured studies with human motor neurons have shown that mutant SOD1 microglia are reactive and sufficient to decrease neuronal survival[112].

Intriguingly, many ALS/FTD related genes have been implicated in regulation of immune function and specifically in microglial biology. FTD-related genes *GRN* and *TREM2* play a major role in immune cells and are connected to control of activation states in microglia[111]. Specifically, TREM2 has been shown to be one of the main regulators of reactive states in microglia[110] and GRN deficient mice develop reactive microglia that associate with TDP43 aggregates[113]. Not only, recent studies have shown that *TREM2*, *GRN*, *TBK1* and *C9orf72* are highly expressed in microglia[16]. Mouse models of C9orf72 have proven that loss of function in this gene results in several immune phenotypes[114], many of which are connected to neuroinflammation[115], as shown also in patients[116], and these changes are a result of aberrant lysosomal trafficking[117]. Given the role of *GRN* in lysosomal biology[113] and of *TBK1* in autophagy and vesicle trafficking specifically in microglia[118], it is interesting to speculate how these pathways might be differently regulated by ALS/FTD-related genes in immune cells and neurons. Moreover, the recent knowledge that interferon signalling, regulated by TBK1[119], is dysregulated in familial and sporadic ALS patients through C9ORF72 dysfunction[120] centres microglia as one of the main players in all kinds of ALS/FTD and not only C9-ALS/FTD.

5.5 Other cells and factors

Many studies have shown that other cell types can be involved and impacted by disease pathogenesis. Several cells of the peripheral immune system have been identified in post-mortem samples in brains of ALS patients[100,105], where they normally would not reside. These infiltrations of NK cells, peripheral myeloid cells, CD4$^+$and CD8$^+$ T cells have also been seen in mouse models[115] and suggest that peripheral immunity might play a role in ALS disease progression[115]. Furthermore, evidence has shown that even cells residing in the periphery, like macrophages along motor neurons axons and at the neuromuscular junction (NMJ), can be affected by ALS and modulate disease progression[121]. Moreover, other groups have also suggested that blood-brain-barrier and endothelial cells are dysfunctional in mouse ALS models[115,122]. This might explain, not only the infiltration of peripheral cells mentioned above, but also the increasing relevance recently demonstrated for environmental factors and microbiota as disease modifiers[115].

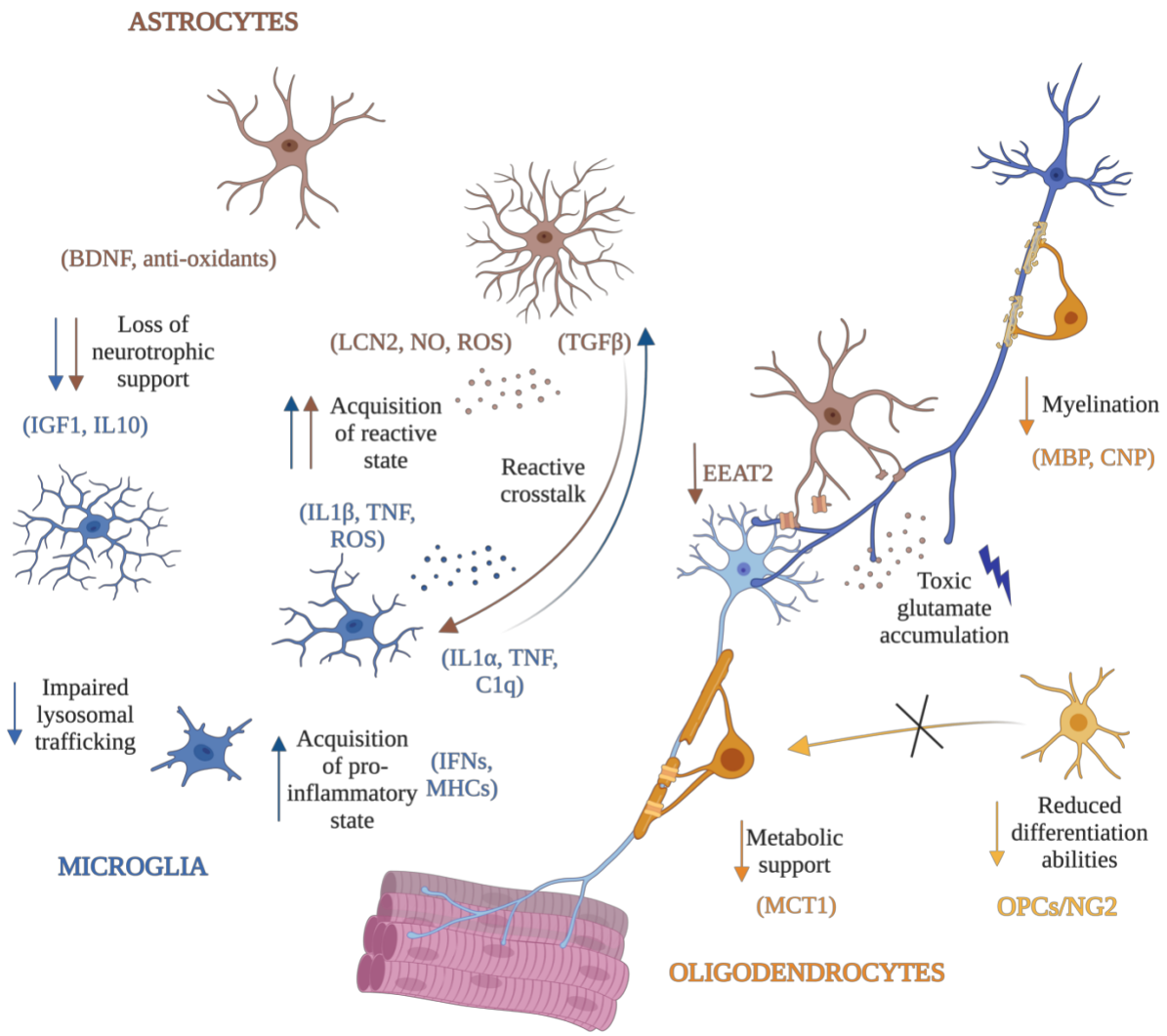


Figure 5 Pathophysiological interactions of glial cells and neurons in Amyotrophic Lateral Sclerosis. *Neuronal dysfunctions described have been shown to be associated with activation states of glial cells. Phenotypes associated with ALS in glial cells entail loss of supportive roles for neurons: loss of neurotrophic support in microglia and astrocytes (top left), loss of proper differentiation abilities of OPCs/NG2 cells (right), loss of myelination properties and metabolic support in oligodendrocytes (right). But also, the acquisition of toxic functions: acquisition of activation states in astrocytes and microglia that release neurotoxic factors (middle), reactive crosstalk between astrocytes and microglia (middle), impaired lysosomal trafficking in microglia resulting in pro-inflammatory response (bottom left). Partially adapted from Taylor et al. 2016[1] and Vahsen et al. 2021[42].*

6. Understanding ALS: transcriptomic analyses of post-mortem tissue

Although we have accumulated immense knowledge around mechanisms of neurodegeneration in ALS, it remains unclear why and how certain subtypes of motor neurons might be more susceptible to the disease. Moreover, the heterogeneity in aetiology still casts many doubts on how sporadic cases might occur and also the complexity of genetics, partly highly penetrable partly driven by several disease modifiers, adds questions on what pathways and molecular mechanisms are more relevant or first to be disrupted in the disease. In addition, many of the pathways identified are disrupted in very unique, specific cell type whose

nuances in diversity and functions are even now not fully understood, especially if we take into account that many of these pathways are ubiquitously present in brain cells and might be either disruptive or beneficial in different cell types. Fundamentally, the field is lacking a way to connect the dots in between primary disease seen in patients, known genetic causes and insights from laboratory research that would allow the potential selection of a few molecular culprits that could be targeted for disease-modifying therapies, biomarkers discovery and, eventually, a cure.

In order to supersede the lack of knowledge on what is happening in the CNS of patients, a few groups have undertaken sequencing studies on primary samples to try to start drawing a line in between the dots that are scattered in the field. Most of the initial studies relied on bulk-RNA sequencing of either cortices or spinal cords from ALS-patients and controls. A few studies analysed cortices from ALS patients and age-matched controls[123-125]. They all identified proteostatic stress and UPR as upregulated in cortices of patients, in connection to oxidative stress and mitochondrial dysfunction and the dysregulation of RNA control[125] and alternative splicing[123] connected to TDP-43, thus unbiasedly confirming some of the pathways discerned from genetics and mouse models. Interestingly, Prudencio et al. found changes in synaptic biology and inflammation to be particularly prominent in ALS patients[123] and Tam et al. corroborated upregulation of inflammation and reactive glial biology in a subset of patients[125], underscoring both the heterogeneity of ALS and the involvement of different cell types in neurodegenerative manifestation. A few more groups undertook similar approaches on samples from spinal cords of ALS patients[126-128], confirming the role of splicing defects and inflammation in disease pathogenesis. In particular, D'Erchia and colleagues confirmed alterations in synaptic molecules in spinal cords from ALS patients and highlighted the point that different cell types might be contributing to the changes in transcriptomic signatures identified mostly motor neurons, oligodendrocytes and microglia[128], once again confirming the multicellular component of ALS.

These studies contributed immensely to the field by confirming many of the known disrupted pathways in an unbiased way using primary samples. However, they also accentuated the need to look at cell type specific changes that were not possible to discern using bulk RNA-sequencing technology. As of today, only one study has reported single nucleus RNA-sequencing analysis of cortices from FTD patients and strongly underlined that different, very rare cell types might be contributors of the disease[129]. On top of that, the strong signature identified in TDP-43-dependent RNA regulation underscored that even though mouse studies have elucidated many insights into neurodegeneration, human specific models need to be implemented in order to fully understand specific alterations created by TDP-43 dysfunction in the context of human pathology. Not to mention that control of the corticospinal motor circuit, the main player in ALS, is evolutionarily quite divergent in human and mice,

where in the murine CNS corticospinal motor neurons descend tracts through the spinal cord and contact spinal interneurons that then relay inputs to spinal motor neurons, whereas primates show direct monosynaptic connections between the motor neurons of the cortex and of the spinal cord[130]. These differences are also connected to notable divergence in neuromuscular junction morphology in the two species, rendering the whole motor system architecture quite different between the two species[131]. Even though mouse models of fALS have generated important contributions to the understanding of the disease, we need to remember that 90% of cases are sporadic in origin and that findings in these models might not fully translate into human biology[132].

7. Modelling ALS: human Pluripotent Stem Cells as *in vitro* models

One solution to bridge the gap between *Mus* and *Homo* is coupling scalable human *in vitro* models with the prominent advent of disease modelling through pluripotent stem cell (PSC) technologies[133]. At almost 25 years since their isolation[134], human pluripotent stem cells have proven to be one of the most versatile tools in the hands of molecular and cellular biologists allowing the construction of physiologically relevant models of human cell types that would otherwise be inaccessible, such as tissue of the nervous system[135]. Specifically, the discovery of induced PSC (iPSCs) proved pivotal for the development of models that could be obtained directly from patients through reprogramming of somatic cells, maintaining their genetic make-up and offer insights into disease specific mechanisms[133].

hiPSC are directed towards a neuronal fate first by removing conditions that support maintenance of pluripotency and "stemness"[135]. Secondly, neuralization process is usually aided by inhibition of TGFb and BMPs pathways, so-called "dual-Smad inhibition"[136], which is often coupled with developmental cues that support diversification into specific subtypes of progenitor cells and then of diverse neuronal and glial subtypes[135-137]. Other methods couple hiPSC-technology with genetic manipulations that allow the overexpression of transcription factors that can generate specific cell types[137,138]. Generating the plethora of diverse cell types of the brain is extremely important in understanding the disruptions that are triggered in different cell types in ALS (described in previous chapters).

Several studies have implemented these methods to model ALS *in vitro* using human cells and discovered quintessential disease-related phenotypes especially for familial ALS cases[12]. Because hiPSCs more easily and spontaneously differentiate into neurons[135], most studies have focused on ALS-related disruptions in cells harbouring specific mutations and comparing them to non-diseased counterparts. Notably, many groups have confirmed generation of RNA foci and dipeptide repeats in neurons generated from *C9orf72* hiPSCs[139-141] and reported their reduced firing capacity, impaired vesicle trafficking and synaptic

function[56,139,141,142]. Others have shown reduced survival and axonal branching in SOD1-neurons[143] and similar dysregulations in axonal and neurofilament dynamics in TDP-43 mutated cells[59] and coupled hiPSC technologies with CRISPR/cas9 genetic manipulations to further dissect TDP-43-related RNA dynamics in neurons[59,60].

Even though these studies have shed lights on molecular phenotypes disrupted by specific mutations they fail to explain how these mechanisms might convergent in similar mechanisms and how these might be affected in sporadic cases. That is why subsequent reports have focused on running parallel comparisons between cells derived from hiPSC harbouring different mutations uncovering shared hyperexcitability phenotypes between *SOD1*, *TDP-43* and *C9orf72* mutants[144] and shared mitochondrial and oxidative stress dynamics[143,145]. And even compared neurons derived from one of the biggest collections of ALS-hiPSCs biobank from both familial and sporadic cases and identified multiple cellular phenotypes demonstrating great variability across genotypes and *in vitro* phenotypes[146]. More recently, our group and others have started to couple hiPSCs with novel genetic manipulation technologies like CRIPSR-cas9 to understand basic function of ALS-related genes and further underlined the involvement of cytoskeletal[59,60] and synaptic biology[61,62].

However, as per mouse models, modelling of sporadic ALS is only at its beginnings and still is not standardised to levels that might allow reproducible findings to translate into the clinic. Moreover, most of the studies in the field have focused on the modelling of neuronal cells[12,147] but, as described in previous chapters of this work, the interplay of several brain cell types has a major role in ALS pathogenesis. Only a few studies have started dissecting the multicellular interplay in ALS and mostly focused on astrocytes. Only two peer-reviewed studies have as of today reported that astrocytes with *TDP-43*[148] and *C9orf72*[149] manipulations show alterations in various aspects of neuronal support altering electrophysiological properties of MNs in co-cultures.

A lot more needs to be done to build human *in vitro* systems where complex ALS alterations can be studied. First of all, dissecting changes in cell types other than neurons. But also, how these different cell types interactions might change in a disease context. Not to mention the fact that ALS manifests in mid-to-late life and that hiPSC-modelling is based on phenotypes grown *in vitro* for weeks and other manipulations might be necessary to trigger phenotypes seen in patients[147]. Therefore, the complexity of multi-cellular interactions must be achieved *in vitro*, in a reproducible manner and in scalable systems that might allow differentiation of hiPSCs from big cohort of patients and controls in order to understand the complexity of human pathogenesis in sporadic ALS.

Scope of this thesis

Tackling ALS: a multidisciplinary approach

Despite the gargantuan strives into developing more over-growing knowledge on the disease, we still cannot match the ongoing efforts that are put into developing a cure. Fundamental obstacles remain in the field of ALS in order to mechanistically understand disease causation and progression in the aim to finally nominate pathways for successful drug targeting and discovery.

With this piece of work, we would like to provide some answers to some of these main barriers. In chapter 3, we widen our understanding of which molecular pathways are disrupted in disease-relevant primary tissues at a single cell level through the analysis of single-nuclei RNA sequencing dataset of ALS patients and age-matched, unaffected individuals. Our study shed a light on the intrinsically higher expression of ALS/FTD genetic causes in upper MNs that is accompanied by selective vulnerability of several subsets of cortical motor neurons. In this work, we use hiPSC-derived *in vitro* system to model some of the molecular changes identifie din primary samples. These changes are found in concomitance with alterations in myelinating cells and microglia that widen our knowledge of cell-to-cell interactions in ALS.

In chapter 4, we would like to then offer a wider view on how to expand and build better models of human brain cells in a dish with the hope it will encompass protocols useful for modelling complex cell-to-cell interactions in ALS.

In chapter 5, we go on to provide a new, human *in vitro* systems for the study of motor neuron biology that is highly reproducible and scalable for high-throughput studies using human induced Pluripotent Stem Cells. This new method, that allows the assessment of multiple cell lines in the same dish, could provide insights into heterogeneity seen in patients in a human specific context and amplify our knowledge mechanisms disrupted in sporadic disease. This study is followed by chapter 6, where we used some of the models built in chapter 5 and to further dissect molecular mechanisms disrupted in disease.

Finally, in our conclusion, we will undertake a discussion onto the future of the field and hopefully the opening to a more holistic approach to the understanding of ALS, where multi-disciplinary techniques and the use of different models might expand our perspectives on the disease.

References

Parts of this chapter have been adapted from the original works:

Limone F*, Mordes D*, Couto A, Burberry A, Pietiläinen O, Joseph B, Ghosh S, Meyer D, Goldman M, Bortolin L, Kadiu I, McCarroll S, Eggan K. "Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neuron to degeneration in ALS". Under revision*, Nature Aging, 2022*.
bioRxiv, https://doi.org/10.1101/2021.07.12.452054

Limone F ***corresp.***, Mitchell JM, Guerra San Juan I, Ghosh S, Smith JLM, Raghunathan K, Couto A, Meyer D, Mello C, Nemesh J, Smith BM, McCarroll S, Pietiläinen O, Nehme R, Eggan K[corresp.]. "Efficient generation of lower induced Motor Neurons by coupling Ngn2 expression with developmental cues". *Cell Reports*, *2022*, https://doi.org/10.1016/j.celrep.2022.111896

Limone F, Klim JR, Mordes D. "Pluripotent Stem Cell Strategies to Rebuild the Human Brain". *Frontiers in Aging Neuroscience*, *2022*. 10.3389/fnagi.2022.1017299

Guerra San Juan I, Nash L, Smith K, Qian M, Burberry A, Dorr A, Couto A, Klim J, Pintacuda G, ***Limone F***, Whisenant E, Noble C, Melnik V, Potter D, Holmes A, Verhage M, Eggan K. "Loss of STMN2 Function Causes Motor Neuropathy". *Neuron*, *2022,* 10.1016/j.neuron.2022.02.011.

Klim J[*], Williams L[*], ***Limone F***, Guerra San Juan I, Davis-Dusenbery B, Mordes D, Burberry A, Steinbaugh MJ, Gamage K, Kirchner R, Moccia R, Cassel S, Chen K, Wainger B, Woolf C, Eggan K. "*ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair*". *Nature Neuroscience*, *2019* February;22(2):167-179. 10.1038/s41593-018-0300-4

1 Taylor, J. P., Brown, R. H., Jr. & Cleveland, D. W. Decoding ALS: from genes to mechanism. *Nature* **539**, 197-206, sdoi:10.1038/nature20413 (2016).
2 Clarac, F., Massion, J. & Smith, A. M. Duchenne, Charcot and Babinski, three neurologists of La Salpetriere Hospital, and their contribution to concepts of the central organization of motor synergy. *J Physiol Paris* **103**, 361-376, doi:10.1016/j.jphysparis.2009.09.001 (2009).
3 Rowland, L. P. How amyotrophic lateral sclerosis got its name: the clinical-pathologic genius of Jean-Martin Charcot. *Arch Neurol* **58**, 512-515, doi:10.1001/archneur.58.3.512 (2001).
4 Charcot, J. M. De la sclérose latérale amyotrophique. *Prog. Med.*, 341-453 (1874).
5 M., C. J. & A., J. Deux cas d'atrophie musculaire progressive: avec lésions de la substance grise et des faisceaux antérolatéraux de la moelle épinière. *Masson* **1869** (1869).
6 Duyckaerts, C., Maisonoble, T., Hauw, J. J. & Seilhean, D. Charcot identifies and illustrates amyotrophic lateral scelrosis. *Free Neuropathology*, doi:doi.org/10.17879/freeneuropathology-2021-3323 (2021).
7 Wainger, B. J. & Lagier-Tourenne, C. Taking on the Elephant in the Tissue Culture Room: iPSC Modeling for Sporadic ALS. *Cell Stem Cell* **23**, 466-467, doi:10.1016/j.stem.2018.09.015 (2018).
8 Brown, R. H. & Al-Chalabi, A. Amyotrophic Lateral Sclerosis. *N Engl J Med* **377**, 162-172, doi:10.1056/NEJMra1603471 (2017).
9 Rowland, L. P. & Shneider, N. A. Amyotrophic lateral sclerosis. *N Engl J Med* **344**, 1688-1700, doi:10.1056/NEJM200105313442207 (2001).

10 Bensimon, G., Lacomblez, L. & Meininger, V. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med* **330**, 585-591, doi:10.1056/NEJM199403033300901 (1994).

11 Kiernan, M. C. *et al.* Improving clinical trial outcomes in amyotrophic lateral sclerosis. *Nat Rev Neurol* **17**, 104-118, doi:10.1038/s41582-020-00434-z (2021).

12 Giacomelli, E. *et al.* Human stem cell models of neurodegeneration: From basic science of amyotrophic lateral sclerosis to clinical translation. *Cell Stem Cell* **29**, 11-35, doi:10.1016/j.stem.2021.12.008 (2022).

13 Wainger, B. J. *et al.* Effect of Ezogabine on Cortical and Spinal Motor Neuron Excitability in Amyotrophic Lateral Sclerosis: A Randomized Clinical Trial. *JAMA Neurol* **78**, 186-196, doi:10.1001/jamaneurol.2020.4300 (2021).

14 Ling, S. C., Polymenidou, M. & Cleveland, D. W. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron* **79**, 416-438, doi:10.1016/j.neuron.2013.07.033 (2013).

15 Renton, A. E., Chio, A. & Traynor, B. J. State of play in amyotrophic lateral sclerosis genetics. *Nat Neurosci* **17**, 17-23, doi:10.1038/nn.3584 (2014).

16 Mejzini, R. *et al.* ALS Genetics, Mechanisms, and Therapeutics: Where Are We Now? *Front Neurosci* **13**, 1310, doi:10.3389/fnins.2019.01310 (2019).

17 Rosen, D. R. *et al.* Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**, 59-62, doi:10.1038/362059a0 (1993).

18 Gurney, M. E. *et al.* Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **264**, 1772-1775, doi:10.1126/science.8209258 (1994).

19 Wong, P. C. *et al.* An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* **14**, 1105-1116, doi:10.1016/0896-6273(95)90259-7 (1995).

20 DeJesus-Hernandez, M. *et al.* Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* **72**, 245-256, doi:10.1016/j.neuron.2011.09.011 (2011).

21 Renton, A. E. *et al.* A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron* **72**, 257-268, doi:10.1016/j.neuron.2011.09.010 (2011).

22 Sreedharan, J. *et al.* TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* **319**, 1668-1672, doi:10.1126/science.1154584 (2008).

23 Kabashi, E. *et al.* TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat Genet* **40**, 572-574, doi:10.1038/ng.132 (2008).

24 Vance, C. *et al.* Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* **323**, 1208-1211, doi:10.1126/science.1165942 (2009).

25 Kwiatkowski, T. J., Jr. *et al.* Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* **323**, 1205-1208, doi:10.1126/science.1166066 (2009).

26 Klim, J. R., Pintacuda, G., Nash, L. A., Guerra San Juan, I. & Eggan, K. Connecting TDP-43 Pathology with Neuropathy. *Trends Neurosci* **44**, 424-440, doi:10.1016/j.tins.2021.02.008 (2021).

27 Therrien, M., Dion, P. A. & Rouleau, G. A. ALS: Recent Developments from Genetics Studies. *Curr Neurol Neurosci Rep* **16**, 59, doi:10.1007/s11910-016-0658-1 (2016).

28 Cirulli, E. T. *et al.* Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways. *Science* **347**, 1436-1441, doi:10.1126/science.aaa3650 (2015).

29 van Rheenen, W. *et al.* Genome-wide association analyses identify new risk variants and the genetic architecture of amyotrophic lateral sclerosis. *Nat Genet* **48**, 1043-1048, doi:10.1038/ng.3622 (2016).

30 van Rheenen, W. *et al.* Common and rare variant association analyses in amyotrophic lateral sclerosis identify 15 risk loci with distinct genetic architectures and neuron-specific biology. *Nat Genet* **53**, 1636-1648, doi:10.1038/s41588-021-00973-1 (2021).

31 Nguyen, H. P., Van Broeckhoven, C. & van der Zee, J. ALS Genes in the Genomic Era and their Implications for FTD. *Trends Genet* **34**, 404-423, doi:10.1016/j.tig.2018.03.001 (2018).

32 Al-Chalabi, A. *et al.* An estimate of amyotrophic lateral sclerosis heritability using twin data. *J Neurol Neurosurg Psychiatry* **81**, 1324-1326, doi:10.1136/jnnp.2010.207464 (2010).

33 Al-Chalabi, A. & Lewis, C. M. Modelling the effects of penetrance and family size on rates of sporadic and familial disease. *Hum Hered* **71**, 281-288, doi:10.1159/000330167 (2011).

34 van Blitterswijk, M. *et al.* Evidence for an oligogenic basis of amyotrophic lateral sclerosis. *Hum Mol Genet* **21**, 3776-3784, doi:10.1093/hmg/dds199 (2012).

35 Leigh, P. N. *et al.* Ubiquitin-immunoreactive intraneuronal inclusions in amyotrophic lateral sclerosis. Morphology, distribution, and specificity. *Brain* **114 (Pt 2)**, 775-788, doi:10.1093/brain/114.2.775 (1991).

36 Neumann, M. *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**, 130-133, doi:10.1126/science.1134108 (2006).

37 Al-Sarraj, S. *et al.* p62 positive, TDP-43 negative, neuronal cytoplasmic and intranuclear inclusions in the cerebellum and hippocampus define the pathology of C9orf72-linked FTLD and MND/ALS. *Acta Neuropathol* **122**, 691-702, doi:10.1007/s00401-011-0911-2 (2011).

38 Sieben, A. *et al.* Hippocampal Sclerosis in Frontotemporal Dementia: When Vascular Pathology Meets Neurodegeneration. *J Neuropathol Exp Neurol* **80**, 313-324, doi:10.1093/jnen/nlab010 (2021).

39 Chare, L. *et al.* New criteria for frontotemporal dementia syndromes: clinical and pathological diagnostic implications. *J Neurol Neurosurg Psychiatry* **85**, 865-870, doi:10.1136/jnnp-2013-306948 (2014).

40 Burrell, J. R. *et al.* The frontotemporal dementia-motor neuron disease continuum. *Lancet* **388**, 919-931, doi:10.1016/S0140-6736(16)00737-6 (2016).

41 Talbot, K., Feneberg, E., Scaber, J., Thompson, A. G. & Turner, M. R. Amyotrophic lateral sclerosis: the complex path to precision medicine. *J Neurol* **265**, 2454-2462, doi:10.1007/s00415-018-8983-8 (2018).

42 Al-Chalabi, A. & Hardiman, O. The epidemiology of ALS: a conspiracy of genes, environment and time. *Nat Rev Neurol* **9**, 617-628, doi:10.1038/nrneurol.2013.203 (2013).

43 Vahsen, B. F. *et al.* Non-neuronal cells in amyotrophic lateral sclerosis - from pathogenesis to biomarkers. *Nat Rev Neurol* **17**, 333-348, doi:10.1038/s41582-021-00487-8 (2021).

44 Saberi, S., Stauffer, J. E., Schulte, D. J. & Ravits, J. Neuropathology of Amyotrophic Lateral Sclerosis and Its Variants. *Neurol Clin* **33**, 855-876, doi:10.1016/j.ncl.2015.07.012 (2015).

45 K., B. Vergleichende Lokalisationslehre der Grosshirnrinde in ihren Prinzipien dargestellt auf Grund des Zellenbaues. *Barth: 1909* (1909).

46 Lowe, J. *et al.* A filamentous inclusion body within anterior horn neurones in motor neurone disease defined by immunocytochemical localisation of ubiquitin. *Neurosci Lett* **94**, 203-210, doi:10.1016/0304-3940(88)90296-0 (1988).

47 Arai, T. *et al.* TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun* **351**, 602-611, doi:10.1016/j.bbrc.2006.10.093 (2006).

48 Hanson, K. A., Kim, S. H. & Tibbetts, R. S. RNA-binding proteins in neurodegenerative disease: TDP-43 and beyond. *Wiley Interdiscip Rev RNA* **3**, 265-285, doi:10.1002/wrna.111 (2012).

49 Kim, H. J. *et al.* Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature* **495**, 467-473, doi:10.1038/nature11922 (2013).

50 Johnson, J. O. *et al.* Mutations in the Matrin 3 gene cause familial amyotrophic lateral sclerosis. *Nat Neurosci* **17**, 664-666, doi:10.1038/nn.3688 (2014).

51 Elden, A. C. *et al.* Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. *Nature* **466**, 1069-1075, doi:10.1038/nature09320 (2010).

52 Neumann, M. *et al.* FET proteins TAF15 and EWS are selective markers that distinguish FTLD with FUS pathology from amyotrophic lateral sclerosis with FUS mutations. *Brain* **134**, 2595-2609, doi:10.1093/brain/awr201 (2011).

53 Molliex, A. *et al.* Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* **163**, 123-133, doi:10.1016/j.cell.2015.09.015 (2015).

54 Patel, A. *et al.* A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell* **162**, 1066-1077, doi:10.1016/j.cell.2015.07.047 (2015).

55 Freibaum, B. D. *et al.* GGGGCC repeat expansion in C9orf72 compromises nucleocytoplasmic transport. *Nature* **525**, 129-133, doi:10.1038/nature14974 (2015).

56 Zhang, K. *et al.* The C9orf72 repeat expansion disrupts nucleocytoplasmic transport. *Nature* **525**, 56-61, doi:10.1038/nature14973 (2015).

57 Lagier-Tourenne, C. *et al.* Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. *Nat Neurosci* **15**, 1488-1497, doi:10.1038/nn.3230 (2012).

58 Polymenidou, M. *et al.* Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. *Nat Neurosci* **14**, 459-468, doi:10.1038/nn.2779 (2011).

59 Klim, J. R. *et al.* ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair. *Nat Neurosci* **22**, 167-179, doi:10.1038/s41593-018-0300-4 (2019).

60 Melamed, Z. *et al.* Premature polyadenylation-mediated loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration. *Nat Neurosci* **22**, 180-190, doi:10.1038/s41593-018-0293-z (2019).

61 Ma, X. R. *et al.* TDP-43 represses cryptic exon inclusion in the FTD-ALS gene UNC13A. *Nature* **603**, 124-130, doi:10.1038/s41586-022-04424-7 (2022).

62 Brown, A. L. *et al.* TDP-43 loss and ALS-risk SNPs drive mis-splicing and depletion of UNC13A. *Nature* **603**, 131-137, doi:10.1038/s41586-022-04436-3 (2022).

63 Alami, N. H. *et al.* Axonal transport of TDP-43 mRNA granules is impaired by ALS-causing mutations. *Neuron* **81**, 536-543, doi:10.1016/j.neuron.2013.12.018 (2014).

64 Chew, J. *et al.* Neurodegeneration. C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits. *Science* **348**, 1151-1154, doi:10.1126/science.aaa9344 (2015).

65 La Spada, A. R. & Taylor, J. P. Repeat expansion disease: progress and puzzles in disease pathogenesis. *Nat Rev Genet* **11**, 247-258, doi:10.1038/nrg2748 (2010).

66 Saxena, S., Cabuy, E. & Caroni, P. A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice. *Nat Neurosci* **12**, 627-636, doi:10.1038/nn.2297 (2009).

67 Deng, H. X. *et al.* Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature* **477**, 211-215, doi:10.1038/nature10353 (2011).

68 Fecto, F. *et al.* SQSTM1 mutations in familial and sporadic amyotrophic lateral sclerosis. *Arch Neurol* **68**, 1440-1446, doi:10.1001/archneurol.2011.250 (2011).

69 Maruyama, H. *et al.* Mutations of optineurin in amyotrophic lateral sclerosis. *Nature* **465**, 223-226, doi:10.1038/nature08971 (2010).

70 Johnson, J. O. *et al.* Exome sequencing reveals VCP mutations as a cause of familial ALS. *Neuron* **68**, 857-864, doi:10.1016/j.neuron.2010.11.036 (2010).

71 Parkinson, N. *et al.* ALS phenotypes with mutations in CHMP2B (charged multivesicular body protein 2B). *Neurology* **67**, 1074-1077, doi:10.1212/01.wnl.0000231510.89311.8b (2006).

72 Chen, H. J. *et al.* Characterization of the properties of a novel mutation in VAPB in familial amyotrophic lateral sclerosis. *J Biol Chem* **285**, 40266-40281, doi:10.1074/jbc.M110.161398 (2010).

73 Chow, C. Y. *et al.* Deleterious variants of FIG4, a phosphoinositide phosphatase, in patients with ALS. *Am J Hum Genet* **84**, 85-88, doi:10.1016/j.ajhg.2008.12.010 (2009).

74 Chen-Plotkin, A. S. *et al.* Variations in the progranulin gene affect global gene expression in frontotemporal lobar degeneration. *Hum Mol Genet* **17**, 1349-1362, doi:10.1093/hmg/ddn023 (2008).

75 Guo, Q. *et al.* In Situ Structure of Neuronal C9orf72 Poly-GA Aggregates Reveals Proteasome Recruitment. *Cell* **172**, 696-705 e612, doi:10.1016/j.cell.2017.12.030 (2018).

76 Shibata, N. *et al.* Morphological evidence for lipid peroxidation and protein glycoxidation in spinal cords from sporadic amyotrophic lateral sclerosis patients. *Brain Res* **917**, 97-104, doi:10.1016/s0006-8993(01)02926-2 (2001).

77 Bannwarth, S. *et al.* A mitochondrial origin for frontotemporal dementia and amyotrophic lateral sclerosis through CHCHD10 involvement. *Brain* **137**, 2329-2345, doi:10.1093/brain/awu138 (2014).

78 Pensato, V. *et al.* TUBA4A gene analysis in sporadic amyotrophic lateral sclerosis: identification of novel mutations. *J Neurol* **262**, 1376-1378, doi:10.1007/s00415-015-7739-y (2015).

79 Puls, I. *et al.* Mutant dynactin in motor neuron disease. *Nat Genet* **33**, 455-456, doi:10.1038/ng1123 (2003).

80 Wu, C. H. *et al.* Mutations in the profilin 1 gene cause familial amyotrophic lateral sclerosis. *Nature* **488**, 499-503, doi:10.1038/nature11280 (2012).

81 Nicolas, A. *et al.* Genome-wide Analyses Identify KIF5A as a Novel ALS Gene. *Neuron* **97**, 1268-1283 e1266, doi:10.1016/j.neuron.2018.02.027 (2018).

82 Al-Chalabi, A. *et al.* Deletions of the heavy neurofilament subunit tail in amyotrophic lateral sclerosis. *Hum Mol Genet* **8**, 157-164, doi:10.1093/hmg/8.2.157 (1999).

83 Corrado, L. *et al.* A novel peripherin gene (PRPH) mutation identified in one sporadic amyotrophic lateral sclerosis patient. *Neurobiol Aging* **32**, 552 e551-556, doi:10.1016/j.neurobiolaging.2010.02.011 (2011).

84 Nishimura, A. L. *et al.* A novel locus for late onset amyotrophic lateral sclerosis/motor neurone disease variant at 20q13. *J Med Genet* **41**, 315-320, doi:10.1136/jmg.2003.013029 (2004).

85 van Es, M. A. *et al.* Genome-wide association study identifies 19p13.3 (UNC13A) and 9p21.2 as susceptibility loci for sporadic amyotrophic lateral sclerosis. *Nat Genet* **41**, 1083-1087, doi:10.1038/ng.442 (2009).

86 Nakata, M. *et al.* Distal excitability changes in motor axons in amyotrophic lateral sclerosis. *Clin Neurophysiol* **117**, 1444-1448, doi:10.1016/j.clinph.2006.04.005 (2006).

87 Kanai, K. *et al.* Altered axonal excitability properties in amyotrophic lateral sclerosis: impaired potassium channel function related to disease stage. *Brain* **129**, 953-962, doi:10.1093/brain/awl024 (2006).

88 De Schaepdryver, M. *et al.* Comparison of elevated phosphorylated neurofilament heavy chains in serum and cerebrospinal fluid of patients with amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry* **89**, 367-373, doi:10.1136/jnnp-2017-316605 (2018).

89 Schiffer, D., Cordera, S., Cavalla, P. & Migheli, A. Reactive astrogliosis of the spinal cord in amyotrophic lateral sclerosis. *J Neurol Sci* **139 Suppl**, 27-33, doi:10.1016/0022-510x(96)00073-1 (1996).

90 Nagy, D., Kato, T. & Kushner, P. D. Reactive astrocytes are widespread in the cortical gray matter of amyotrophic lateral sclerosis. *J Neurosci Res* **38**, 336-347, doi:10.1002/jnr.490380312 (1994).

91 Rothstein, J. D., Van Kammen, M., Levey, A. I., Martin, L. J. & Kuncl, R. W. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann Neurol* **38**, 73-84, doi:10.1002/ana.410380114 (1995).

92 Ferraiuolo, L. & Maragakis, N. J. Mini-Review: Induced pluripotent stem cells and the search for new cell-specific ALS therapeutic targets. *Neurosci Lett* **755**, 135911, doi:10.1016/j.neulet.2021.135911 (2021).

93 Wang, L., Gutmann, D. H. & Roos, R. P. Astrocyte loss of mutant SOD1 delays ALS disease onset and progression in G85R transgenic mice. *Hum Mol Genet* **20**, 286-293, doi:10.1093/hmg/ddq463 (2011).

94 Guttenplan, K. A. *et al.* Neurotoxic Reactive Astrocytes Drive Neuronal Death after Retinal Injury. *Cell Rep* **31**, 107776, doi:10.1016/j.celrep.2020.107776 (2020).

95 Haidet-Phillips, A. M. *et al.* Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. *Nat Biotechnol* **29**, 824-828, doi:10.1038/nbt.1957 (2011).

96 Re, D. B. *et al.* Necroptosis drives motor neuron death in models of both sporadic and familial ALS. *Neuron* **81**, 1001-1008, doi:10.1016/j.neuron.2014.01.011 (2014).

97 Di Giorgio, F. P., Carrasco, M. A., Siao, M. C., Maniatis, T. & Eggan, K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci* **10**, 608-614, doi:10.1038/nn1885 (2007).

98 Nagai, M. *et al.* Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* **10**, 615-622, doi:10.1038/nn1876 (2007).

99 Tomassy, G. S. *et al.* Distinct profiles of myelin distribution along single axons of pyramidal neurons in the neocortex. *Science* **344**, 319-324, doi:10.1126/science.1249766 (2014).

100 Kawamata, T., Akiyama, H., Yamada, T. & McGeer, P. L. Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord tissue. *Am J Pathol* **140**, 691-707 (1992).

101 Nolan, M. *et al.* Quantitative patterns of motor cortex proteinopathy across ALS genotypes. *Acta Neuropathol Commun* **8**, 98, doi:10.1186/s40478-020-00961-2 (2020).

102 Lee, Y. *et al.* Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* **487**, 443-448, doi:10.1038/nature11314 (2012).

103 Kang, S. H. *et al.* Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. *Nat Neurosci* **16**, 571-579, doi:10.1038/nn.3357 (2013).

104 Irwin, D. J. *et al.* Myelin oligodendrocyte basic protein and prognosis in behavioral-variant frontotemporal dementia. *Neurology* **83**, 502-509, doi:10.1212/WNL.0000000000000668 (2014).

105 Henkel, J. S. *et al.* Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. *Ann Neurol* **55**, 221-235, doi:10.1002/ana.10805 (2004).

106 Zurcher, N. R. *et al.* Increased in vivo glial activation in patients with amyotrophic lateral sclerosis: assessed with [(11)C]-PBR28. *Neuroimage Clin* **7**, 409-414, doi:10.1016/j.nicl.2015.01.009 (2015).

107 Dols-Icardo, O. *et al.* Motor cortex transcriptome reveals microglial key events in amyotrophic lateral sclerosis. *Neurol Neuroimmunol Neuroinflamm* **7**, doi:10.1212/NXI.0000000000000829 (2020).

108 Hall, E. D., Oostveen, J. A. & Gurney, M. E. Relationship of microglial and astrocytic activation to disease onset and progression in a transgenic model of familial ALS. *Glia* **23**, 249-256, doi:10.1002/(sici)1098-1136(199807)23:3<249::aid-glia7>3.0.co;2-# (1998).

109 Boillee, S. *et al.* Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* **312**, 1389-1392, doi:10.1126/science.1123511 (2006).

110 Keren-Shaul, H. *et al.* A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* **169**, 1276-1290 e1217, doi:10.1016/j.cell.2017.05.018 (2017).

111 Deczkowska, A. *et al.* Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell* **173**, 1073-1081, doi:10.1016/j.cell.2018.05.003 (2018).

112 de Boer, A. S. *et al.* Genetic validation of a therapeutic target in a mouse model of ALS. *Sci Transl Med* **6**, 248ra104, doi:10.1126/scitranslmed.3009351 (2014).

113 Zhang, J. *et al.* Neurotoxic microglia promote TDP-43 proteinopathy in progranulin deficiency. *Nature* **588**, 459-465, doi:10.1038/s41586-020-2709-7 (2020).

114 Burberry, A. *et al.* Loss-of-function mutations in the C9ORF72 mouse ortholog cause fatal autoimmune disease. *Sci Transl Med* **8**, 347ra393, doi:10.1126/scitranslmed.aaf6038 (2016).

115 Burberry, A. *et al.* C9orf72 suppresses systemic and neural inflammation induced by gut bacteria. *Nature* **582**, 89-94, doi:10.1038/s41586-020-2288-7 (2020).

116 O'Rourke, J. G. *et al.* C9orf72 is required for proper macrophage and microglial function in mice. *Science* **351**, 1324-1329, doi:10.1126/science.aaf1064 (2016).

117 Zhang, Y. *et al.* The C9orf72-interacting protein Smcr8 is a negative regulator of autoimmunity and lysosomal exocytosis. *Genes Dev* **32**, 929-943, doi:10.1101/gad.313932.118 (2018).

118 Gerbino, V. *et al.* The Loss of TBK1 Kinase Activity in Motor Neurons or in All Cell Types Differentially Impacts ALS Disease Progression in SOD1 Mice. *Neuron* **106**, 789-805 e785, doi:10.1016/j.neuron.2020.03.005 (2020).

119 Yu, C. H. *et al.* TDP-43 Triggers Mitochondrial DNA Release via mPTP to Activate cGAS/STING in ALS. *Cell* **183**, 636-649 e618, doi:10.1016/j.cell.2020.09.020 (2020).

120 McCauley, M. E. *et al.* C9orf72 in myeloid cells suppresses STING-induced inflammation. *Nature* **585**, 96-101, doi:10.1038/s41586-020-2625-x (2020).

121 Chiot, A. *et al.* Modifying macrophages at the periphery has the capacity to change microglial reactivity and to extend ALS survival. *Nat Neurosci* **23**, 1339-1351, doi:10.1038/s41593-020-00718-z (2020).

122 Zhong, Z. *et al.* ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degeneration. *Nat Neurosci* **11**, 420-422, doi:10.1038/nn2073 (2008).

123 Prudencio, M. *et al.* Distinct brain transcriptome profiles in C9orf72-associated and sporadic ALS. *Nat Neurosci* **18**, 1175-1182, doi:10.1038/nn.4065 (2015).

124 Mordes, D. A. *et al.* Dipeptide repeat proteins activate a heat shock response found in C9ORF72-ALS/FTLD patients. *Acta Neuropathol Commun* **6**, 55, doi:10.1186/s40478-018-0555-8 (2018).

125 Tam, O. H. *et al.* Postmortem Cortex Samples Identify Distinct Molecular Subtypes of ALS: Retrotransposon Activation, Oxidative Stress, and Activated Glia. *Cell Rep* **29**, 1164-1177 e1165, doi:10.1016/j.celrep.2019.09.066 (2019).

126 Rabin, S. J. *et al.* Sporadic ALS has compartment-specific aberrant exon splicing and altered cell-matrix adhesion biology. *Hum Mol Genet* **19**, 313-328, doi:10.1093/hmg/ddp498 (2010).

127 Brohawn, D. G., O'Brien, L. C. & Bennett, J. P., Jr. RNAseq Analyses Identify Tumor Necrosis Factor-Mediated Inflammation as a Major Abnormality in ALS Spinal Cord. *PLoS One* **11**, e0160520, doi:10.1371/journal.pone.0160520 (2016).

128 D'Erchia, A. M. *et al.* Massive transcriptome sequencing of human spinal cord tissues provides new insights into motor neuron degeneration in ALS. *Sci Rep* **7**, 10046, doi:10.1038/s41598-017-10488-7 (2017).

129 Gerrits, E. *et al.* Neurovascular dysfunction in GRN-associated frontotemporal dementia identified by single-nucleus RNA sequencing of human cerebral cortex. *Nat Neurosci* **25**, 1034-1048, doi:10.1038/s41593-022-01124-3 (2022).

130 Lemon, R. N. Descending pathways in motor control. *Annu Rev Neurosci* **31**, 195-218, doi:10.1146/annurev.neuro.31.060407.125547 (2008).

131 Jones, R. A. *et al.* Cellular and Molecular Anatomy of the Human Neuromuscular Junction. *Cell Rep* **21**, 2348-2356, doi:10.1016/j.celrep.2017.11.008 (2017).

132 Todd, T. W. & Petrucelli, L. Modelling amyotrophic lateral sclerosis in rodents. *Nat Rev Neurosci* **23**, 231-251, doi:10.1038/s41583-022-00564-x (2022).

133 Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872, doi:10.1016/j.cell.2007.11.019 (2007).

134 Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147, doi:10.1126/science.282.5391.1145 (1998).

135 Tao, Y. & Zhang, S. C. Neural Subtype Specification from Human Pluripotent Stem Cells. *Cell Stem Cell* **19**, 573-586, doi:10.1016/j.stem.2016.10.015 (2016).

136 Chambers, S. M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* **27**, 275-280, doi:10.1038/nbt.1529 (2009).

137 Limone, F., Klim, J. R. & Mordes, D. A. Pluripotent stem cell strategies for rebuilding the human brain. *Front Aging Neurosci* **14**, 1017299, doi:10.3389/fnagi.2022.1017299 (2022).

138 Mertens, J., Marchetto, M. C., Bardy, C. & Gage, F. H. Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience. *Nat Rev Neurosci* **17**, 424-437, doi:10.1038/nrn.2016.46 (2016).

139 Donnelly, C. J. *et al.* RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. *Neuron* **80**, 415-428, doi:10.1016/j.neuron.2013.10.015 (2013).

140 Gendron, T. F. *et al.* Poly(GP) proteins are a useful pharmacodynamic marker for C9ORF72-associated amyotrophic lateral sclerosis. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aai7866 (2017).

141 Sareen, D. *et al.* Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion. *Sci Transl Med* **5**, 208ra149, doi:10.1126/scitranslmed.3007529 (2013).

142 Shi, Y. *et al.* Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons. *Nat Med* **24**, 313-325, doi:10.1038/nm.4490 (2018).

143 Kiskinis, E. *et al.* Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. *Cell Stem Cell* **14**, 781-795, doi:10.1016/j.stem.2014.03.004 (2014).

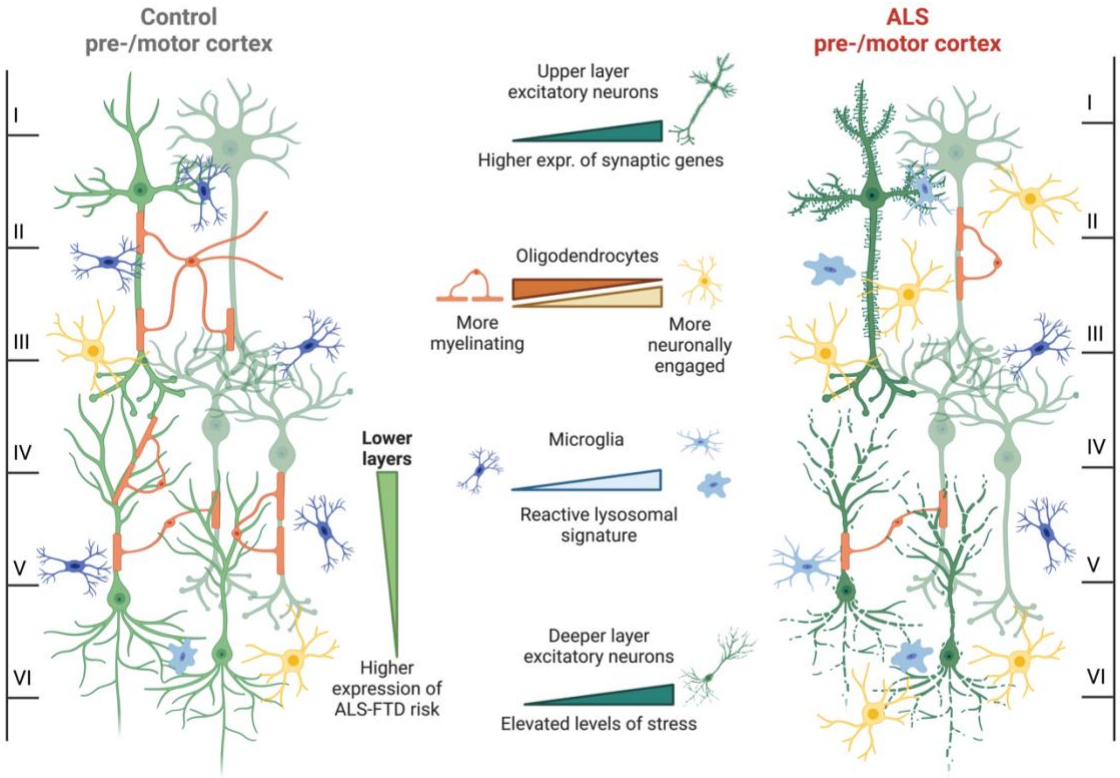
144 Wainger, B. J. *et al.* Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. *Cell Rep* **7**, 1-11, doi:10.1016/j.celrep.2014.03.019 (2014).

145 Egawa, N. *et al.* Drug screening for ALS using patient-specific induced pluripotent stem cells. *Sci Transl Med* **4**, 145ra104, doi:10.1126/scitranslmed.3004052 (2012).
146 Fujimori, K. *et al.* Modeling sporadic ALS in iPSC-derived motor neurons identifies a potential therapeutic agent. *Nat Med* **24**, 1579-1589, doi:10.1038/s41591-018-0140-5 (2018).
147 Hawrot, J., Imhof, S. & Wainger, B. J. Modeling cell-autonomous motor neuron phenotypes in ALS using iPSCs. *Neurobiol Dis* **134**, 104680, doi:10.1016/j.nbd.2019.104680 (2020).
148 Smethurst, P. *et al.* Distinct responses of neurons and astrocytes to TDP-43 proteinopathy in amyotrophic lateral sclerosis. *Brain* **143**, 430-440, doi:10.1093/brain/awz419 (2020).
149 Zhao, C. *et al.* Mutant C9orf72 human iPSC-derived astrocytes cause non-cell autonomous motor neuron pathophysiology. *Glia* **68**, 1046-1064, doi:10.1002/glia.23761 (2020).

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.

This work is under consideration at *Nature Aging.*

Earlier versions of this chapter can be found at bioRxiv https://doi.org/10.1101/2021.07.12.452054

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

Francesco Limone[1,2,3,10], Daniel A. Mordes[1,2,4,10], Alexander Couto[1], Brian J. Joseph[1], Jana M. Mitchell[1,2], Martine Therrien[2,6], Sulagna Dia Ghosh[1,2,7], Daniel Meyer[2,7], Yingying Zhang[1,2], Melissa Goldman[2,7], Laura Bortolin[2,7], Inma Cobos[4], Irena Kadiu[8], Steven A. McCarroll[2,7], Beth Stevens[2,6], Olli Pietiläinen[1,2,5], Aaron Burberry[1,2,9] and Kevin Eggan[1,2,*]

[1]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA, USA. [2]Stanley Centre for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA, USA. [3]Leiden University Medical Center, LUMC, 2333 ZA Leiden, The Netherlands. [4]Department of Pathology, Massachusetts general Hospital, Boston, MA, USA. [5]Neuroscience Center, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland. [6]Boston Children's Hospital, F.M. Kirby Neurobiology Center, Boston, MA, USA. [7]Department of Genetics, Harvard Medical School, Boston MA, USA. [8]Neuroscience Therapeutic Area, New Medicines, UCB Pharma, Braine-l'Alleud, Belgium. [9]Department of Pathology, School of Medicine, Case Western Reserve University, Cleveland, OH, USA. [10]These authors contributed equally: Francesco Limone, Daniel Mordes. *e-mail: kevin.eggan@bmrn.com

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 50s[1]. Although specific genetic causes have been identified, most cases are sporadic (~90%), have no family history and unknown etiology[2], rendering modelling of non-genetic forms of the

disease difficult[3]. 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 familial[6] 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 neurons[9], 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 disease[14-17].

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 diseases[18-27]. 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 un 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 technology[28] (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 Seurat[29], single-cell analysis package, to cluster and annotate groups according to canonical markers of brain cell types[30]: 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 studies[31] 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).

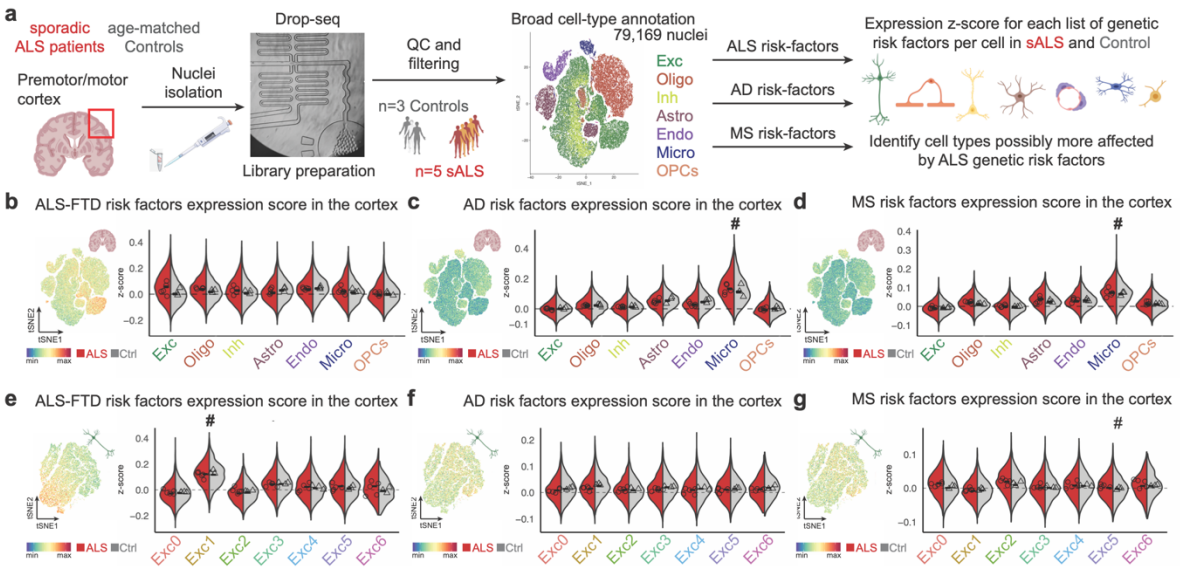


Figure 1 Cellular susceptibility to ALS-FTD in the human cortex. a, Diagram of workflow for isolation of nuclei from cortices of ALS patients and age-matched controls followed by single-nucleus RNA sequencing and assessment of expression of gene modules associated with neurodegenerative diseases. **b-d**, 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 field[2] (Extended Data Fig. 2a). We then computed a "module score" for this set of genes[32]; 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 MS[35] (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 neurodegeneration[33-37] and shown by other reports[30] (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 V[18] and used as a reporter for CSMNs[39], 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 Cortex[41]. We detected expression of their markers in these groups with Exc1 expressing *SERPINE2* and *POU3F1*, specific of ETNs[41], 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 areas[43], we plotted markers specific to layer V neurons of regions adjacent to the Motor Cortex like von Economo cells[44], affected in FTD[45], 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 cortex[47]. 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 dataset[47],

confirmed that the top 10 ALS/FTD-associated genes most high 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 mouse[50] 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.

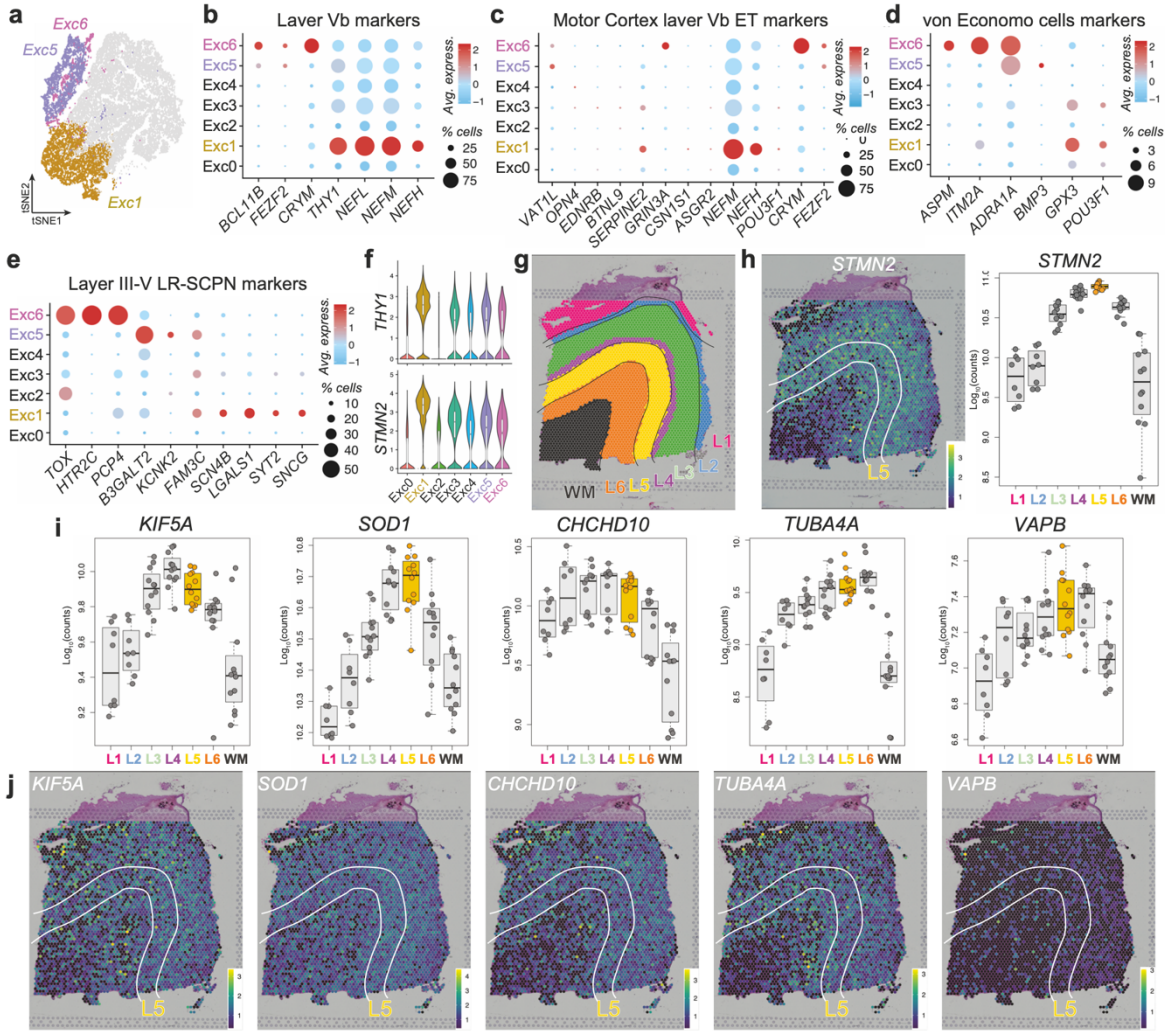


Figure 2 ALS-FTD susceptible neurons are L5-ET Neurons. a, t-SNE projection of presumptive layer V neurons. **b**, Dotplot representing expression of Layer V markers **c**, Dotplot for markers of LVb Extratelencephalic neurons of human Motor Cortex. **d**, Dotplot representing expression of von Economo 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 ALS[51].

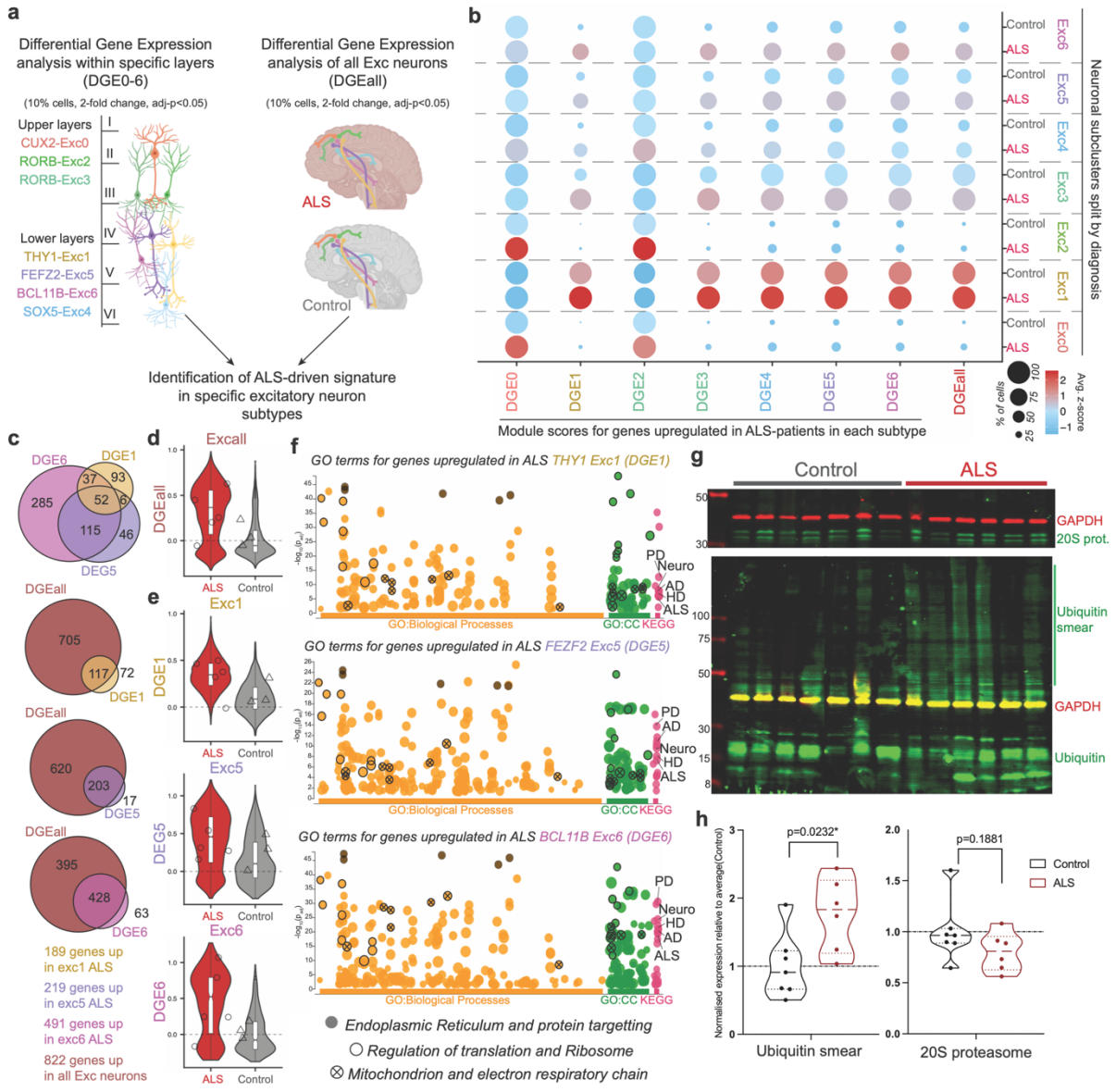


Figure 3 ALS excitatory neurons present increased expression of stress-related pathways. a, Schematic of Differential Gene Expression Analysis. **b**, Dotplot representing scores for genes upregulated in each subgroup of Exc neurons (DGE0-6) and globally upregulated in all Exc (DGEall). **c**, Comparison 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 interquantile 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 interquantile 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 patients[52] (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 patients[18] but not neurons from AD patients[20] (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 standardization[53-55], 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 cells[49], phase separation[56], stress granules[57] 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 neurons[58]

(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 proteosomal 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 integrity[60] and because others detected changes in myelination in ALS motor cortex[16] and in FTD frontal cortex[27], 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 cortex[41].

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 (GPRCs) that mark developmental milestones: *GPR56*, expressed in OPCs[61], and *GPR37*, expressed in myelinating cells[62], 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 patients[16].

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).

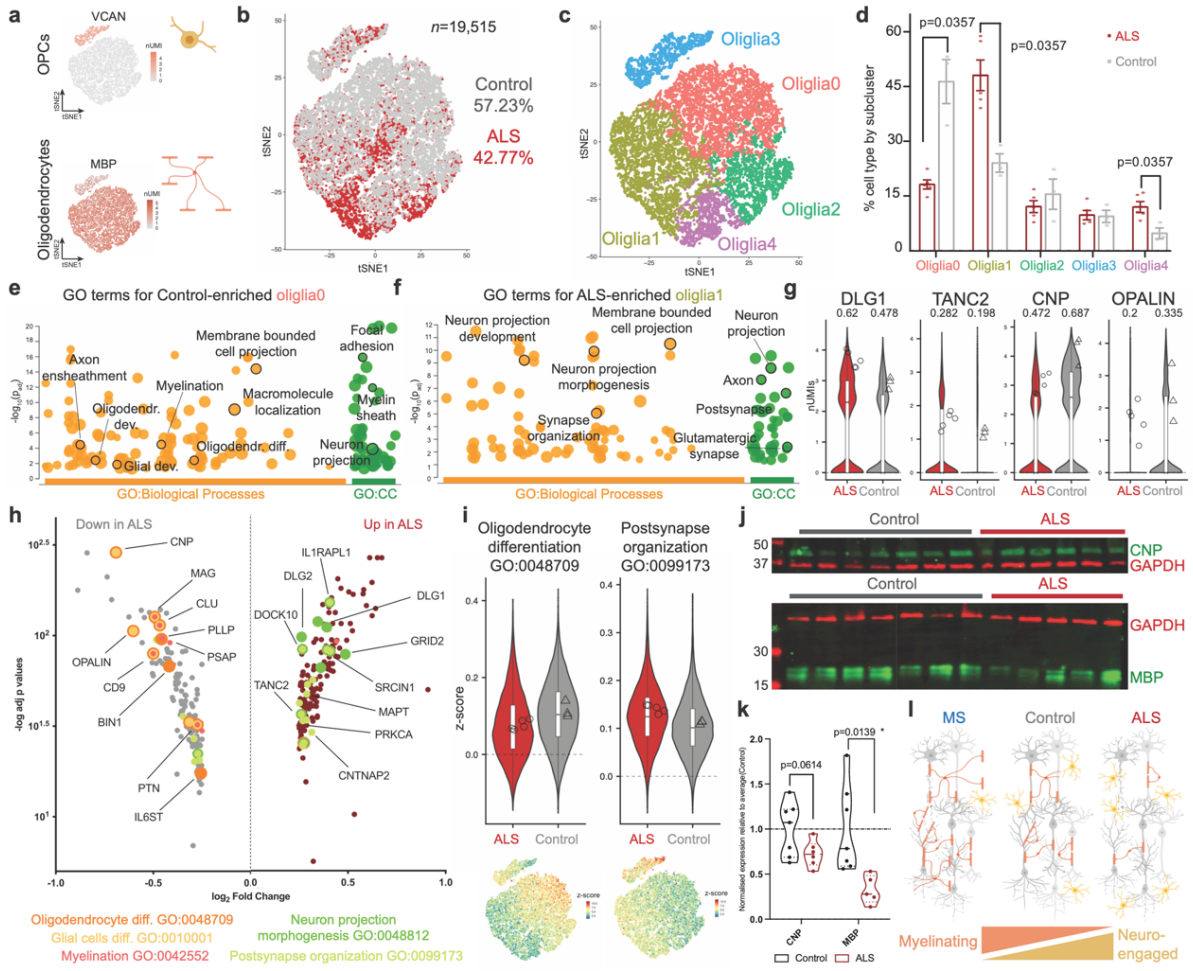


Figure 4 In ALS, oligodendroglial cells decrease their myelinating machinery in favour of a neuro-engaged state. a, t-SNE projection of OPCs and oligodendrocytes markers. **b**, t-SNE projection of oligodendroglia (ALS n=8,372 nuclei, Control n=11,168 nuclei). **c**, t-SNE projection of subclusters within 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 interquantile 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 interquantile 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 models[63], patient samples[6] and function of ALS-related genes in myeloid cells[64-66] 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) pipeline[67], 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 inflammation[67] (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 AD[20] and MS[69], 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*, *ASAH1*, *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.

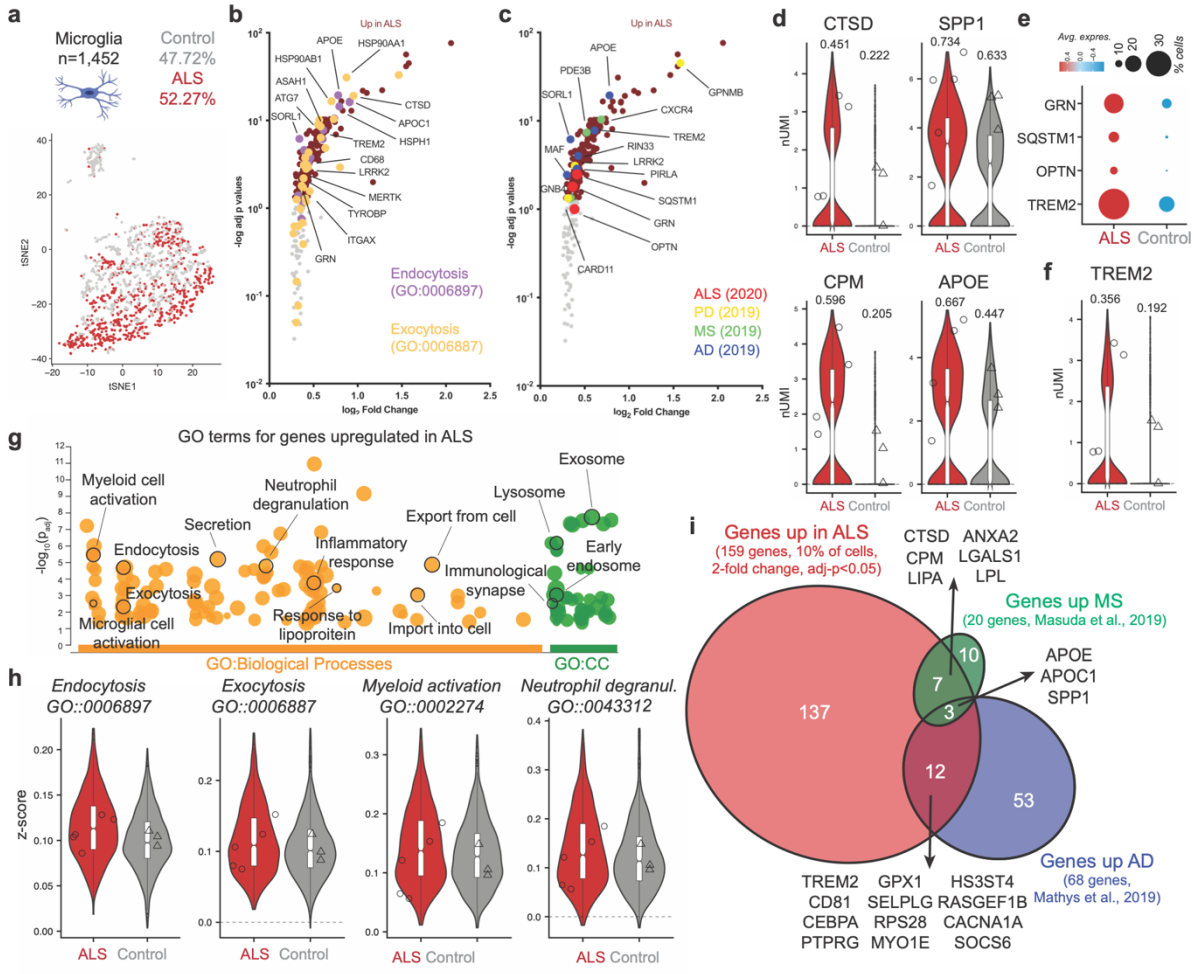


Figure 5 Disease-Associated Microglia signature in ALS. a, t-SNE projection of microglia (ALS n=759 nuclei, Control n=693 nuclei). **b,c**, Volcano plot of genes upregulated in microglia from ALS. Genes identified in GO terms for endocytosis and exocytosis (b), genes associated to neurodegenerative diseases in (c). **d**, Violin plots of representative genes upregulated in ALS patients associated with reactive microglia (geometric boxplots represent median and interquantile 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 interquantile 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 interquantile 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 diseases[70]. 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/FTD[10]. This enrichment is not recapitulated for risk factors connected to AD and MS, related to immune processes and are more enriched in microglia[30]. One study suggested that ALS-associated variants connected to autophagy and protein clearing are most highly expressed in glutamatergic neurons[51] and these findings were later corroborated adding the importance of neuronal morphology and ribonucleotide metabolism[52], 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 ALS[71]. 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 MS[18]; ventral dopaminergic neurons in Parkinson's Disease[23] 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

AD[73]. 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 survival[16]. 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 MS[19]. 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 cortex[41] and regional distribution of different types of oligodendrocyte in the nervous system might explain differential responses to disease[76]. The upregulation of synaptic transcripts in oligodendrocytes of ALS patients might represent phagocytic activity of this cell type in neurodegenerative contexts[77] or the need for synaptic proteins in the formation of myelin sheath[78]. 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 ALS[79]. 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 neurodegeneration[27]. 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 disease[52]. 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 activity[80].

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 patients[81]. 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*-ALS[82], 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 MS[69], 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 contexts[83], 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 ALS[84]. 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 heterogenous disease[7], and a smaller cohort size cannot fully recapitulate the diversity identified in patients. However, only recently biobanks have been able 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 contaminations[85]? As mentioned in the text, oligodendrocytes in the motor cortex have been shown to express synaptic transcripts[41], 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.

References

1 Taylor, J. P., Brown, R. H., Jr. & Cleveland, D. W. Decoding ALS: from genes to mechanism. *Nature* **539**, 197-206, doi:10.1038/nature20413 (2016).

2 Brown, R. H. & Al-Chalabi, A. Amyotrophic Lateral Sclerosis. *N Engl J Med* **377**, 162-172, doi:10.1056/NEJMra1603471 (2017).

3 Wainger, B. J. & Lagier-Tourenne, C. Taking on the Elephant in the Tissue Culture Room: iPSC Modeling for Sporadic ALS. *Cell Stem Cell* **23**, 466-467, doi:10.1016/j.stem.2018.09.015 (2018).

4 Prudencio, M. *et al.* Distinct brain transcriptome profiles in C9orf72-associated and sporadic ALS. *Nat Neurosci* **18**, 1175-1182, doi:10.1038/nn.4065 (2015).

5 Mordes, D. A. *et al.* Dipeptide repeat proteins activate a heat shock response found in C9ORF72-ALS/FTLD patients. *Acta Neuropathol Commun* **6**, 55, doi:10.1186/s40478-018-0555-8 (2018).

6 D'Erchia, A. M. *et al.* Massive transcriptome sequencing of human spinal cord tissues provides new insights into motor neuron degeneration in ALS. *Sci Rep* **7**, 10046, doi:10.1038/s41598-017-10488-7 (2017).

7 Tam, O. H. *et al.* Postmortem Cortex Samples Identify Distinct Molecular Subtypes of ALS: Retrotransposon Activation, Oxidative Stress, and Activated Glia. *Cell Rep* **29**, 1164-1177 e1165, doi:10.1016/j.celrep.2019.09.066 (2019).

8 Eshima, J. *et al.* Molecular subtypes of ALS are associated with differences in patient prognosis. *Nat Commun* **14**, 95, doi:10.1038/s41467-022-35494-w (2023).

9 Neumann, M. *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**, 130-133, doi:10.1126/science.1134108 (2006).

10 Hammer, R. P., Jr., Tomiyasu, U. & Scheibel, A. B. Degeneration of the human Betz cell due to amyotrophic lateral sclerosis. *Exp Neurol* **63**, 336-346, doi:10.1016/0014-4886(79)90129-8 (1979).

11 Giordana, M. T. *et al.* TDP-43 redistribution is an early event in sporadic amyotrophic lateral sclerosis. *Brain Pathol* **20**, 351-360, doi:10.1111/j.1750-3639.2009.00284.x (2010).

12 Seeley, W. W. *et al.* Early frontotemporal dementia targets neurons unique to apes and humans. *Ann Neurol* **60**, 660-667, doi:10.1002/ana.21055 (2006).

13 Nana, A. L. *et al.* Neurons selectively targeted in frontotemporal dementia reveal early stage TDP-43 pathobiology. *Acta Neuropathol* **137**, 27-46, doi:10.1007/s00401-018-1942-8 (2019).

14 Suzuki, N. *et al.* The mouse C9ORF72 ortholog is enriched in neurons known to degenerate in ALS and FTD. *Nat Neurosci* **16**, 1725-1727, doi:10.1038/nn.3566 (2013).

15 Ransohoff, R. M. How neuroinflammation contributes to neurodegeneration. *Science* **353**, 777-783, doi:10.1126/science.aag2590 (2016).

16 Kang, S. H. *et al.* Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. *Nat Neurosci* **16**, 571-579, doi:10.1038/nn.3357 (2013).

17 Boillee, S. *et al.* Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* **312**, 1389-1392, doi:10.1126/science.1123511 (2006).

18 Schirmer, L. *et al.* Neuronal vulnerability and multilineage diversity in multiple sclerosis. *Nature* **573**, 75-82, doi:10.1038/s41586-019-1404-z (2019).

19 Jakel, S. *et al.* Altered human oligodendrocyte heterogeneity in multiple sclerosis. *Nature* **566**, 543-547, doi:10.1038/s41586-019-0903-2 (2019).

20 Mathys, H. *et al.* Single-cell transcriptomic analysis of Alzheimer's disease. *Nature* **570**, 332-337, doi:10.1038/s41586-019-1195-2 (2019).

21 Zhou, Y. *et al.* Human and mouse single-nucleus transcriptomics reveal TREM2-dependent and TREM2-independent cellular responses in Alzheimer's disease. *Nat Med* **26**, 131-142, doi:10.1038/s41591-019-0695-9 (2020).

22 Leng, K. *et al.* Molecular characterization of selectively vulnerable neurons in Alzheimer's disease. *Nat Neurosci*, doi:10.1038/s41593-020-00764-7 (2021).

23 Kamath, T. *et al.* Single-cell genomic profiling of human dopamine neurons identifies a population that selectively degenerates in Parkinson's disease. *Nat Neurosci* **25**, 588-595, doi:10.1038/s41593-022-01061-1 (2022).

24 Sadick, J. S. *et al.* Astrocytes and oligodendrocytes undergo subtype-specific transcriptional changes in Alzheimer's disease. *Neuron* **110**, 1788-1805 e1710, doi:10.1016/j.neuron.2022.03.008 (2022).

25 Grubman, A. *et al.* A single-cell atlas of entorhinal cortex from individuals with Alzheimer's disease reveals cell-type-specific gene expression regulation. *Nat Neurosci* **22**, 2087-2097, doi:10.1038/s41593-019-0539-4 (2019).

26 Lau, S. F., Cao, H., Fu, A. K. Y. & Ip, N. Y. Single-nucleus transcriptome analysis reveals dysregulation of angiogenic endothelial cells and neuroprotective glia in Alzheimer's disease. *Proc Natl Acad Sci U S A* **117**, 25800-25809, doi:10.1073/pnas.2008762117 (2020).

27 Gerrits, E. *et al.* Neurovascular dysfunction in GRN-associated frontotemporal dementia identified by single-nucleus RNA sequencing of human cerebral cortex. *Nat Neurosci* **25**, 1034-1048, doi:10.1038/s41593-022-01124-3 (2022).

28 Macosko, E. Z. *et al.* Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell* **161**, 1202-1214, doi:10.1016/j.cell.2015.05.002 (2015).

29 Stuart, T. *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-1902 e1821, doi:10.1016/j.cell.2019.05.031 (2019).

30 Kelley, K. W., Nakao-Inoue, H., Molofsky, A. V. & Oldham, M. C. Variation among intact tissue samples reveals the core transcriptional features of human CNS cell classes. *Nat Neurosci* **21**, 1171-1184, doi:10.1038/s41593-018-0216-z (2018).

31 Lake, B. B. *et al.* Integrative single-cell analysis of transcriptional and epigenetic states in the human adult brain. *Nat Biotechnol* **36**, 70-80, doi:10.1038/nbt.4038 (2018).

32 Tirosh, I. *et al.* Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* **352**, 189-196, doi:10.1126/science.aad0501 (2016).

33 Kunkle, B. W. *et al.* Genetic meta-analysis of diagnosed Alzheimer's disease identifies new risk loci and implicates Abeta, tau, immunity and lipid processing. *Nat Genet* **51**, 414-430, doi:10.1038/s41588-019-0358-2 (2019).

34 Jansen, I. E. *et al.* Genome-wide meta-analysis identifies new loci and functional pathways influencing Alzheimer's disease risk. *Nat Genet* **51**, 404-413, doi:10.1038/s41588-018-0311-9 (2019).

35 International Multiple Sclerosis Genetics, C. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science* **365**, doi:10.1126/science.aav7188 (2019).

36 Paolicelli, R. C. *et al.* Microglia states and nomenclature: A field at its crossroads. *Neuron* **110**, 3458-3483, doi:10.1016/j.neuron.2022.10.020 (2022).

37 Dolan, M. J. *et al.* A resource for generating and manipulating human microglial states in vitro. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2022.05.02.490100 (2022).

38 Arlotta, P. *et al.* Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron* **45**, 207-221, doi:10.1016/j.neuron.2004.12.036 (2005).

39 Ozdinler, P. H. *et al.* Corticospinal motor neurons and related subcerebral projection neurons undergo early and specific neurodegeneration in hSOD1G(9)(3)A transgenic ALS mice. *J Neurosci* **31**, 4166-4177, doi:10.1523/JNEUROSCI.4184-10.2011 (2011).

40 Tsang, Y. M., Chiong, F., Kuznetsov, D., Kasarskis, E. & Geula, C. Motor neurons are rich in non-phosphorylated neurofilaments: cross-species comparison and alterations in ALS. *Brain Res* **861**, 45-58, doi:10.1016/s0006-8993(00)01954-5 (2000).

41 Bakken, T. E. *et al.* Comparative cellular analysis of motor cortex in human, marmoset and mouse. *Nature* **598**, 111-119, doi:10.1038/s41586-021-03465-8 (2021).

42 Guerra San Juan, I. *et al.* Loss of mouse Stmn2 function causes motor neuropathy. *Neuron*, doi:10.1016/j.neuron.2022.02.011 (2022).

43 Rivara, C. B., Sherwood, C. C., Bouras, C. & Hof, P. R. Stereologic characterization and spatial distribution patterns of Betz cells in the human primary motor cortex. *Anat Rec A Discov Mol Cell Evol Biol* **270**, 137-151, doi:10.1002/ar.a.10015 (2003).

44 Hodge, R. D. *et al.* Transcriptomic evidence that von Economo neurons are regionally specialized extratelencephalic-projecting excitatory neurons. *Nat Commun* **11**, 1172, doi:10.1038/s41467-020-14952-3 (2020).

45 Cobos, I. & Seeley, W. W. Human von Economo neurons express transcription factors associated with Layer V subcerebral projection neurons. *Cereb Cortex* **25**, 213-220, doi:10.1093/cercor/bht219 (2015).

46 Zeng, H. *et al.* Large-scale cellular-resolution gene profiling in human neocortex reveals species-specific molecular signatures. *Cell* **149**, 483-496, doi:10.1016/j.cell.2012.02.052 (2012).

47 Maynard, K. R. *et al.* Transcriptome-scale spatial gene expression in the human dorsolateral prefrontal cortex. *Nat Neurosci* **24**, 425-436, doi:10.1038/s41593-020-00787-0 (2021).

48 Velmeshev, D. *et al.* Single-cell genomics identifies cell type-specific molecular changes in autism. *Science* **364**, 685-689, doi:10.1126/science.aav8130 (2019).

49 Braak, H., Ludolph, A. C., Neumann, M., Ravits, J. & Del Tredici, K. Pathological TDP-43 changes in Betz cells differ from those in bulbar and spinal alpha-motoneurons in sporadic amyotrophic lateral sclerosis. *Acta Neuropathol* **133**, 79-90, doi:10.1007/s00401-016-1633-2 (2017).

50 Porta, S. *et al.* Patient-derived frontotemporal lobar degeneration brain extracts induce formation and spreading of TDP-43 pathology in vivo. *Nat Commun* **9**, 4220, doi:10.1038/s41467-018-06548-9 (2018).

51 van Rheenen, W. *et al.* Common and rare variant association analyses in amyotrophic lateral sclerosis identify 15 risk loci with distinct genetic architectures and neuron-specific biology. *Nat Genet* **53**, 1636-1648, doi:10.1038/s41588-021-00973-1 (2021).

52 Saez-Atienzar, S. *et al.* Genetic analysis of amyotrophic lateral sclerosis identifies contributing pathways and cell types. *Sci Adv* **7**, doi:10.1126/sciadv.abd9036 (2021).

53 Baxi, E. G. *et al.* Answer ALS, a large-scale resource for sporadic and familial ALS combining clinical and multi-omics data from induced pluripotent cell lines. *Nat Neurosci* **25**, 226-237, doi:10.1038/s41593-021-01006-0 (2022).

54 Giacomelli, E. *et al.* Human stem cell models of neurodegeneration: From basic science of amyotrophic lateral sclerosis to clinical translation. *Cell Stem Cell* **29**, 11-35, doi:10.1016/j.stem.2021.12.008 (2022).

55 Limone, F., Klim, J. R. & Mordes, D. A. Pluripotent stem cell strategies for rebuilding the human brain. *Front Aging Neurosci* **14**, 1017299, doi:10.3389/fnagi.2022.1017299 (2022).

56 Yu, H. *et al.* HSP70 chaperones RNA-free TDP-43 into anisotropic intranuclear liquid spherical shells. *Science* **371**, doi:10.1126/science.abb4309 (2021).

57 Gwon, Y. *et al.* Ubiquitination of G3BP1 mediates stress granule disassembly in a context-specific manner. *Science* **372**, eabf6548, doi:10.1126/science.abf6548 (2021).

58 Klim, J. R. *et al.* ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair. *Nat Neurosci* **22**, 167-179, doi:10.1038/s41593-018-0300-4 (2019).

59 Limone, F. *et al.* Efficient generation of lower induced motor neurons by coupling Ngn2 expression with developmental cues. *Cell Rep*, 111896, doi:10.1016/j.celrep.2022.111896 (2022).

60 Tomassy, G. S. *et al.* Distinct profiles of myelin distribution along single axons of pyramidal neurons in the neocortex. *Science* **344**, 319-324, doi:10.1126/science.1249766 (2014).

61 Giera, S. *et al.* The adhesion G protein-coupled receptor GPR56 is a cell-autonomous regulator of oligodendrocyte development. *Nat Commun* **6**, 6121, doi:10.1038/ncomms7121 (2015).

62 Yang, H. J., Vainshtein, A., Maik-Rachline, G. & Peles, E. G protein-coupled receptor 37 is a negative regulator of oligodendrocyte differentiation and myelination. *Nat Commun* **7**, 10884, doi:10.1038/ncomms10884 (2016).

63 Keren-Shaul, H. *et al.* A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* **169**, 1276-1290 e1217, doi:10.1016/j.cell.2017.05.018 (2017).

64 de Boer, A. S. *et al.* Genetic validation of a therapeutic target in a mouse model of ALS. *Sci Transl Med* **6**, 248ra104, doi:10.1126/scitranslmed.3009351 (2014).

65 Zhang, Y. *et al.* The C9orf72-interacting protein Smcr8 is a negative regulator of autoimmunity and lysosomal exocytosis. *Genes Dev* **32**, 929-943, doi:10.1101/gad.313932.118 (2018).

66 Burberry, A. *et al.* C9orf72 suppresses systemic and neural inflammation induced by gut bacteria. *Nature* **582**, 89-94, doi:10.1038/s41586-020-2288-7 (2020).
67 Subramanian, A. *et al.* A Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles. *Cell* **171**, 1437-1452 e1417, doi:10.1016/j.cell.2017.10.049 (2017).
68 Abud, E. M. *et al.* iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron* **94**, 278-293 e279, doi:10.1016/j.neuron.2017.03.042 (2017).
69 Masuda, T. *et al.* Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature* **566**, 388-392, doi:10.1038/s41586-019-0924-x (2019).
70 Blum, J. A. & Gitler, A. D. Singling out motor neurons in the age of single-cell transcriptomics. *Trends Genet*, doi:10.1016/j.tig.2022.03.016 (2022).
71 Farhan, S. M. K. *et al.* Exome sequencing in amyotrophic lateral sclerosis implicates a novel gene, DNAJC7, encoding a heat-shock protein. *Nat Neurosci* **22**, 1966-1974, doi:10.1038/s41593-019-0530-0 (2019).
72 Melamed, Z. *et al.* Premature polyadenylation-mediated loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration. *Nat Neurosci* **22**, 180-190, doi:10.1038/s41593-018-0293-z (2019).
73 Otero-Garcia, M. *et al.* Molecular signatures underlying neurofibrillary tangle susceptibility in Alzheimer's disease. *Neuron* **110**, 2929-2948 e2928, doi:10.1016/j.neuron.2022.06.021 (2022).
74 Pineda, S. S. *et al.* Single-cell profiling of the human primary motor cortex in ALS and FTLD. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2021.07.07.451374 (2021).
75 Yadav, A. *et al.* A Cellular Taxonomy of the Adult Human Spinal Cord. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2022.03.25.485808 (2022).
76 Floriddia, E. M. *et al.* Distinct oligodendrocyte populations have spatial preference and different responses to spinal cord injury. *Nat Commun* **11**, 5860, doi:10.1038/s41467-020-19453-x (2020).
77 Falcao, A. M. *et al.* Disease-specific oligodendrocyte lineage cells arise in multiple sclerosis. *Nat Med* **24**, 1837-1844, doi:10.1038/s41591-018-0236-y (2018).
78 Hughes, A. N. & Appel, B. Oligodendrocytes express synaptic proteins that modulate myelin sheath formation. *Nat Commun* **10**, 4125, doi:10.1038/s41467-019-12059-y (2019).
79 Genc, B. *et al.* Apical dendrite degeneration, a novel cellular pathology for Betz cells in ALS. *Sci Rep* **7**, 41765, doi:10.1038/srep41765 (2017).
80 Wainger, B. J. *et al.* Effect of Ezogabine on Cortical and Spinal Motor Neuron Excitability in Amyotrophic Lateral Sclerosis: A Randomized Clinical Trial. *JAMA Neurol* **78**, 186-196, doi:10.1001/jamaneurol.2020.4300 (2021).
81 O'Rourke, J. G. *et al.* C9orf72 is required for proper macrophage and microglial function in mice. *Science* **351**, 1324-1329, doi:10.1126/science.aaf1064 (2016).
82 McCauley, M. E. *et al.* C9orf72 in myeloid cells suppresses STING-induced inflammation. *Nature* **585**, 96-101, doi:10.1038/s41586-020-2625-x (2020).
83 Ennerfelt, H. *et al.* SYK coordinates neuroprotective microglial responses in neurodegenerative disease. *Cell* **185**, 4135-4152 e4122, doi:10.1016/j.cell.2022.09.030 (2022).
84 Pandya, V. A. & Patani, R. Decoding the relationship between ageing and amyotrophic lateral sclerosis: a cellular perspective. *Brain* **143**, 1057-1072, doi:10.1093/brain/awz360 (2020).
85 Marsh, S. E. *et al.* Dissection of artifactual and confounding glial signatures by single-cell sequencing of mouse and human brain. *Nat Neurosci* **25**, 306-316, doi:10.1038/s41593-022-01022-8 (2022).

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 -80ºC.

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 described[86]. 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.15[28]. 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 algorithm[91] 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 pipeline[92] 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 SynGO[93] (12th of June 2020). Interactome map was built using STRING[94] 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 MS[35].

Generation of Microglia-like Cells. Microglial-like cells were differentiated as described[68] 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), ITSG-X 2% v/v (ThermoFisher), L-ascorbic acid 2-Phosphate (64 ug/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 μM) and LiCl (2mM) and transferred in hypoxic incubator (20% O_2, 5% CO_2, 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% O_2, 5% CO_2, 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 2μM H_2O_2 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 sonicated[99]. 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).

References

86 Krienen, F. M. *et al.* Innovations present in the primate interneuron repertoire. *Nature* **586**, 262-269, doi:10.1038/s41586-020-2781-z (2020).

87 Saunders, A. *et al.* Molecular Diversity and Specializations among the Cells of the Adult Mouse Brain. *Cell* **174**, 1015-1030 e1016, doi:10.1016/j.cell.2018.07.028 (2018).

88 Mitchell, J. M. *et al.* Mapping genetic effects on cellular phenotypes with “cell villages”. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2020.06.29.174383 (2020).

89 Limone, F. *et al.* Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neurons to degeneration in ALS. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2021.07.12.452054 (2021).

90 Rapino, F. *et al.* Small-molecule screen reveals pathways that regulate C4 secretion in stem cell-derived astrocytes. *Stem Cell Reports* **18**, 237-253, doi:10.1016/j.stemcr.2022.11.018 (2023).

91 Finak, G. *et al.* MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. *Genome Biol* **16**, 278, doi:10.1186/s13059-015-0844-5 (2015).

92 Raudvere, U. *et al.* g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res* **47**, W191-W198, doi:10.1093/nar/gkz369 (2019).

93 Koopmans, F. *et al.* SynGO: An Evidence-Based, Expert-Curated Knowledge Base for the Synapse. *Neuron* **103**, 217-234 e214, doi:10.1016/j.neuron.2019.05.002 (2019).

94 Szklarczyk, D. *et al.* STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res* **47**, D607-D613, doi:10.1093/nar/gky1131 (2019).

95 Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550, doi:10.1073/pnas.0506580102 (2005).

96 Nehme, R. *et al.* Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission. *Cell Rep* **23**, 2509-2523, doi:10.1016/j.celrep.2018.04.066 (2018).

97 Pietilainen, O. *et al.* Astrocytic cell adhesion genes linked to schizophrenia correlate with synaptic programs in neurons. *Cell Rep* **42**, 111988, doi:10.1016/j.celrep.2022.111988 (2023).

98 Fukuda, A. *et al.* De novo DNA methyltransferases DNMT3A and DNMT3B are essential for XIST silencing for erosion of dosage compensation in pluripotent stem cells. *Stem Cell Reports* **16**, 2138-2148, doi:10.1016/j.stemcr.2021.07.015 (2021).

99 Hao, J. *et al.* Loss of TBK1 activity leads to TDP-43 proteinopathy through lysosomal dysfunction in human motor neurons. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2021.10.11.464011 (2021).

Acknowledgements

We thank the study participants and staff at Massachusetts Alzheimer’s Disease Research Center for sequencing (NIA P50 AG005134). We thank the study participants and Kathleen Wilsbach, Lyle Ostrow and staff at Target ALS Neuropathology core and associated institutions for validation cohort samples. We thank UCB Pharma for partially funding these studies. D.M. acknowledges support from Massachusetts ADRC (5P50AG005134) pilot project and the NINDS (K08NS104270). We would also like to thank Dr Paul Tesar and his group for invaluable discussions on oligodendroglial biology. Some figures created with BioRender.com.

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

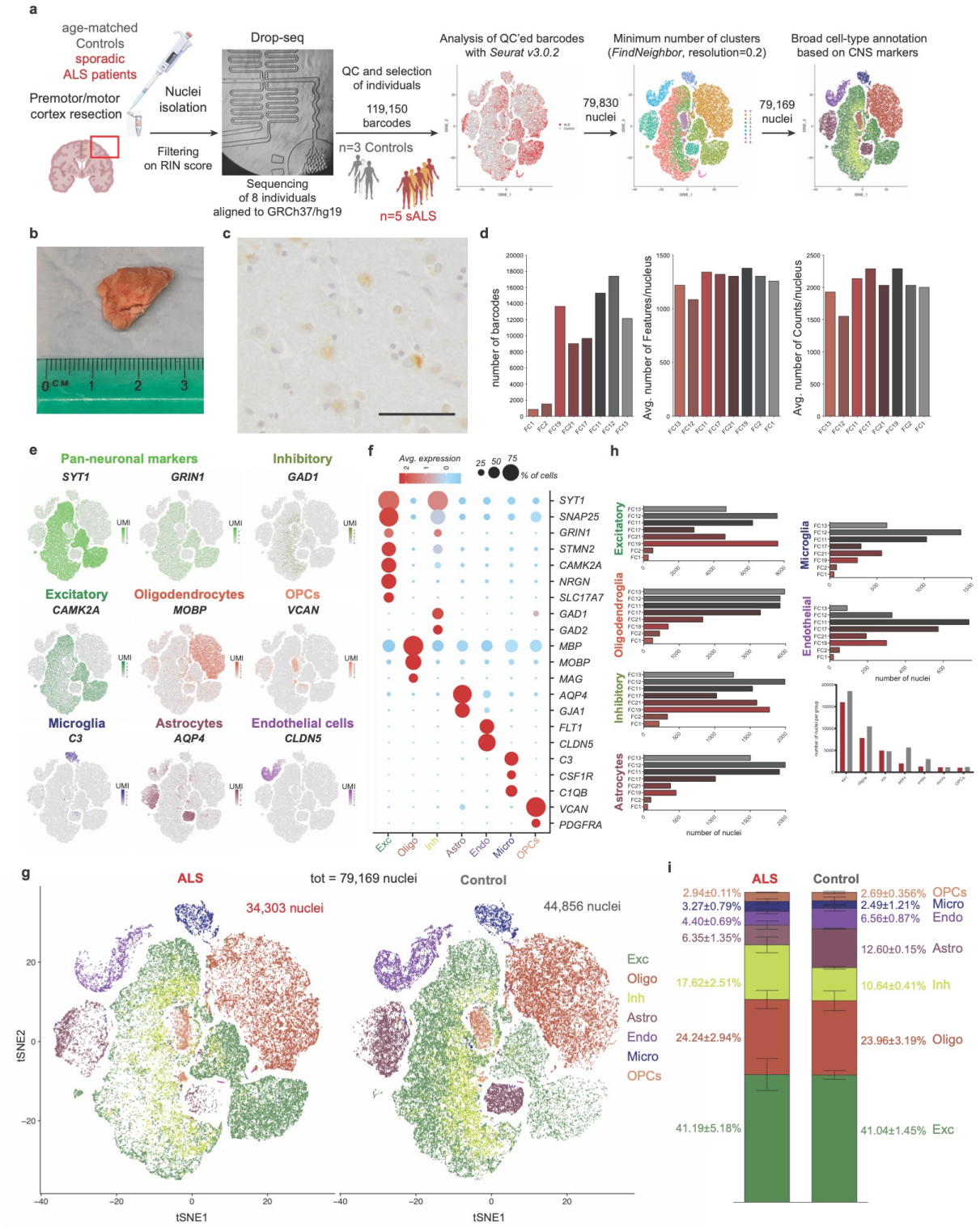
protein and RNA validation with cellular models; J.M.M. performed analysis of published datasets; M.T. and B.S. contributed to microglial biology section; K.E. acquired primary funding.

Competing interests

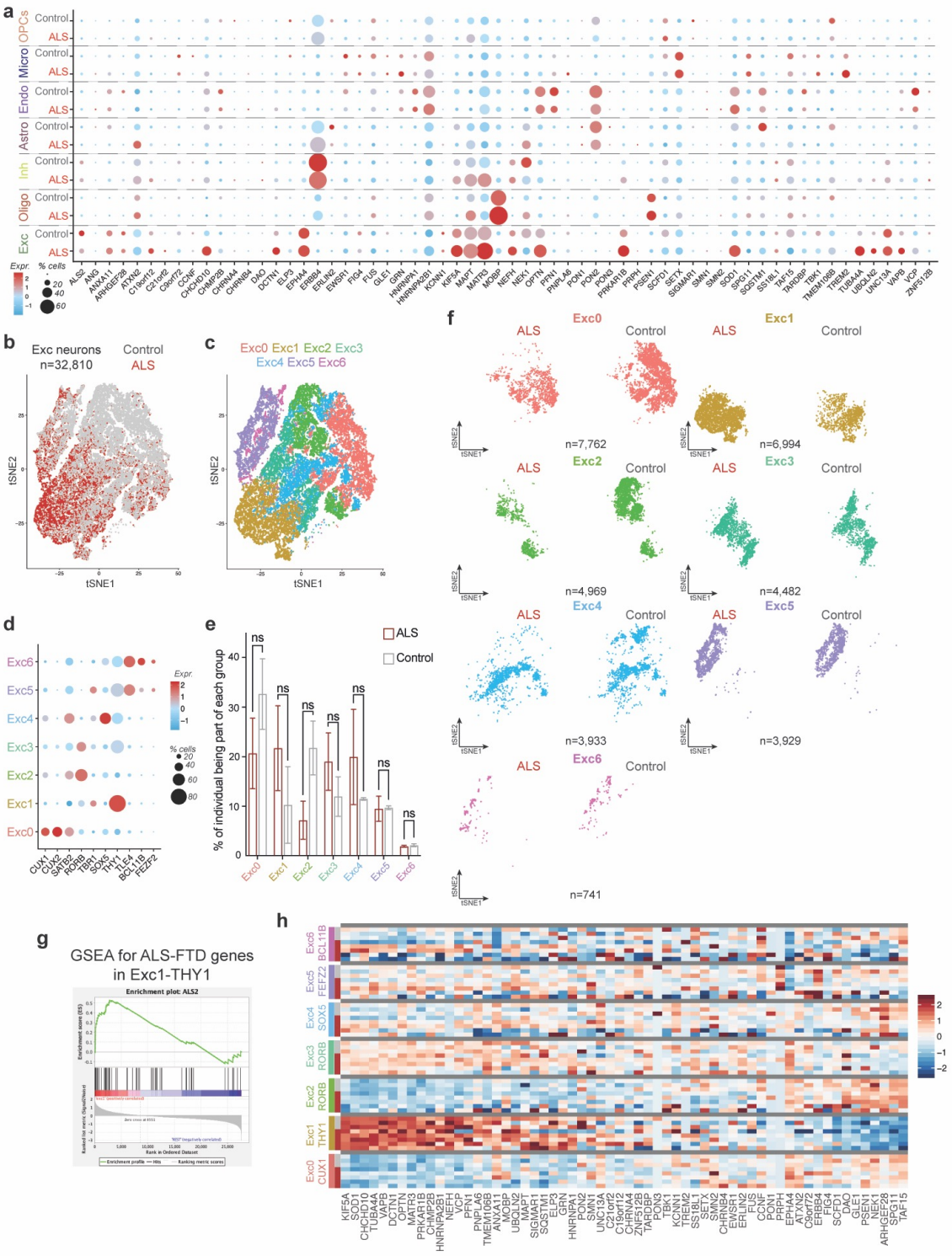
I.K. is an employee at UCB Pharma and holds stock options. K.E. is a cofounder of Q-State Biosciences, Quralis, Enclear Therapies and is group vice-president at BioMarin Pharmaceutical. K.E. and F.L. are authors on a pending patent “Single-nuclei characterization of amyotrophic lateral sclerosis frontal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

Additional information

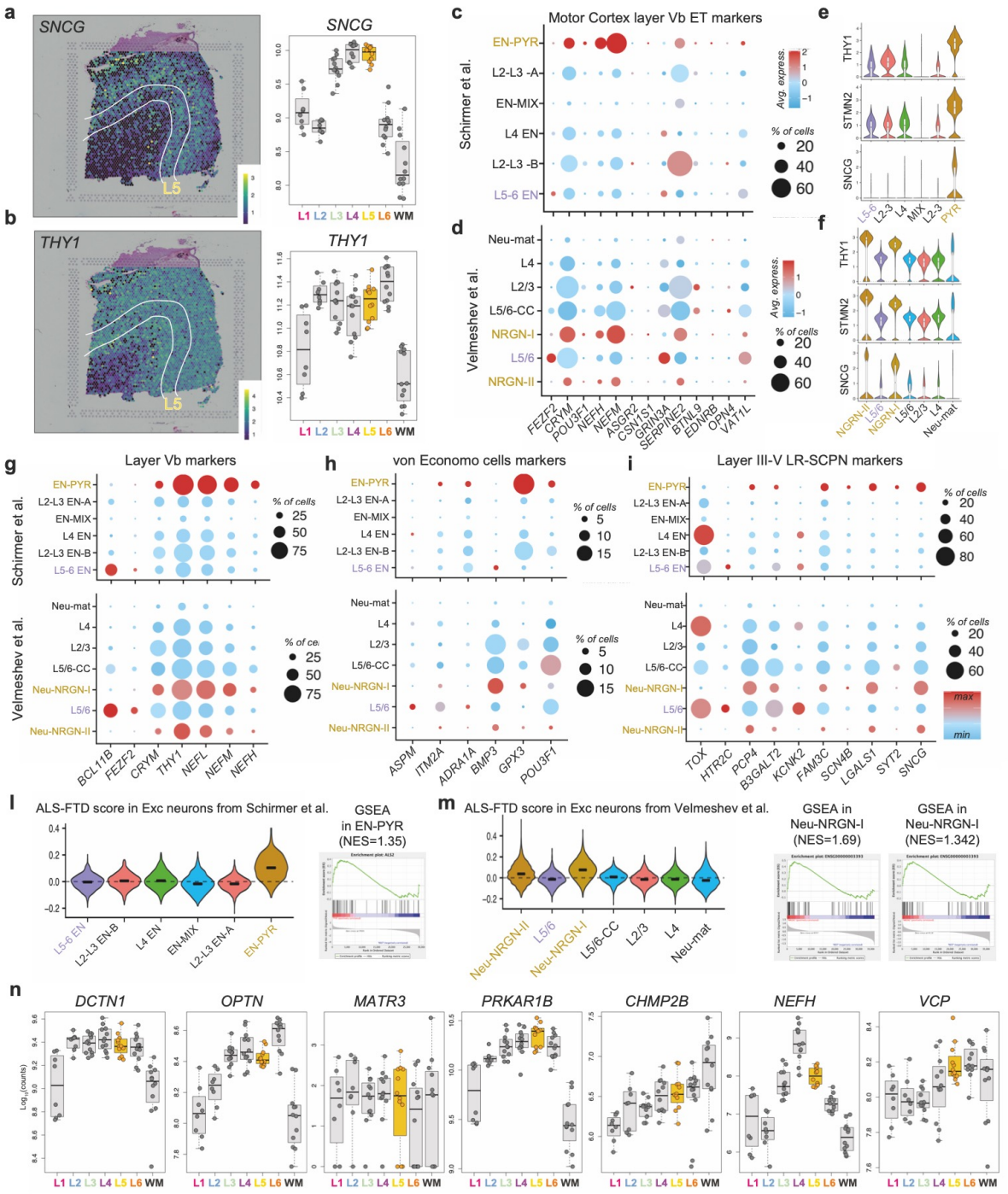
We worked to ensure diversity in experimental samples through the selection of genomic datasets. One or more of the authors self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.



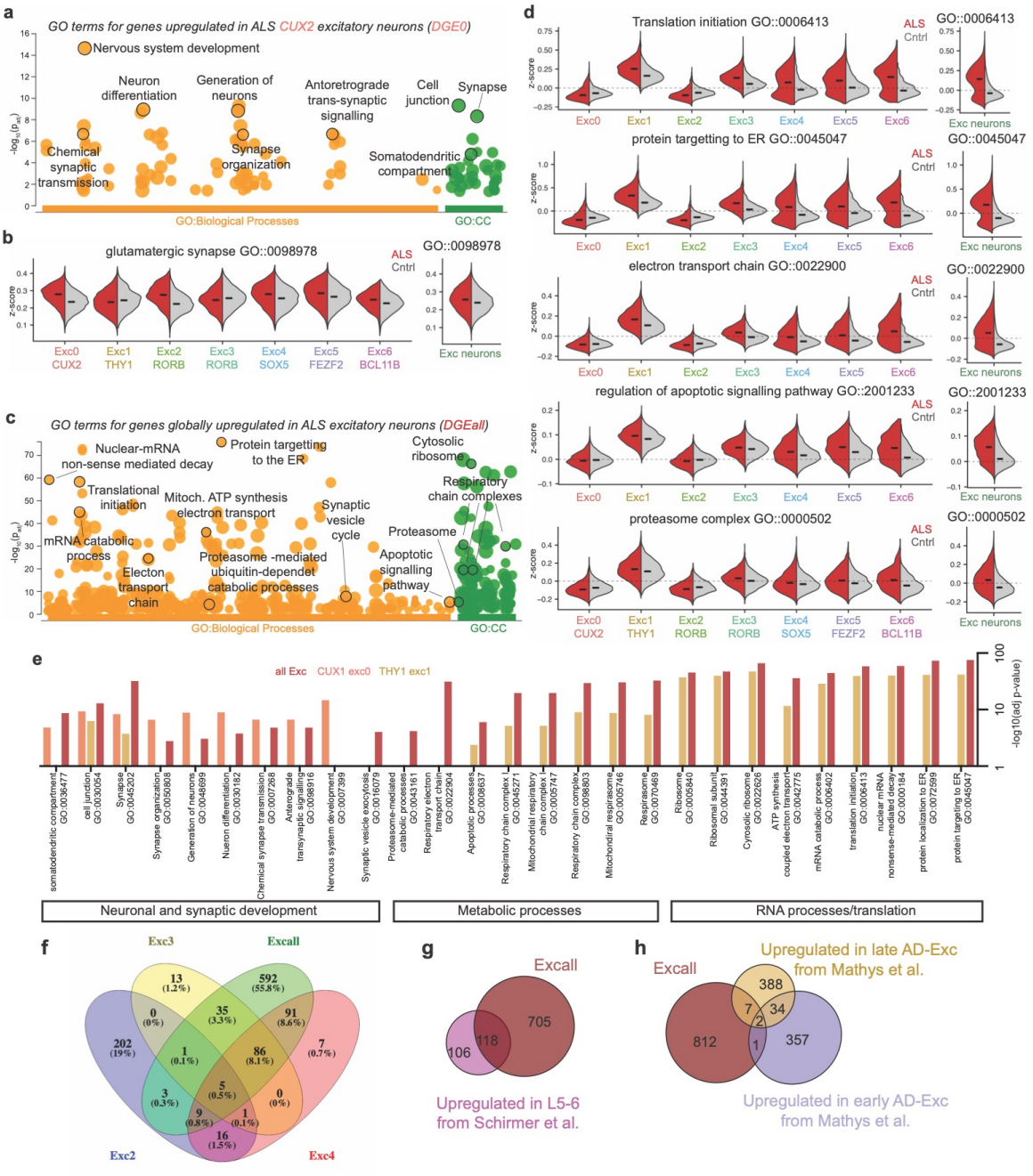
Extended Data Fig. 1 | Technical parameters of snRNAseq and cell-type distribution across individuals. a. Schematic diagram of workflow for isolation of nuclei from cortices of ALS patients and age-matched controls followed by single-cell RNA sequencing by DropSeq, library generation and Quality Controls 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 25!m). **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. **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.



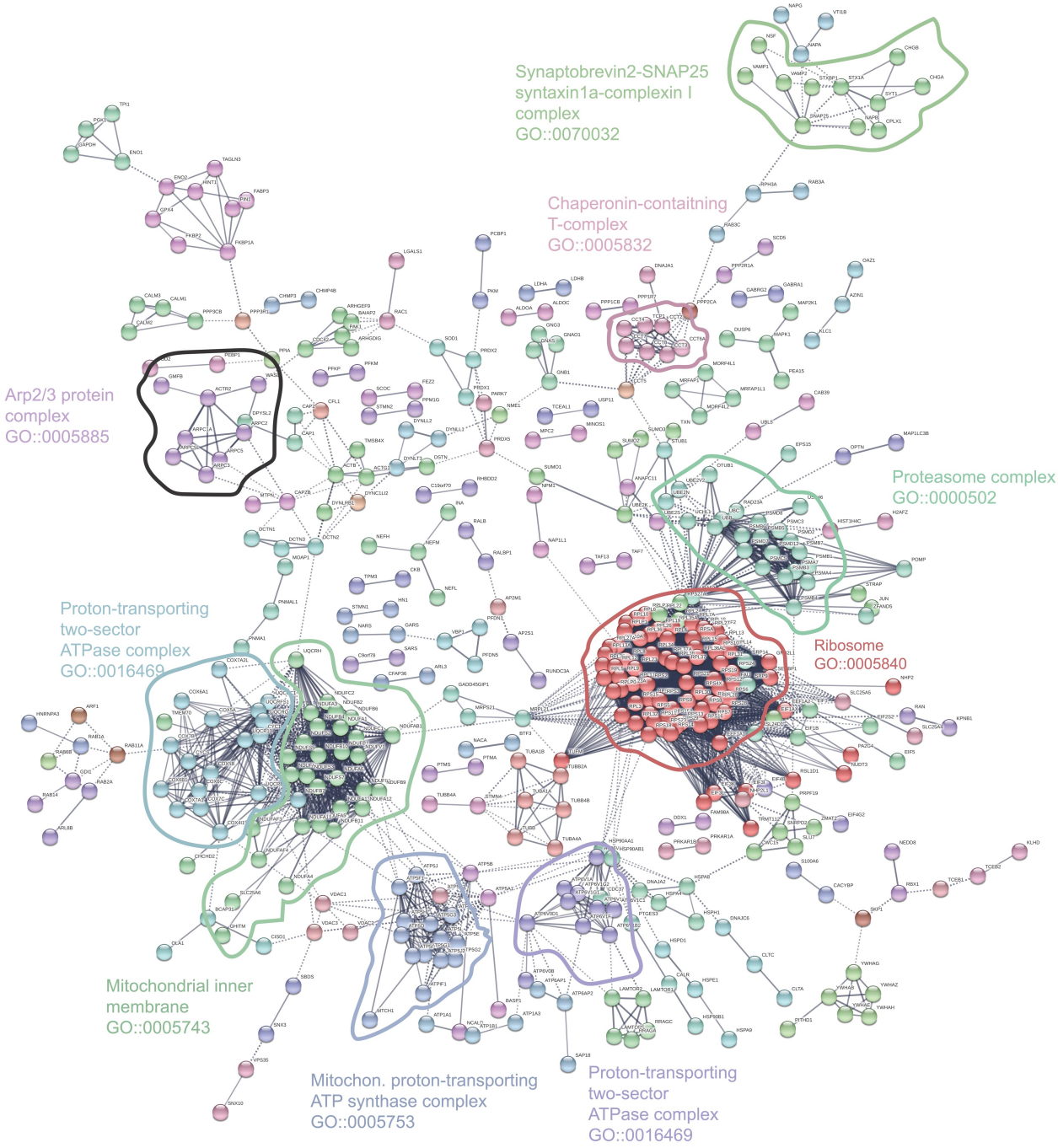
Extended Data Fig. 2 | Expression of ALS-FTD associated genes in different cellular subtypes and excitatory neurons subtypes. a. Dotplot representing expression of gene associated with the ALS-FTD spectrum in each cell type identified in the whole cortex split by diagnosis. **b.** *t*-SNE projection 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.



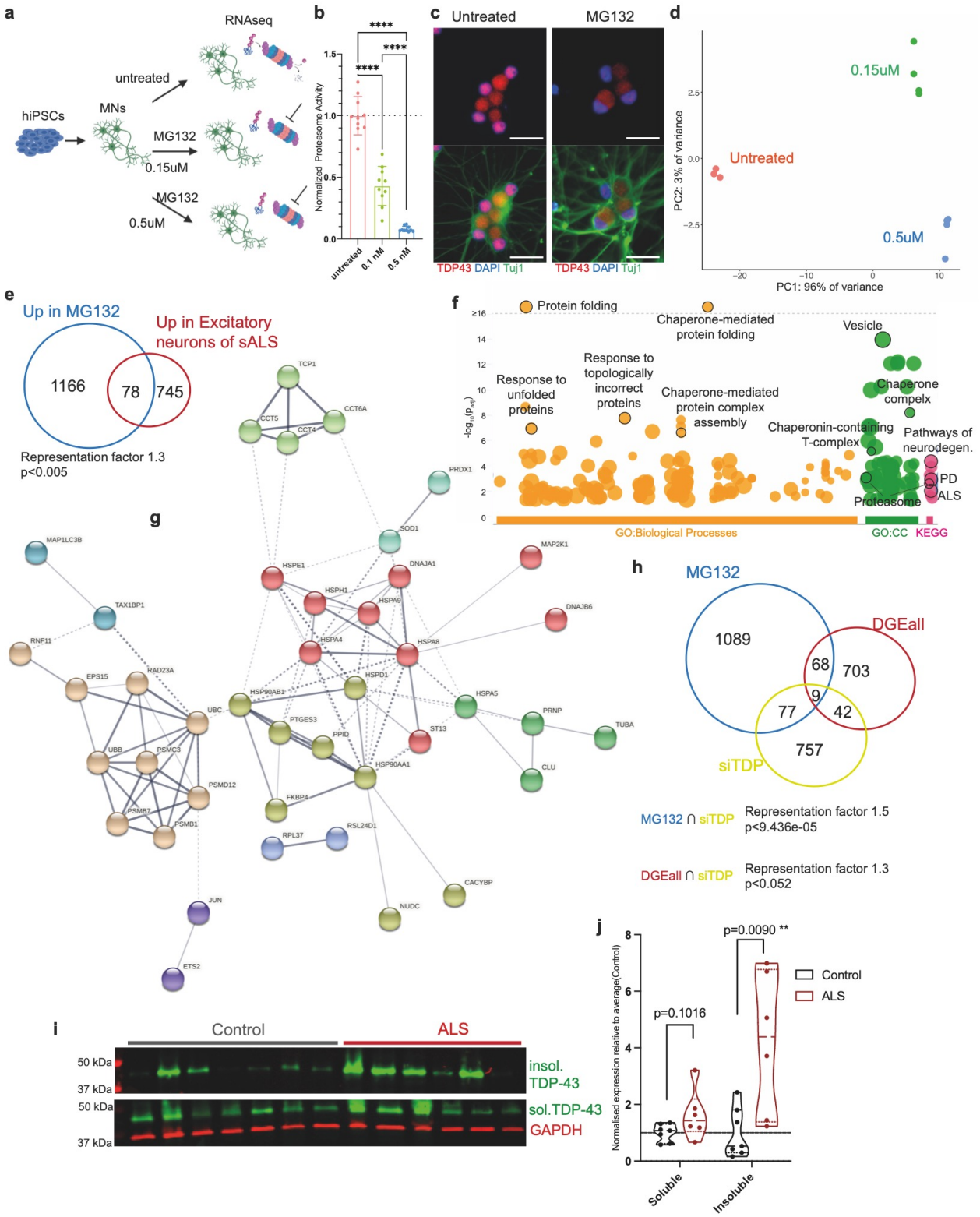
Extended Data Fig. 3 | L5-ETNs/CSMNs-like neurons express higher levels of ALS-FTD related genes. a,b, Spotplot and corresponding boxplot from Maynard et al. for the expression of layer Vb Motor Cortex marker, *SNCG* and *THY1*, identified as enriched in Exc1. **c,d.** Dotplot and representative Violin 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. **l.** Violin plots and corresponding Gene Set Enrichment Analysis of z-scores for expression of ALS-FTD-associated genes in THY1-neurons identified by Schimer 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.



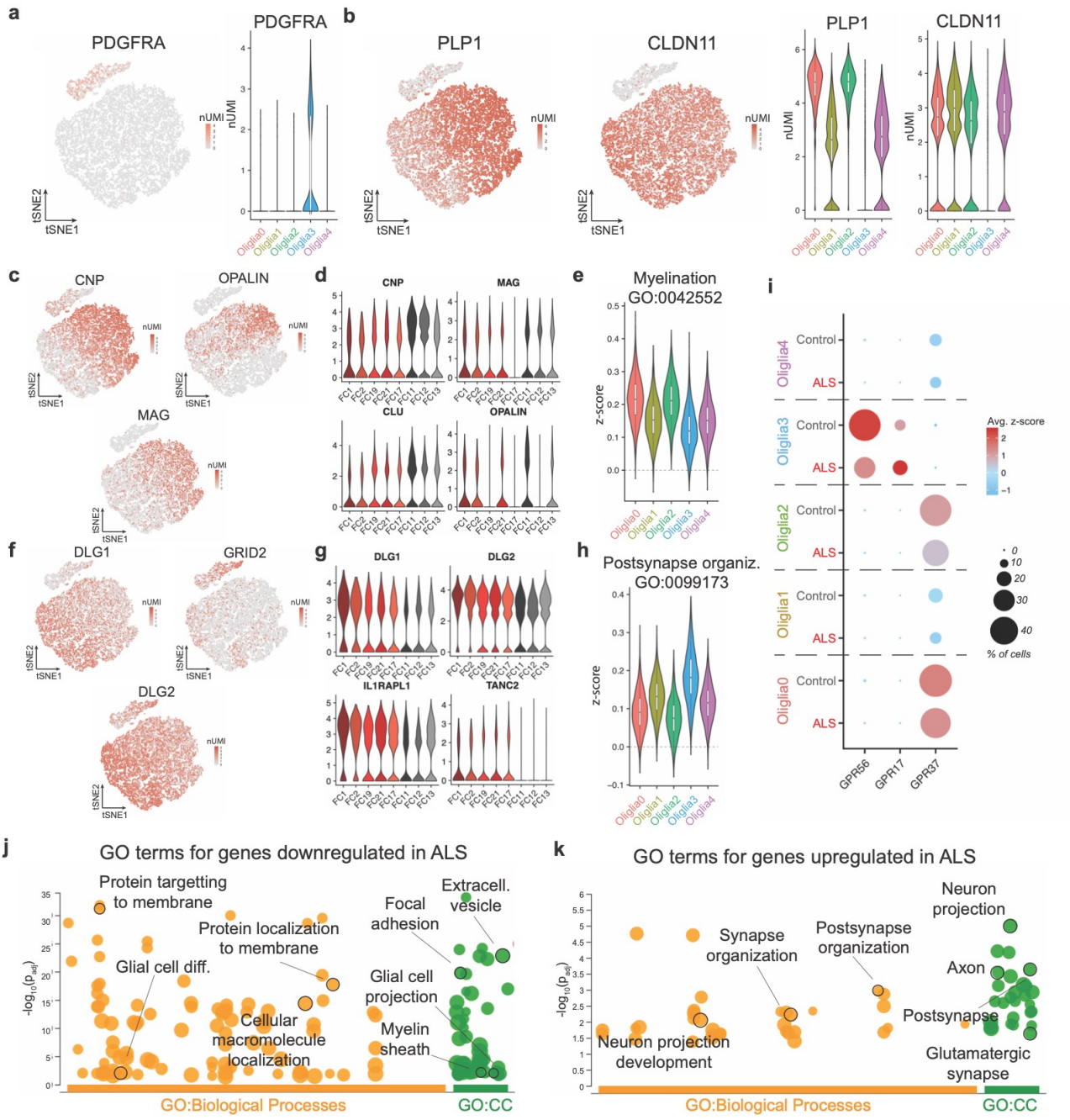
Extended Data Fig. 4 | Classes of L5-ETNs express higher levels of stress pathways. a. Gene Ontology analysis for genes globally upregulated in ALS excitatory neurons (DGEall). **b.** Violin plots representing z-scores for selected, statistically significant GO terms upregulated in lower layers in each 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 -log10(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.



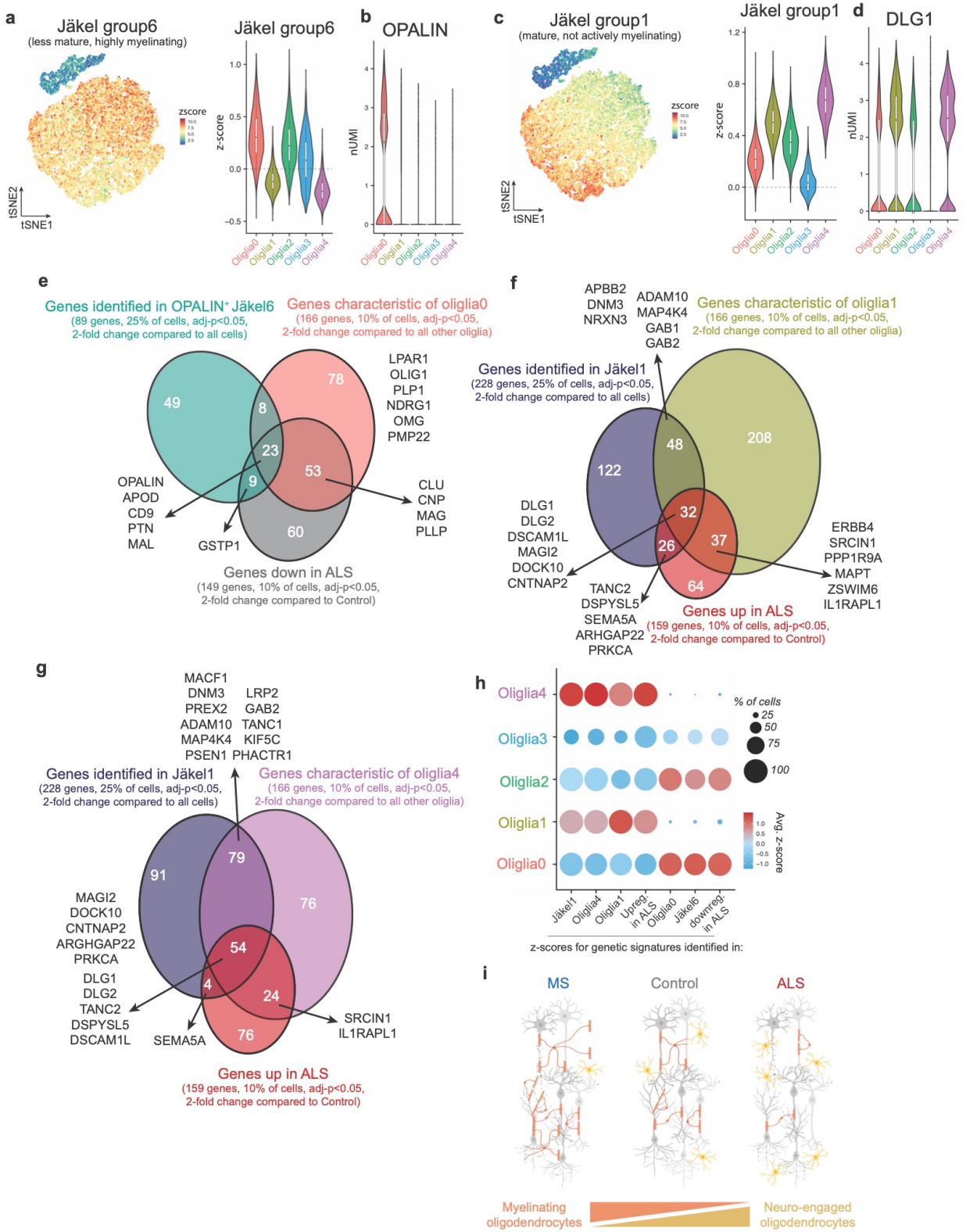
Extended Data Fig. 5 | Global protein-protein interaction network for genes upregulated in ALS excitatory neurons. Color-coding derived from MLC clustering (4) to identified closely related groups of proteins.



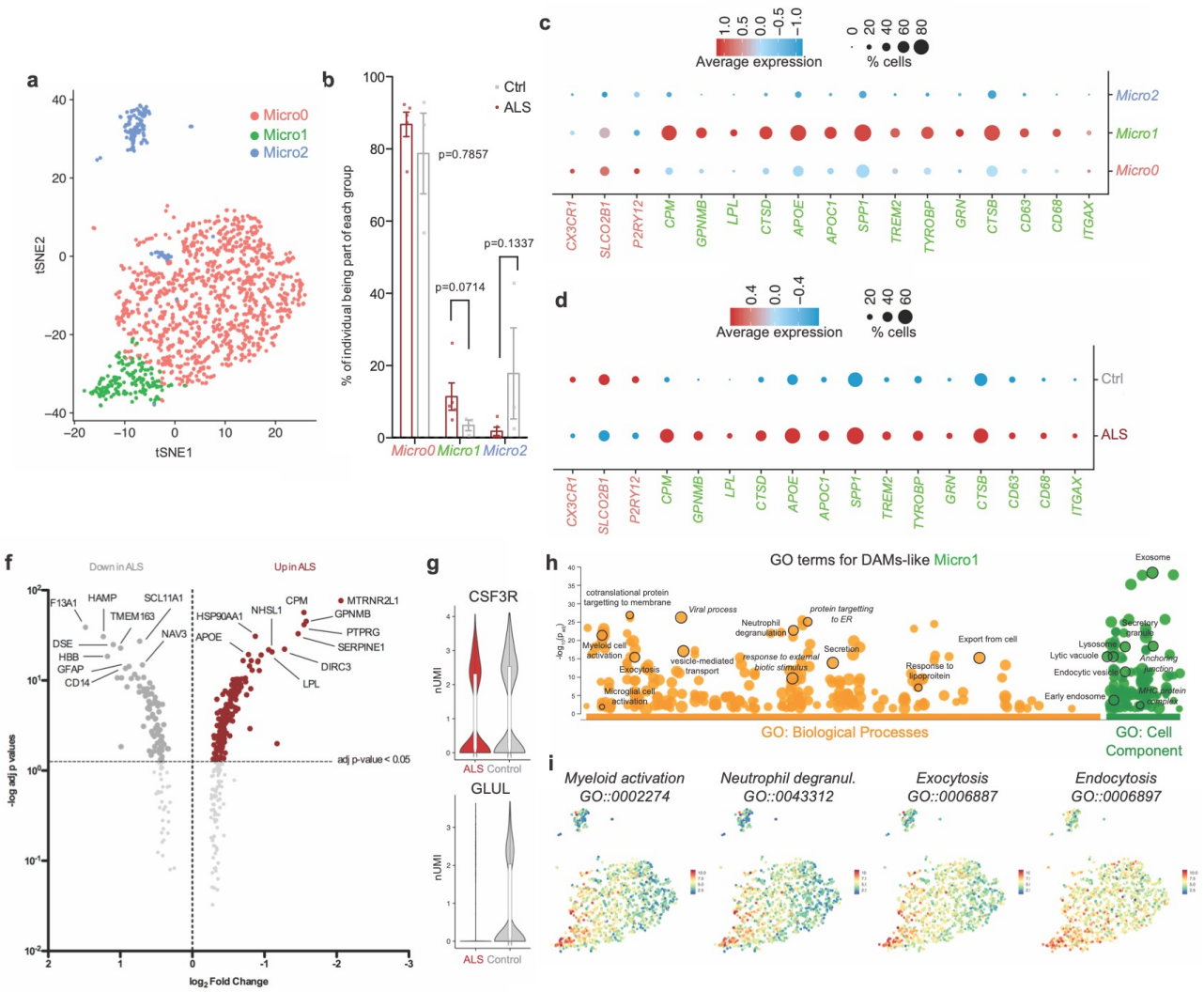
Extended Data Fig. 6 | Proteostatic stress in hPSC-derived neurons resembles changes in excitatory neurons from brain of ALS patients. a. Diagram of neuronal differentiation from Pluripotent Stem Cells and treatment with proteasome inhibitors for bulk RNA-sequencing. **b.** Quantification of proteasome 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.



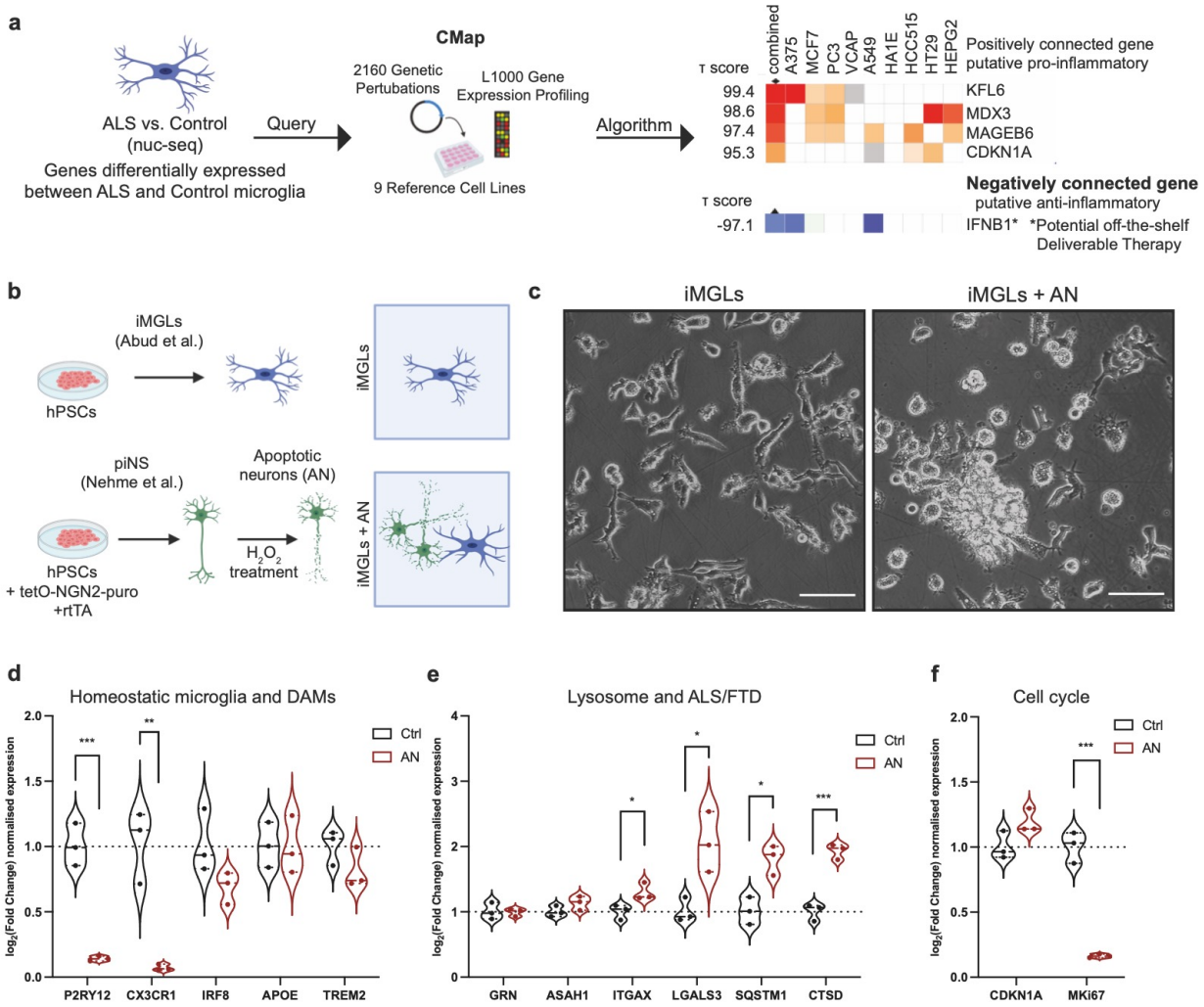
Extended Data Fig. 7 | Oligodendrocytes polarize between myelinating and neuro-engaged states. a. *t*-SNE projection and Violin plot of markers of Oligodendrocyte Progenitor Cells (OPCs). **b.** *t*-SNE projection and Violin plots of markers of mature oligodendrocytes. **c-d.** *t*-SNE projection and 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 (oliglia0) 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 (oliglia1 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 oligodendrocytes 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 selectedALS-FTD-associated and lysosomal genes 24h after feeding iMGLs with apoptotic neurons. **e.** RT-qPCR quantification of homeostatic and DAMs genes after feeding. **e.** RT-qPCR quantification of cell cycle-associated genes after feeding.

Chapter 3:

Pluripotent Stem Cell Strategies for Rebuilding the Human Brain

In this chapter we describe protocols to differentiate hPSCs into different brain cell types that could be used to further dissect the multicellular contribution to ALS.

This work is published in *Frontiers in Aging Neuroscience,* 2022
10.3389/fnagi.2022.1017299

Pluripotent Stem Cell Strategies for Rebuilding the Human Brain

Francesco Limone[1,2,3], Joseph R. Klim[4,], Daniel A. Mordes[5,*]*

[1]Department of Stem Cell and Regenerative Biology, Department of Molecular and Cellular Biology, and Harvard Stem Cell Institute, Cambridge, Massachusetts 02138, USA. [2]Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, USA. [3]Leiden University Medical Center, LUMC, 2333 ZA Leiden, The Netherlands. [4]Faze Medicines, Cambridge, MA 02142, USA [5]Institute for Neurodegenerative Diseases, Department of Pathology, University of California, San Francisco, San Francisco, CA 94158, USA *Correspondence: Daniel.Mordes@ucsf.edu (D.A. Mordes) or jklim@fazemed.com (J.R. Klim)

Keywords: neurodegeneration, aging, cell replacement therapies, regenerative medicine, pluripotent stem cells, developmental neuroscience, brain regeneration, neurological diseases

Neurodegenerative disorders have been extremely challenging to treat with traditional drug-based approaches and curative therapies are lacking. Given continued progress in stem cell technologies, cell replacement strategies have emerged as concrete and potentially viable therapeutic options. In this review, we cover advances in methods used to differentiate human pluripotent stem cells into several highly specialized types of neurons, including cholinergic, dopaminergic, and motor neurons, and the potential clinical applications of stem cell-derived neurons for common neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, ataxia, and amyotrophic lateral sclerosis. Additionally, we summarize cellular differentiation techniques for generating glial cell populations, including oligodendrocytes and microglia, and their conceivable translational roles in supporting neural function. Clinical trials of specific cell replacement therapies in the nervous system are already underway, and several attractive avenues in regenerative medicine warrant further investigation.

INTRODUCTION

Age – it's the one mountain you can't overcome, and as the average life expectancy extends into the eighth decade, neurodegenerative diseases are becoming increasingly prevalent. Despite their increasing incidence, preventative or disease-modifying strategies for these emotionally and financially draining disorders are lacking. Due to the fundamental lack of regeneration within the central nervous system (CNS), neurodegenerative diseases relentlessly attacking discrete populations of neurons are excellent candidates for cell replacement therapies. Here, we review the current prospects on the application of pluripotent stem cell-derived cell types for the treatment of neurodegenerative disease.

Pluripotent stem cells provide a uniquely scalable source of functional somatic cells, including cells of the CNS, that can potentially replace damaged or diseased tissues. Although prospects for using stem cell derivatives seemed fanciful at the start of the millennium, approximately two decades later several clinical trials using cellular products of pluripotent

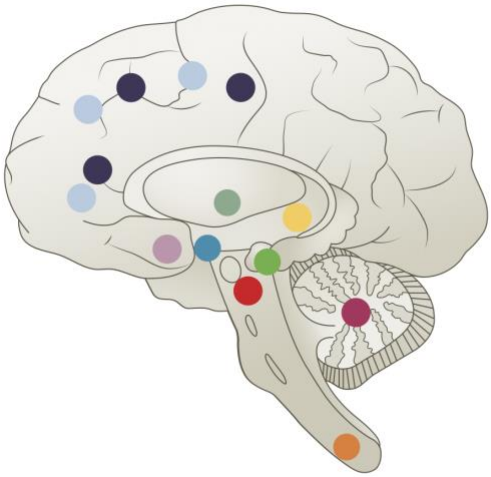
stem cells are underway or about to reach the clinic (Gage and Temple, 2013; Kimbrel and Lanza, 2015; Steinbeck and Studer, 2015; Tao and Zhang, 2016; Trounson and DeWitt, 2016). This progress has been facilitated through the development of robust methods for converting human pluripotent stem cells into the specific cell types that are lost in disease. Most techniques are based on fundamental principles learned from developmental biology and aim to recapitulate cell fate determination pathways in the culture dish, and these methods have been thoroughly reviewed elsewhere (Tao and Zhang, 2016). More recently, exogenous over-expression of transcription factors (TFs) has provided an alternative route to directed differentiation methodologies for generating specific classes of neurons. When appropriate, we will highlight both approaches that advance the field towards producing defined cellular populations, which are the ideal candidate for cell replacement therapies.

In this review, we summarize recent progress toward generating specific cell types from human pluripotent stem cells for regenerative medicine. The examples described herein are not intended to be all-inclusive, and readers are encouraged to examine other reviews on the clinical development of stem cell-based therapies (Gage and Temple, 2013; Kimbrel and Lanza, 2015; Steinbeck and Studer, 2015; Tao and Zhang, 2016; Trounson and DeWitt, 2016). Rather, we focus on recent biotechnological advances in the derivation of human cells and their application as cell therapies in the field of neurodegeneration (**Table 1**). These selected studies illustrate the biological concepts, experimental approaches, and therapeutic possibilities of *in vitro* stem cell-derived cells of the neural and glial lineages. We conclude our review with a discussion of emerging technologies in the field, current limitations, and remaining challenges for regenerative medicine in translational neurosciences.

Table 1. *Common neurodegenerative diseases characterized by selective vulnerability.*

Disease	Prevalence	Main symptoms	Key brain regions affected	Main vulnerable neuronal subtypes	Pathological hallmarks (associated protein)	Therapies (symptomatic treatments)	Regenerative medicine cell-based approaches
Alzheimer Dementia (AD)	~5M	Cognitive impairments in memory, language & behaviour	Hippocampus, Basal Forebrain, Locus coeruleus (pons), Cortex	Pyramidal neurons, Cholinergic neurons	Neurofibrillary tangles (tau); neuritic plaques (beta-amyloid & tau)	acetylcholinesterase inhibitors, memantine	Cholinergic neurons, GABAergic Inhibitory neurons
Parkinson Disease (PD) and Parkinson Disease with Dementia (PDD)	~1M	Tremor, stiffness, slow movements, autonomic dysfunction, sleep problems, cognitive decline	Substantia nigra (midbrain), locus coeruleus (pons), Cortex (especially the cingulate)	Dopaminergic neurons	Lewy bodies and Lewy neurites (alpha-synuclein)	Levodopa, COMT inhibitors, dopamine agonists, deep brain stimulation	Dopaminergic neurons
Huntington Disease (HD)	~30K	Uncontrolled movements (chorea), neuropsychiatric	Neostriatum, especially caudate (basal ganglia), cortex	Spiny neurons	Intranuclear & cytoplasmic neuronal inclusions (Htt)	Tetrabenazine, neuroleptics (off-label), antidepressants	Spiny neurons

Spinocerebellar Ataxias (SCAs)	~150K	Difficulty with walking and speech, lack of coordination	Cerebellum, brainstem, spinal cord (dorsal)	Purkinje neurons, pontine nuclei neurons	Intranuclear & cytoplasmic neuronal inclusions (various, e.g., ataxins)	Limited, physical therapy	Purkinje neurons
Amyotrophic Lateral sclerosis (ALS)	~20 K	Progressive weakness and muscle atrophy	Spinal cord (ventral), brainstem (motor nuclei), & frontal cortex	Upper and lower motor neurons	TDP-43 positive cytoplasmic neuronal inclusions	Riluzole, edaravone	Lower motor neurons



Neuronal Type	Basal Forebrain Cholinergic	Cortical Glutamatergic Pyramidal	GABAergic Inhibitory
Disease	AD	AD, FTD	AD, Epilepsy
Pathological Markers	β-amyloid TAU	β-amyloid TAU FUS TDP-43	β-amyloid TAU
Differentiation Method	1-4	5-9	111-15
Transplant Studies	2	5-7, 10	16-17

Neuronal Type	Dopaminergic	Medium Spiny	Hypothalamic	Hippocampal	Serotonergic	Purkinje	Motor
Disease	PD	HD	Narcolepsy	AD, Psychiatric diseases	Psychiatric diseases	Ataxias	ALS, SMA
Pathological Markers	α-synuclein Parkin	Huntingtin	Reduced hypothalamic grey matter	β-amyloid		poly-Q repeats	TDP-43 SOD1
Differentiation Methods	18-22	26-29	30-33	34-36	37-39	41-45	47-55
Transplant Studies	23-25	26-27, 29	30	34	40	46	55-56

Figure 1 Basal forebrain cholinergic: *diff.* 1-4 Bissonnette et al., 2011; Liu et al., 2013a; Hu et al., 2016; Liu et al., 2013b; *transpl.* 2 Liu et al., 2013a. **Cortical Glutamatergic:** *diff.* 5-9 Espuny-Camacho et al., 2013; Cao et al., 2017; Qi et al., 2017; Zhang et al., 2013; Nehme et al., 2018; *transpl.* 5, 6, 7, 10 Espuny-Camacho et al., 2013; Qi et al., 2017; Zhang et al., 2013; Espuny-Camacho et al., 2017. **GABAergic inhibitory**: *diff.* 11-15 Maroof et al., 2013; Nicholas et al., 2013; Chanda et al., 2014 ; Sun et al., 2016 ; Yuan et al., 2018; *transpl.* 16-17 Anderson et al., 2018; Cunningham et al., 2014. **Dopaminergic**: *diff.* 18-22 Kriks et al., 2011; Kim et al., 2021; Cai et al., 2009; Caiazzo et al., 2011; Pfisterer et al., 2011; *transpl.* 23-25 Kikuchi et al., 2017; Wakeman et al., 2017; Grealish et al., 2014. **Medium Spiny**: *diff.* 26-29 Aubry et al., 2008; Carri et al., 2012; Ma et al., 2022; Victor et al., 2014; *transpl.* 26, 27, 29 Aubry et al., 2008; Carri et al., 2012; Victor et al., 2014. **Hypothalamic**: *diff.* 30-33 Merkle et al., 2015; Kirwan et al., 2017 ; Wang et al., 2015 ; Rajamani et al., 2018; *transpl.* 30 Merkle et al. 2015. **Hippocampal**: *diff.* 34-36 Yu et al., 2014; Sakaguchi et al., 2015 ; Hiragi et al., 2017; *transpl.* 34 Yu et al., 2014. **Serotonergic**: *diff.* 37-39 Lu et al., 2016 ; Vadodaria et al., 2016 ; Xu et al., 2016; *transpl.* 40 Carlsson et al., 2009. **Purkinje**: *diff.* 41-45 Muguruma et al., 2015; Wang et al., 2015; Watson et al., 2018; Silva et al., 2020; Ishida et al., 2016; *transpl.* 46 Higuera et al., 2017. **Motor**: *diff.* 47-55 Amoroso et al., 2013; Du et al., 2015; Maury et al. 2015; Klim et al., 2019; Son et al., 2011; Hester et al., 2011; Goto et al., 2017; Limone et al., 2022; Lippman et al., 2015; *transpl.* 55-56 Yohn et al., 2008; Corti et al., 2012.

PARKINSON'S DISEASE

Parkinson's disease is characterized by the degeneration of several neuronal subtypes, most notably the dopaminergic neurons of the substantia nigra pars compacta (SNpc), located in the ventral midbrain. These neurons project to the dorsal striatum of the basal ganglia and function in motor control, and their loss of these neurons contributes to the movement symptoms observed in the initial stages of Parkinson's disease. Fetal-derived dopamine neurons have had promising clinical benefits for Parkinson's disease patients (Hallett et al., 2014). To avoid the ethical and logistical issues associated with fetal tissue transplants, the application of pluripotent stem cells to generate dopaminergic neurons has been a long-standing goal. Indeed, translational research to bring these specific neurons to the clinic has far exceeded the other cell replacement strategies discussed here and recent advances have extensively been discussed elsewhere (Barker et al., 2017; Kim et al., 2020). In this section we will provide a summary of the most relevant discoveries that led to the first transplantation studies with hiPSC-derived cells that established a road map for the field.

Dopaminergic neurons

From the initial basic science studies that furnished the directed differentiation strategies of dopaminergic neurons to their large-scale production in GMP-facilities for transplantation studies, the research program for midbrain dopamine neurons has made excellent progress. Several groups developed methods to produce FOXA2/LMX1A-positive midbrain neurons capable of releasing dopamine (Arenas et al., 2015). For example, the Studer group has developed a highly efficient protocol for producing these neurons by combining dual-SMAD inhibition with activation of SHH and FGF8 signaling. The critical step in midbrain specification is the strong activation of WNT signaling achieved using a GSK3β inhibitor (Kim et al., 2021; Kriks et al., 2011). Transcription factors, such as LMX1A, can also be used to enhance directed differentiation approaches (Cai et al., 2009), or for the direct reprogramming of fibroblasts into dopaminergic neurons (Caiazzo et al., 2011; Pfisterer et al., 2011), and combined with cell sorting methods to further enrich for midbrain dopaminergic neurons (Arenas et al., 2015). Preclinical studies demonstrate that human iPS cell-derived dopaminergic neurons are safe and efficacious in both rodent and primate Parkinson's disease model (Kikuchi et al., 2017; Wakeman et al., 2017) with similar efficacy to fetal-derived tissue (Grealish et al., 2014). A number of clinical trials with stem cell-based therapies are currently being planned with their details summarized at a recent consortium meeting (Barker et al., 2017). Although PD patients receiving the stem cell-derived dopaminergic neurons will likely show improvements in movement symptoms, their additional symptoms, including depression, fatigue, visual hallucinations, and sleep disturbances, might persist due to continued degeneration of other neuronal types. This has led to some to propose serotonergic neurons (Lu et al., 2016; Vadodaria et al., 2016; Xu et al., 2016) as an additive cellular therapy

for PD (Politis and Loane, 2011). A delicate balance must be struck between dopaminergic and serotonergic neurons, however, as fetal grafts with high levels of serotonergic neurons have been associated with graft-induced dyskinesias in parkinsonian rats (Carlsson et al., 2009).

DEMENTIA

Neurological conditions involving both memory loss and impaired judgement are classified as dementia (Yue and Jing, 2015). Alzheimer's disease is the most common type of dementia in individuals older than 65 years old and the most prevalent neurodegenerative disease (**Table 1**). The incidence of Alzheimer's disease (AD) dramatically increases with age, and with the aging US population, it is estimated that approximately 14 million individuals will be affected by 2050. AD often first manifests clinically as impairments with short-term memory, and later affects behavior and language. Current treatments are aimed at ameliorating these symptoms without substantially affecting disease course. Cognitive decline is associated with progressive degeneration of neurons in the limbic system (especially the hippocampus and connected entorhinal cortex), the basal forebrain, and neocortical areas. Histologically, patient brains are characterized by the accumulation of extracellular beta-amyloid depositions and intracellular tau-positive neurofibrillary tangles as well as neuritic plaques that contain both tau within dystrophic neurites and beta-amyloid. Neuropathological studies strongly suggest that AD has well-defined and consistent spatiotemporal pattern of neurofibrillary degeneration, in most cases, that begins in the entorhinal cortex and spreads to pyramidal neurons in the hippocampus and then neocortical areas, with association areas affected sooner and more severely. Currently, there is no effective therapy to block the progression of AD making it a major looming public heath challenge.

Basal Forebrain Cholinergic Neurons

One of the earliest cell types perturbed by AD is the basal forebrain cholinergic neuron (BFCN). These neurons, which arise from the median ganglionic eminence (MGE) during development, are responsible for various aspects of cognition including learning, memory, and attention. At the molecular level, BFCNs are primary cholinergic neurons and innervate the cerebral cortex, hippocampus, and amygdala, and play critical roles in processing information related to cognitive function (Martinez et al., 2021). Transplantation of fetal cholinergic tissue from rats into the cortex of lesioned primates has been shown to restore memory deficits suggesting a potentially therapeutic roles for these cells (Ridley et al., 1994).

Several methods to differentiate pluripotent stem cells into BFCNs have been described (Bissonnette et al., 2011; Hu et al., 2016; Liu et al., 2013a; Liu et al., 2013b). Typically, first forebrain neural progenitors are obtained and then treated with a SHH (sonic

hedgehog) agonist and FGF8 to coax the cells into expressing the transcription factors Nkx2.1, consistent with a ventral medial ganglionic eminence (MGE) neural progenitor identity. Subsequent culture of these progenitor cells on glia or treatment with BMP9 then yields a mixture of neurons containing BFCNs (Bissonnette et al., 2011; Hu et al., 2016; Liu et al., 2013a; Liu et al., 2013b). Alternatively, overexpression of the transcription factors Lhx8 and Gbx1 can convert the progenitors into BFCNs (Bissonnette et al., 2011). Cells produced using these methods express markers consistent with a cholinergic identity and exhibit expected electrophysiological profiles. In one study, MGE-progenitor cells transplanted into mouse brains differentiated into neurons, including BFCNs, and formed synaptic connections (Liu et al., 2013b). More importantly, injection of these precursor cells led to learning and memory improvements in lesioned mice (Liu et al., 2013b). Whether these improvements were the specific result of the BFCNs or other cell types remains to be determined but this study provides an important proof-of-principle for the use of stem cell-based therapy to improve cognition.

Cortical glutamatergic pyramidal neurons

Cerebral cortex development consists of three major processes: cell proliferation, neuronal migration, and cortical organization into multiple well-defined layers. The cerebral cortex contains two major classes of neurons; a majority population of excitatory glutamatergic projections neurons that arise during development from the dorsal telencephalon, which is the developmental precursor to the cerebral cortex, and a minor population of inhibitory interneurons. Through successive waves of neurogenesis, these neurons generate the six layers of the neocortex, which can be further functionally divided based on specific patterns of axonal output and dendritic input. Due to their abundance and ability to project long distances, cortical pyramidal neurons, named for their shape, are able to integrate and send information across the entire nervous system (Bekkers, 2011).

The production of pyramidal neurons from pluripotent stem cells is considered to be a default differentiation fate because it occurs in the absence of exogenous signaling factors (Espuny-Camacho et al., 2013). Inhibiting certain signaling pathways, however, can enhance the yield of cortical glutamatergic neurons by suppressing the emergence of inhibitory interneurons (Cao et al., 2017). More recently, accelerated methods for generating cortical neurons have been reported. One method relies on a cocktail of molecules to both pattern the cells to dorsal forebrain lineage and then inhibit neural stem cell self-renewal to drive neurogenesis, which preliminary data suggests can be timed to achieve the production of neurons of different cortical layers (Qi et al., 2017). Forced expression of the transcription factor Ngn2 in stem cells further accelerates the differentiation to yield very pure populations of glutamatergic neurons (Zhang et al., 2013) that can be enhanced with the addition of developmental cues (Nehme et al., 2018). Transcriptional studies suggest this method favors

the production of upper layer neurons, therefore additional methods to achieve the full diversity of cortical layers may still be necessary. After injecting into the postnatal mouse brain, human cortical neurons generated using the methodologies described above displayed proper, long-distance projection patterns and integrated functionally within the host's circuitry (Espuny-Camacho et al., 2013; Qi et al., 2017; Zhang et al., 2013). Whether they can ameliorate disease phenotypes in animal models remains an unanswered question, but neurons transplanted into a murine AD model display pathological hallmarks of the disease including altered tau biochemistry (Espuny-Camacho et al., 2017).

GABAergic inhibitory neurons

In both the brain and spinal cord, gamma-aminobutyric acid (GABA)-releasing interneurons are the major class of inhibitory neurons and play crucial roles in modulating neural circuits. There are many distinct subtypes of interneurons that differ in their synaptic connections, expression of neuropeptides, neurotransmitter machinery, and developmental origin with some immature interneurons having the remarkable ability to migrate and disperse long distances to integrate throughout the CNS (Southwell et al., 2014). This integrative property makes interneurons a promising candidate for cell replacement therapies.

Several groups have developed directed differentiation approaches for producing interneurons from human pluripotent stem cells (Liu et al., 2013a; Maroof et al., 2013; Nicholas et al., 2013). These approaches typically inhibit both branches of SMAD signaling as well as WNT signaling using small molecules to achieve robust forebrain induction into cells resembling the MGE, as suggested by expression of the transcription factor Nkx2.1. Careful timing of SHH activation then allows for induction of ventral cell fate in these progenitor cells that develop into GABAergic interneurons as opposed to basal forebrain cholinergic neurons (Liu et al., 2013a). In addition to directed differentiation approaches, transcription factor-mediated inductions of interneurons from stem cells have also been described (Chanda et al., 2014; Sun et al., 2016; Yuan et al., 2018). Minimally, transient expression of ASCL1 and DLX2 can convert stem cells into GABAergic interneurons. When injected into the mouse brain, these cells, migrated, integrated, and matured into a variety of interneuronal subtypes, including expression of the mature subtype markers parvalbumin or somatostatin. Further studies, such as single-cell transcriptomic approaches, are needed to characterize the full repertoire of subtypes of interneurons that can be obtained from pluripotent stem cells. Impressive studies have gone on to show that transplanted interneurons were capable of improving memory (Anderson et al., 2018) and in some cases suppressing seizures and abnormal behaviors in an epileptic mouse model (Cunningham et al., 2014). Based on these promising studies, one biotech company, Neurona Therapeutics, is pioneering the clinical uses for interneuron-based cell therapies for epilepsy and neuropathic pain.

Hippocampal neurons

Composed of granule and pyramidal neurons, the hippocampus plays a critical role in learning and memory. It is also an area of the brain that deteriorates in Alzheimer's disease, additional forms of dementia, and other age-related cognitive declines of distinct etiologies. Interestingly, in addition to the subventricular zone, the dentate gyrus of the hippocampus is a unique site of adult neurogenesis (although the absolute rate of neurogenesis remains controversial). Therefore, incorporation of immature stem cell-derived neurons into existing neural circuity beyond embryonic development is a hopeful prospect.

To generate hippocampal neurons, stem cells are patterned to dorsal forebrain progenitors by inhibiting both branches of the SMAD signaling (dual-SMAD inhibition) as well as factors to promote WNT and SHH signaling. Subsequently, WNT3a is applied along with BDNF to drive the neurogenesis of hippocampal granule neurons (Hiragi et al., 2017; Sakaguchi et al., 2015; Yu et al., 2014). Initial findings indicate concurrent WNT and BMP activation can drive the differentiation of the dorsal forebrain progenitors into pyramidal neurons (Sakaguchi et al., 2015). Rodent transplantation studies with hippocampal neural precursors revealed that the human neurons could integrate into the dentate gyrus (Yu et al., 2014), but it remains to be determined if these xenografts can affect disease-related phenotypes in animal models.

HUNTINGTON'S DISEASE

Huntington's disease is caused by a CAG trinucleotide repeat expansion within the coding region of the *HTT* gene, resulting in an extended polyglutamine (polyQ) tract within the Huntingtin protein. The progressive loss of neurons and gross atrophy in the neostriatum (caudate nucleus and putamen) disrupts neuronal circuits involving the basal ganglia and leads to gradually worsening motor impairment and, as additional brain regions are affected, significant cognitive and psychiatric symptoms.

Medium spiny neurons

Medium spiny neurons that reside in the striatum, contribute to the complex circuits that control movement and are particularly vulnerable in Huntington's disease. During development, these inhibitory neurons arise from the lateral ganglionic eminence (LGE) and are marked by the expression of DARPP32 (dopamine- and cAMP-regulated phosphoprotein Mr~32 kDa) (Fjodorova et al., 2015). The relatively specific loss of DARPP32+ medium spiny class of neurons in the neostriatum makes Huntington's disease a strong candidate for cell replacement therapies. Like for Parkinson's disease, fetal transplants have paved the way for stem cell-derived therapies for HD (Freeman et al., 2000).

Numerous groups have validated directed differentiation approaches for producing medium spiny neurons from stem cells (Aubry et al., 2008; Carri et al., 2012; Ma et al., 2012).

Like the methods for producing other inhibitory neurons from the neighboring MGE, combinatorial SHH/WNT signaling modulation induces an anterior-ventral fate. Of note, reduced activation of SHH signaling and the addition of Activin A can favor a LGE fate while inhibiting a MGE fate (Fjodorova et al., 2015). A direct conversion method has also recently been described for transforming fibroblasts into medium spiny neurons, specifically, with a combination of 4 transcription factors (CTIP2, DLX1, DLX2, and MYT1L) and two microRNAs (miR-9/9 and miR-124) (Victor et al., 2014). Whether these direct programming methods can be applied to pluripotent stem cells remains to be determined but could be used to improve the yield of medium spiny neurons from stem cells, which are at best ~50%. When transplanted into a murine striatum, the neurons integrate into the host circuit and project to the proper anatomical targets. In some cases, the transplanted cells neurons can rescue motor deficits in quinolinic acid, an excitotoxin, striatal-lesioned mice, a model of HD (Carri et al., 2012; Victor et al., 2014). In another study, however, the transplanted cells also resulted in cellular overgrowth (Aubry et al., 2008). Based on these studies, refined purification methods to yield more homogenous neuron populations followed by additional animal model studies seem warranted.

ATAXIAS

Spinocerebellar ataxias (SCAs) are a clinically and genetically heterogenous group of neurological disorders associated with impairments in motor coordination due to degeneration of the cerebellum and connected neuronal pathways. Many SCAs are caused by CAG nucleotide repeat expansions within certain genes leading to the production of polyglutamine (polyQ)-containing proteins with putative toxic gain-of-function effects. For instance, an autosomal dominantly-inherited, abnormally long (>33 CAG repeats) trinucleotide repeat expansion within *ATXN-2* results in SCA2 that can manifest with ataxia, loss of neurological reflexes, and Parkinsonian symptoms. Ataxias can be associated with other inherited disorders. For examples, an autosomal recessively-inherited GAA trinucleotide repeat expansions in *FXN*, encoding frataxin, cause Friedrich's ataxia, which is characterized by progressive ataxia, impaired speech, loss of vibratory and proprioceptive sensation due to degeneration of spinal cord neurons and nerve fiber tracts connecting to the cerebellum. There are no effective treatments for these debilitating and often fatal diseases.

Purkinje cells

Purkinje cells are large inhibitory GABAergic neurons with extensive dendritic arbors that reside within the hindbrain structure of the cerebellum. As the output neurons of the cerebellar cortex, they project to neurons within deep cerebellar nuclei and play an important role in motor coordination. Until recently, the differentiation of human PSCs into Purkinje neurons remained elusive, perhaps due to their late emergence during development. An initial

directed differentiation approach for this cell type required several steps and many factors. First, exogenous factors were employed to stimulate endogenous Wnt1 and FGF8 signaling and promote a midbrain/hindbrain identity, and inhibition of SHH signaling was used to pattern cells towards a dorsal identity (Muguruma et al., 2015; Wang et al., 2015). Then, the maturation process could be accomplished through several methods: plating precursors on mouse cerebellar slice cultures (Watson et al., 2018), within self-organizing, polarized cerebellar structures (Muguruma et al., 2015), or more recently in a defined basal medium optimized for cell culture (Bardy et al., 2015; Silva et al., 2020). Studies indicate that the stem cell-derived Purkinje cells are susceptible to genetic insults, such as the trinucleotide CAG repeat in *CACNA1A* associated with SCA6 *(Ishida et al., 2016),* that trigger their selective demise, and that they can also engraft into the mouse cerebellum (Wang et al., 2015). Although more defined and robust methods are needed before cell replacement therapies should be considered clinically, the initial findings have paved the way for producing this neuronal type that is relevant to many neurological disorders.

MOTOR NEURON DISEASES

The specific loss of motor neurons underlies several devastating neurological diseases including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). Both diseases involve the progressive loss of motor function, eventually progressing to fatal paralysis. In nearly all (~97%) of cases of ALS, motor neurons in both the brain and spinal exhibit pathological changes in the cellular localization of the RNA binding protein TDP-43, which include loss of the normal nuclear localization and the formation of cytoplasmic inclusions (Klim et al., 2021).

Spinal Motor Neurons

Motor neurons represent a diverse group of neuronal subtypes and provide the pivotal link between mind and the animation of the body. Generally, there are two types of motor neurons; upper motor neurons that reside in the frontal cortex and project to lower motor neurons, found in the ventral brainstem and spinal cord, which in turn form synapses with the musculature. Decades of developmental studies and genetic analyses have illuminated the molecular underpinnings of lower motor neuron specification during embryo development (Dasen and Jessell, 2009) with the morphological gradients well established (Davis-Dusenbery et al., 2014).

Leveraging this knowledge, stem cell scientists developed methods to generate motor neurons from mouse embryonic stem cells by applying retinoic acid (RA) to caudalize the cells towards a spinal cord (the distal or tail end of the neural tube) identity and activating SHH to ventralize them toward a motor, rather than sensory, identity (Wichterle et al., 2002). Several research groups have advanced these earlier findings to reproducibly convert human

pluripotent stem cells into vast quantities of motor neurons (Amoroso et al., 2013; Du et al., 2015; Klim et al., 2019; Maury et al., 2015). These approaches typically rely on neural induction through small molecule dual-SMAD signaling inhibition, in some cases activation of WNT signaling, accelerated neurogenesis through inhibition of FGF or NOTCH signaling, all coupled with MN patterning described above (RA and SHH). Ichida, Son, and colleagues have used a large cadre of MN-related transcription factors (Isl1, Ascl1, Myt1l, Brn2, Ngn2, Lhx3, and Neurod1) to directly convert fibroblasts into induced motor neurons(Son et al., 2011). Alternatively, simpler protocols were achieved that used a subset these factors to transform human stem cells into motor neurons (Goto et al., 2017; Hester et al., 2011). Recently, we have also shown that transcription factor-based and small molecule approaches could be combined to yield a highly pure population of cervical-like motor neurons from iPSCs with 100% efficiency through the inducible expression of Ngn2 (neurogenin-2) alone coupled with RA and SHH treatments (Limone et al., 2022). Interestingly, carefully varying the timing of retinoid application has been demonstrated to afford more caudal motor neuron fates (Lippmann et al., 2015), but methodologies for producing upper motor neurons, also known as cortical spinal motor neurons (CSMNs), are still lacking. As degeneration of cortical and spinal cord motor regions occur in ALS, a full array of motor neuron subtypes might be needed as a cell replacement therapy.

So far, motor neuron transplant results have been encouraging. For example pioneering transplant studies demonstrate that mES-derived motor neurons injected into tibial nerve of adult mice can form functional NMJs and ameliorate muscle atrophy (Yohn et al., 2008). Another notable study was able to transplant human iPS cell-derived motor neurons into the ventral horns of an SMA mouse model (Corti et al., 2012). The transplanted motor neurons could survive and engraft into the murine spinal cord and could even ameliorate disease phenotypes and extend the life span relative to those receiving a fibroblast transplant (Corti et al., 2012). These exciting initial studies highlight the need for large animal models for testing motor neuron-based cell therapies.

GLIAL CELLS

Although glia are more abundant than neurons, nuances remain in our understanding of how their exact cellular identities are established and how glial developmental pathways can be recapitulated *in vitro* for cell replacement approaches. Three main types of glia exist in the CNS: astrocytes, oligodendrocytes (OLs), and microglia. In brief, astrocytes are responsible for forming and modulating the blood-brain barrier (BBB) and modifying the chemical microenvironment governing synaptic function. Microglia are the resident immune cells of the CNS that function in synaptic pruning during development, immune surveillance, debris clearance and defense from pathogens. Oligodendrocytes are responsible for

myelinating axons in the CNS, thereby maintaining strong electrical connectivity of brain circuitry. Glia have been implicated in almost all neurodegenerative diseases, and their dysfunction in this context are more extensively reviewed elsewhere (Zheng et al., 2018). Glial transplantation for the treatment of neurodegenerative diseases has been explored much less than for neurons, though might be advantageous for ameliorating glial dysfunction as well as mitigating the loss of degenerating neurons by engaging in supportive roles. Like neurons, glial cells can be generated by activating development cues or overexpression of cell type-specific transcription factors. We will discuss a selection of strategies to generate glia and their most promising applications to neurodegenerative diseases.

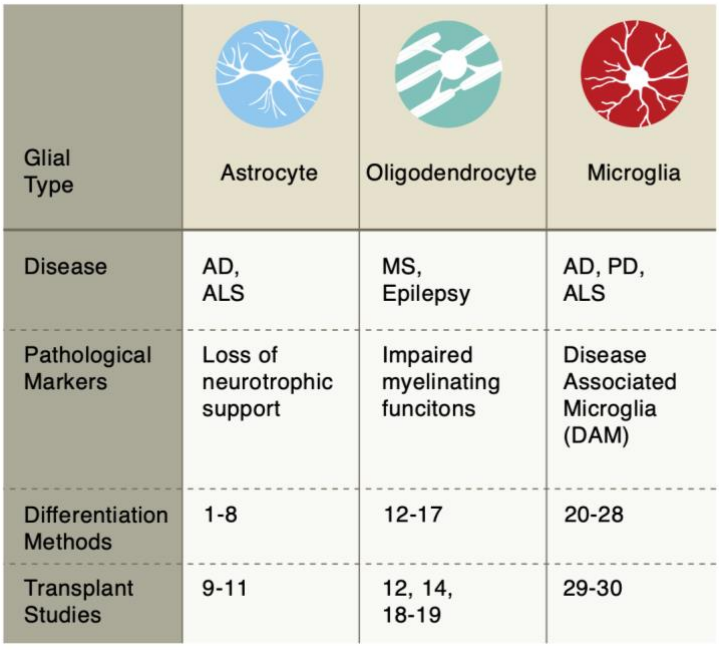
Astrocytes

Astrocytes are star-shaped glial cells that reside in both the brain and spinal cord to maintain BBB integrity, regulate nutrient flow, and govern neuronal function. They arise relatively early in neuronal development from radial glial progenitor cells usually after these cells have generated neurons. Broadly, differentiation protocols recapitulate developmental cues (Krencik et al., 2011; Shaltouki et al., 2013) by promoting neuronal stem cell (NSC) identity via dual SMAD inhibition and then gliogenesis with morphogens (Krencik and Zhang, 2011). Promoting gliogenesis after NSC differentiation has traditionally been a slow rate-limiting step in the generation of astrocytes, but recent transgenic and chemical strategies have greatly accelerated this process. Expansion of NSCs with Activin A, Heregulin 1β (Neuregulin1), and IGFI (Shaltouki et al., 2013; Tcw et al., 2017), flow cytometry-based enrichment strategies (Barbar et al., 2020) or overexpression of TFs NFIA and SOX9 can dramatically shorten differentiation protocols (Canals et al., 2018; Tchieu et al., 2019). hPSC-derived astrocyte-like cells can be generated in as little as 30 days and show functional properties similar to primary astrocytes in that they uptake glutamate, promote neurite outgrowth, propagate calcium waves, and retain their identity in vivo (Krencik and Zhang, 2011; Li et al., 2018; Shaltouki et al., 2013). Many groups have recently developed methods to increase maturity and function of these cells by differentiating them from 3D structures coupled with cell sorting methods (Barbar et al., 2020).

Studies on ALS and PD animal models are laying the foundation for astrocyte transplantation therapies. In ALS models, astrocytes exert toxic gain-of-function effects that can act in a cell non-autonomous manner to contribute to motor neuron degeneration (Di Giorgio et al., 2008; Di Giorgio et al., 2007; Hall et al., 2017; Meyer et al., 2014). For instance, mice expressing human mutant SOD1 in astrocytes in addition to neurons had reduced to survival compared to mice only expressing mutant SOD1 in neurons, in other words, a wild-type astrocyte microenvironment may promote motor neuron survival (Bataveljić et al., 2012). Focal transplantation of glial-restricted NPCs (Neuronal Progenitor Cells) into the cervical spinal cord of SOD1 transgenic rats during disease progression extended survival and

decreased motor neuron death, in part due to the partial rescue of GLT1 expression in astrocytes (Clement et al., 2003). Clinical trials are ongoing to prove the efficacy of transplanted PSC-derived astrocytes to boost neuronal survival and slow disease progression. For instance, a phase 1/2a trial in a small cohort of ALS patients (NCT02943850) has shown that a single injection of human NPCs engineered to produce glial cell line-derived neurotrophic factor (GDNF) into the spinal cord is safe, and viable grafts differentiated into astrocytes that may be neuroprotective through increased GDNF production (Baloh et al., 2022).

Transplantation studies for PD also showed promising results. Co-transplantation of primary fetal NPCs and rat astrocytes increased long-term engraftment of mature midbrain dopaminergic neurons and increase anti-inflammatory markers in the brains of PD rats (Lepore et al., 2008). Transplantation of primary astrocytes into the SNpc increase synaptosomal dopamine uptake in the striatum, reduce ROS stress, and improved motor deficits of pharmacologically-induced PD rats (Song et al., 2017). These observations suggest hPSC-derived astrocytes may be used to slow disease progression and complement dopaminergic neuron transplantation.



Glial Type	Astrocyte	Oligodendrocyte	Microglia
Disease	AD, ALS	MS, Epilepsy	AD, PD, ALS
Pathological Markers	Loss of neurotrophic support	Impaired myelinating funcitons	Disease Associated Microglia (DAM)
Differentiation Methods	1-8	12-17	20-28
Transplant Studies	9-11	12, 14, 18-19	29-30

Figure 2 Astrocyte: *diff.* 1-8 Krencik et al. 2011; Shaltouki et al. 2013; Krenick et al. 2011; TCW et al. 2017; Barbar et al. 2020; Tchieu et al. 2019; Canals et al. 2018; Li et al. 2018**;** *transpl.* 9-11 Baloh et al. 2022; Lepore et al. 2008; Song et al. 2017**. Oligodendrocyte:** *diff.* 12-17 Wang et al. 2013; Douvaras and Fossati 2015; Douvaras et al. 2014; Marton et al. 2019; Ehrlich et al. 2017; García-Léon et al. 2018**;** *transpl.* 12, 14, 18-19 Wang et al. 2013; Douvaras et al. 2014; Thiruvalluvan et al. 2016; Windrem et al. 2020**. Microglia:** *diff.* 20-28 Muffat et al. 2016; Haenseler et al. 2017; Takata et al. 2017; Abud et al. 2017; Douvaras et al. 2017; Pandya et al. 2017; Dolan et al. 2022; Limone et al. 2021; Chen et al. 2021**;** *transpl.* 29-30 Xu et al. 2020; Svoboda et al. 2019

Oligodendrocytes

Similar to astrocytes, oligodendrocytes are derived in development after neurogenesis. In both the forebrain and the spinal cord, oligodendroglial progenitor cells (OPCs) are generated from Nkx2.1^{+}, SHH-derived progenitors and their differentiation is regulated by TFs Olig1 and Olig2. OPCs have an immense ability to migrate and populate the entire brain and spinal cord where most of them further differentiate into committed, myelinating oligodendrocytes (OLs) while a small subset of them are maintained in a progenitor state. Their great migratory abilities, plasticity and pivotal role in neuronal support render these cells ideal for transplantation studies and replacement therapies.

To generate OLs, hPSCs are first converted to a neural stem cell with small molecules or a neural epithelial identity through SHH activation and are then pushed towards an oligodendrocyte progenitors (OPCs) identity by the addition of PDGF-AA. These OPCs can be matured into OLs by various cocktails of small molecules, often containing IGF-1 and T3 (Douvaras and Fossati, 2015; Douvaras et al., 2014; Wang et al., 2013). Several groups have shown that complete maturation of OPCs into highly myelinating oligodendrocytes can be achieved either by injecting these cells in vivo (Douvaras et al., 2014) or by differentiating these cells in 3D structures (Marton et al., 2019). Protocols relying on overexpression of several transcription factors, including OLIG2, NKX6.2, and SOX10, were developed to be faster and similarly efficient (Ehrlich et al., 2017; García-León et al., 2018). García-León et al. found, however, that overexpression of SOX10 alone in NSCs was the most efficient at generating OLs in as little as 20 days, and the generated OLs were capable of myelinating cortical neurons both *in vitro* and *in vivo* (García-León et al., 2018).

Stem cell-derived OLs hold promise for both demyelinating diseases and spinal cord injury. Multiple sclerosis (MS) is a chronic, autoimmune disease characterized by the loss of myelin and associated oligodendrocytes, often in a remitting and relapsing clinical course that results in gradual neurological decline. MS-iPSC-derived OPCs can myelinate the corpus callosum of immunocompromised hypomyelinated (shiver) mice (Douvaras et al., 2014; Wang et al., 2013), offering a potential regenerative route for re-myelination for cases of MS that are resistant to immune-suppressant treatment. Strikingly, human iPSC-derived OPCs can myelinate axons in a non-human primate marmoset model (Thiruvalluvan et al., 2016). Long term transplantation studies in both shiver mice and demyelinating cuprizone treatment also showed that these cells can not only migrate to distal regions of the CNS farther than previously believed but can also improve behavior and motor function in murine models (Windrem et al., 2020). These results highlight the feasibility of an iPSC-derived OL transplantation therapy for MS and perhaps for other demyelinating diseases.

Microglia

Unlike other glial cells, microglia are immune cells not derived from the neuroectoderm but originate from the embryonic yolk sac in early stages of development and then migrate to the neural tube (Ginhoux et al., 2010; Kierdorf et al., 2013). Chemical differentiation strategies generally generate early myeloid progenitors by isolation of delaminating cells from so-called yolk-sac embryoid bodies (Haenseler et al., 2017; Muffat et al., 2016) or by promoting hematopoiesis with hypoxic conditions and defined medias (Abud et al., 2017). Initial studies used co-cultures of these immature myeloid cells with human neurons or murine brain extracts to generate resident brain-like microglia (Takata et al., 2017). These protocols made scalability challenging so others have devised ways to further push immature myeloid progenitors toward

microglia-like cells (MGLs) with defined medias containing M-CSF to generate myeloid cells coupled with CNS-enriched TGF-beta and CNS-specific, CSF1-receptor ligand IL34 to promote a brain-like specification of these myeloid progenitors. Generated MGLs show competence to phagocytose (Abud et al., 2017; Dolan et al., 2022; Douvaras et al., 2017; Haenseler et al., 2017; Limone et al., 2021; Muffat et al., 2016; Pandya et al., 2017) respond to IFN-γ and LPS stimulation via secretion of pro-inflammatory cytokines (Abud et al., 2017; Muffat et al., 2016), and migrate to sites of injury (Muffat et al., 2016). When co-cultured with neurons, MGLs have also been observed to secrete anti-inflammatory and pro-remodeling cytokines (Haenseler et al., 2017). Like for other glial cells, transcription factor-based protocols may offer increased efficiency and decreased time for the generation of microglial-like cells. One study has shown that overexpression of transcription factors CEBPA and PU.1 coupled with CNS-patterning molecules described above can generate Microglia-like cells from human iPSC (Chen et al., 2021) with a second one showing improved efficiency by overexpressing PU.1 from primitive hematopoietic progenitors (Sonn et al., 2022). A recent study has defined a set of six transcription factors for the generation of microglia-like cells at a scale sufficient for genetic screening (Drager et al., 2022). Following the progress in the derivation of specific neuronal populations, it is plausible that newer approaches might find that just a few transcriptional factors could be sufficient, when coupled with small molecules, for the generation of this cell type.

Long term engraftment studies have been rendered difficult by the lack of homology between murine and human CSF1, which is pivotal for long term microglial survival. However, initial studies have shown the feasibility of transplantation of hiPSC-derived iMGLs in humanized mouse models (Svoboda et al., 2019; Xu et al., 2020) .

TECHNOLOGICAL ADVANCES

Directed differentiation approaches have evolved considerably since the initial derivation of neurons from human embryonic stem cells (Zhang et al., 2001). Although defined culture conditions that primarily employ small molecules instead of poorly defined co-culture systems are more robust, modern directed differentiation approaches still tend to yield highly heterogeneous cultures containing the cell type of interest along with developmentally related cells. Direct conversion strategies like the ones described above typically yield more homogenous cell populations, but viral integration could disrupt normal gene expression and thus might not be amenable to clinical applications. Alternatively, the use of cell surface antibodies for sorting different neural populations has been pioneered to enrich for more defined cell populations (Yuan et al., 2011), or dyes that are selectively taken up by specific cells could theoretically also be used to mark specific cell types as has been demonstrated for neural precursor cells (Yun et al., 2012). These advances have led to several of these

differentiation protocols being used for modeling neurodegeneration in different cell types *in vitro* (Giacomelli et al., 2022), opening the door to their adaptation to transplantation studies in the future. Additionally, several groups have made significant progress in the development of protocols for the generation of 3D structures containing various CNS cell types (known as brain organoids) that can enhance cell type specification and maturation (Del Dosso et al., 2020). Whether this technology can be translated into reproducible, manufacturable products for transplantation studies remains unclear, though it does offer a myriad of intriguing possibilities for the field.

It is unclear whether nascent, immature neurons or elaborate, mature neurons will integrate more successfully into a degenerating brain to provide therapeutic benefit. Either way, the ability to control the functional maturation of stem cell-derived neurons would benefit many applications. For *in vitro* disease modelling studies, we have found that co-culture of human neurons with murine glial cells effectively increased neuronal activity, but co-culture with non-human cells is not an ideal strategy for cell replacement therapies. Instead, Gage and colleagues have developed a defined neuronal medium, BrainPhys, which better mimics the environment present in healthy human brains and enhances both spontaneous electrical and synaptic activity of human neurons (Bardy et al., 2015). Whether increased activity translates into increased survival after transplantation remains an unanswered but fascinating question.

The process of reprogramming adult cells back to the pluripotent state erases many aspects of aging that put vulnerable cells at risk in the first place(Mertens et al., 2018). Although resetting the biological clock makes disease modeling more challenging, it might rid the newly derived cells from the neurodegenerative stimuli of aging when transplanted. Still, there might be aspects of maturation that are critical for neuronal integration or function. Unlike stem cell-derived neurons, for example, neurons directly converted from adult fibroblasts capture the faithful expression of all tau isoforms detected in adult brains at the proper ratios. Direct conversion of adult cells to replace lost neurons might therefore be alternative technology to consider (Capano et al., 2022) and has even been shown to reverse symptoms of Parkinson’s disease in a rodent model by converting midbrain astrocytes to dopaminergic neurons (Qian et al., 2020).

LIMITATIONS AND CHALLENGES

Induced pluripotent stem cell technology marshalled in the possibility of personalized regenerative medicine using therapies based on an individual’s own cells. To this end, investigators in Japan started a clinical trial to treat age-related macular degeneration using autologous transplants, however, the trial was eventually suspended after treating one patient (Mandai et al., 2017). Several hurdles generate significant headwinds for this type of approach

including 1) the time and effort needed to generate iPS cells, 2) genomic instability of pluripotent stem cells, and 3) the cost of personalized therapeutics. Most of these hurdles have several potential solutions that we will describe here briefly.

Despite recent advances, the overall time to move from the collection of fibroblasts via skin biopsy in the clinic, the reprogramming of fibroblasts into PSCs with completion of appropriate quality controls, to the differentiation of individualized stem cells into a personal population of a specific cell type, such as mature motor neurons, remains extensive, and hence possibly beyond the therapeutic window for rapidly progressive neurodegenerative diseases like ALS. To meet the demands of future clinical applications, state-of-the-art technologies for the cryopreservation of differentiated cell types are being tested to provide a ready to go off-the-shelf product (Holm et al., 2010; Nishiyama et al., 2016). Indeed, this approach is being pioneered within the Parkinson's disease cell replacement field, which has demonstrated that cryopreserved iPSC-derived neurons can maintain high viability and the molecular properties of a dopaminergic neuron. Moreover, these cryopreserved cells can be directly transplanted into a rat model of PD to reverse functional deficits (Wakeman et al., 2017).

For cell replacement therapies, even rare proliferating cells are especially worrisome because they could ultimately lead to the growth of tumors. Moreover, genomic instability of pluripotent stem cells has long been a concern for the field as aneuploid cells have readily been observed (Draper et al., 2004). To identify more subtle genetic changes, groups have performed whole-exome sequencing on many of the hES cell lines listed on the US National Institutes of Health registry and reported the acquisition of dominant negative p53 mutations, a mutation associated with many cancers, for several hES cell lines (Merkle et al., 2017), and other genomic changes associated with cancer and tumorigenesis (Merkle et al., 2022). Similar studies have also identified recurrent mutations that can occur during the reprogramming process and subsequent propagation (Pera, 2011). Therefore, thoughtful genetic characterization should be standard before stem cells or any of their derivatives are used in the clinic. This analysis will not only be useful to rule out stem cell lines with potentially dangerous mutations but could also be used after transplant to retrospectively identify the distribution of the donor cells.

To overcome the laborious nature of converting somatic cells into pluripotent stem cells, the New York Stem Cell Foundation has developed an automated platform for the high throughput conversion of skin biopsies into iPS cells (Paull et al., 2015). This high throughput platform can be used in conjunction with synthetic modified RNA to reprogram cells and avoid viral transduction (Warren et al., 2010). Finally, xenofree culture conditions have been developed and are now commercially available for deriving and propagating human pluripotent

stem cells (Chen et al., 2011; Klim et al., 2010). Collectively, these innovations will help expedite the large-scale generation of clinical grade iPS cells.

Finally, widely applicable and efficient cell banking methods are needed to meet the demand of cell transplantation therapies. There are ongoing efforts in both Japan and the United States to screen and bank cells for allogeneic transplantations. Estimates from Cellular Dynamics International suggest that top 183 haplotypes could cover 95% of the US population. To gain maximum population coverage and provide social justice (Ellison, 2016), a universal stem cell donor could be part of the banking effort. This tactic proposes to use genetic engineering to reduce immunogenicity by removing the MHC molecules from the surface of the cells while also introducing well-established tolerance-inducing molecules (Han et al., 2019; Riolobos et al., 2013). Ultimately, stem cell banking will facilitate regenerative therapies by providing a common and less costly off-the-shelf cellular materials that can be thoroughly characterized before regular and repeated clinical use.

CONCLUDING REMARKS

It's an incredibly exciting time for stem cell-based regenerative medicine with a number of clinical trials started and more just on the horizon for neurodegenerative diseases, including one for Parkinson's disease (Kimbrel and Lanza, 2015). The International Society for Stem Cell Research (ISSCR) has established an updated set of guidelines (Daley et al., 2016) for the clinical translation of stem cell research to ensure safety and appropriate rigor while avoiding the real and present dangers of unregulated stem cell therapies (Berkowitz et al., 2016).

The demand for neurodegenerative disease therapeutics continues to grow as populations around the globe age. Currently, no pharmacological strategies exist that can significantly alter disease course for neurodegenerative diseases, thus cell replacement therapies remain an attractive avenue of exploration. Although the prospect of using stem cell-derived neurons to treat many of the diseases discussed above remains abstract, the Parkinson's disease clinical trials, grounded on years of fetal transplant studies and animal models with high fidelity, will provide important guideposts as others venture into these uncharted territories. In this review, we highlighted current methodologies for generating therapeutically relevant neuronal and glial cell types. Although directed differentiation strategies for some of these CNS cell types are in their nascent stage, they represent important first steps towards heralding in a new era of cellular therapeutics.

AUTHOR CONTRIBUTIONS

J.R.K. conceived of the review; J.R.K, D.A.M., and F.L. drafted the original manuscript; D.A.M. drafted the table; J.R.K. drafted the figures; All authors read, edited, and approved the submitted version.

ACKNOWLEDGEMENTS

We would like to thank Katrina M. Wilbur and Renate G. E. Hellmiss from Harvard's MCB Graphics for their graphic design skills in preparing the figures, and Andrew Castillo for assisting in researching glial-related topics. D.A.M. acknowledges support from the NINDS (K08NS104270). J.R.K. is an employee of Faze Medicines and a shareholder of Faze Medicines as well as QurAlis. J.R.K. and F.L. are authors on a patent application that describes methods and compositions for restoring STMN2 levels (US patent application 20220133848), and J.R.K. is an author on a patent that describes surfaces for the long-term culture of pluripotent cells (US patent 8648170). The authors have no other competing conflicts or financial interests.

REFERENCES

Abud, E.M., Ramirez, R.N., Martinez, E.S., Healy, L.M., Nguyen, C.H.H., Newman, S.A., Yeromin, A.V., Scarfone, V.M., Marsh, S.E., Fimbres, C.*, et al.* (2017). iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. Neuron *94*, 278--293.e279. https://doi.org/10.1016/j.neuron.2017.03.042

Amoroso, M.W., Croft, G.F., Williams, D.J., O'Keeffe, S., Carrasco, M.A., Davis, A.R., Roybon, L., Oakley, D.H., Maniatis, T., Henderson, C.E.*, et al.* (2013). Accelerated High-Yield Generation of Limb-Innervating Motor Neurons from Human Stem Cells. The Journal of Neuroscience *33*, 574--586. https://doi.org/10.1523/jneurosci.0906-12.2013

Anderson, N.C., Zandt, M.A.V., Shrestha, S., Lawrence, D.B., Gupta, J., Chen, C.Y., Harrsch, F.A., Boyi, T., Dundes, C.E., Aaron, G.*, et al.* (2018). Pluripotent stem cell-derived interneuron progenitors mature and restore memory deficits but do not suppress seizures in the epileptic mouse brain. Stem Cell Research *33*, 83--94. https://doi.org/10.1016/j.scr.2018.10.007

Arenas, E., Denham, M., and Villaescusa, J.C. (2015). How to make a midbrain dopaminergic neuron. Development *142*, 1918--1936. https://doi.org/10.1242/dev.097394

Aubry, L., Bugi, A., Lefort, N., Rousseau, F., Peschanski, M., and Perrier, A.L. (2008). Striatal progenitors derived from human ES cells mature into DARPP32 neurons in vitro and in quinolinic acid-lesioned rats. Proceedings of the National Academy of Sciences *105*, 16707--16712. https://doi.org/10.1073/pnas.0808488105

Baloh, R.H., Johnson, J.P., Avalos, P., Allred, P., Svendsen, S., Gowing, G., Roxas, K., Wu, A., Donahue, B., Osborne, S.*, et al.* (2022). Transplantation of human neural progenitor cells secreting GDNF into the spinal cord of patients with ALS: a phase 1/2a trial. Nat Med. https://doi.org/10.1038/s41591-022-01956-3

Barbar, L., Jain, T., Zimmer, M., Kruglikov, I., Sadick, J.S., Wang, M., Kalpana, K., Rose, I.V.L., Burstein, S.R., Rusielewicz, T.*, et al.* (2020). CD49f Is a Novel Marker of Functional and Reactive Human iPSC-Derived Astrocytes. Neuron *107*, 436-453 e412. https://doi.org/10.1016/j.neuron.2020.05.014

Bardy, C., Hurk, M.v.d., Eames, T., Marchand, C., Hernandez, R.V., Kellogg, M., Gorris, M., Galet, B., Palomares, V., Brown, J.*, et al.* (2015). Neuronal medium that supports basic synaptic functions and activity of human neurons in vitro. Proceedings of the National Academy of Sciences *112*, E2725--E2734. https://doi.org/10.1073/pnas.1504393112

Barker, R.A., Parmar, M., Studer, L., and Takahashi, J. (2017). Human Trials of Stem Cell-Derived Dopamine Neurons for Parkinson's Disease: Dawn of a New Era. Cell Stem Cell *21*, 569--573. https://doi.org/10.1016/j.stem.2017.09.014

Bataveljić, D., Nikolić, L., Milosević, M., Todorović, N., and Andjus, P.R. (2012). Changes in the astrocytic aquaporin-4 and inwardly rectifying potassium channel expression in the brain of the amyotrophic lateral sclerosis SOD1 G93A rat model. Glia *60*, 1991--2003. https://doi.org/10.1002/glia.22414

Bekkers, J.M. (2011). Pyramidal neurons. Curr Biol *21*, R975. https://doi.org/10.1016/j.cub.2011.10.037

Berkowitz, A.L., Miller, M.B., Mir, S.A., Cagney, D., Chavakula, V., Guleria, I., Aizer, A., Ligon, K.L., and Chi, J.H. (2016). Glioproliferative Lesion of the Spinal Cord as a Complication of "Stem-Cell Tourism". The New England Journal of Medicine *375*, 196--198. https://doi.org/10.1056/nejmc1600188

Bissonnette, C.J., Lyass, L., Bhattacharyya, B.J., Belmadani, A., Miller, R.J., and Kessler, J.A. (2011). The Controlled Generation of Functional Basal Forebrain Cholinergic Neurons from Human Embryonic Stem Cells. Stem Cells *29*, 802--811. https://doi.org/10.1002/stem.626

Cai, J., Donaldson, A., Yang, M., German, M.S., Enikolopov, G., and Iacovitti, L. (2009). The Role of Lmx1a in the Differentiation of Human Embryonic Stem Cells into Midbrain Dopamine Neurons in Culture and After Transplantation into a Parkinson\'s Disease Model. Stem Cells *27*, 220--229. https://doi.org/10.1634/stemcells.2008-0734

Caiazzo, M., Dell'Anno, M.T., Dvoretskova, E., Lazarevic, D., Taverna, S., Leo, D., Sotnikova, T.D., Menegon, A., Roncaglia, P., Colciago, G., *et al.* (2011). Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature *476*, 224--227. https://doi.org/10.1038/nature10284
Canals, I., Ginisty, A., Quist, E., Timmerman, R., Fritze, J., Miskinyte, G., Monni, E., Hansen, M.G., Hidalgo, I., Bryder, D., *et al.* (2018). Rapid and efficient induction of functional astrocytes from human pluripotent stem cells. Nat Methods *15*, 693-696. https://doi.org/10.1038/s41592-018-0103-2
Cao, S.-Y., Hu, Y., Chen, C., Yuan, F., Xu, M., Li, Q., Fang, K.-H., Chen, Y., and Liu, Y. (2017). Enhanced derivation of human pluripotent stem cell-derived cortical glutamatergic neurons by a small molecule. Scientific Reports *7*, 3282. https://doi.org/10.1038/s41598-017-03519-w
Capano, L.S., Sato, C., Ficulle, E., Yu, A., Horie, K., Kwon, J.S., Burbach, K.F., Barthelemy, N.R., Fox, S.G., Karch, C.M., *et al.* (2022). Recapitulation of endogenous 4R tau expression and formation of insoluble tau in directly reprogrammed human neurons. Cell Stem Cell *29*, 918-932 e918. https://doi.org/10.1016/j.stem.2022.04.018
Carlsson, T., Carta, M., Muñoz, A., Mattsson, B., Winkler, C., Kirik, D., and Björklund, A. (2009). Impact of grafted serotonin and dopamine neurons on development of l-DOPA-induced dyskinesias in parkinsonian rats is determined by the extent of dopamine neuron degeneration. Brain *132*, 319--335. https://doi.org/10.1093/brain/awn305
Carri, A.D., Onorati, M., Lelos, M.J., Castiglioni, V., Faedo, A., Menon, R., Camnasio, S., Vuono, R., Spaiardi, P., Talpo, F., *et al.* (2012). Developmentally coordinated extrinsic signals drive human pluripotent stem cell differentiation toward authentic DARPP-32+ medium-sized spiny neurons. Development *140*, 301--312. https://doi.org/10.1242/dev.084608
Chanda, S., Ang, C.E., Davila, J., Pak, C., Mall, M., Lee, Q.Y., Ahlenius, H., Jung, S.W., Südhof, T.C., and Wernig, M. (2014). Generation of Induced Neuronal Cells by the Single Reprogramming Factor ASCL1. Stem Cell Reports *3*, 282--296. https://doi.org/10.1016/j.stemcr.2014.05.020
Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., *et al.* (2011). Chemically defined conditions for human iPSC derivation and culture. Nature Methods *8*, 424--429. https://doi.org/10.1038/nmeth.1593
Chen, S.-W., Hung, Y.-S., Fuh, J.-L., Chen, N.-J., Chu, Y.-S., Chen, S.-C., Fann, M.-J., and Wong, Y.-H. (2021). Efficient conversion of human induced pluripotent stem cells into microglia by defined transcription factors. Stem Cell Reports. https://doi.org/10.1016/j.stemcr.2021.03.010
Clement, A.M., Nguyen, M.D., Roberts, E.A., Garcia, M.L., Boillée, S., Rule, M., McMahon, A.P., Doucette, W., Siwek, D., Ferrante, R.J., *et al.* (2003). Wild-Type Nonneuronal Cells Extend Survival of SOD1 Mutant Motor Neurons in ALS Mice. Science *302*, 113--117. https://doi.org/10.1126/science.1086071
Corti, S., Nizzardo, M., Simone, C., Falcone, M., Nardini, M., Ronchi, D., Donadoni, C., Salani, S., Riboldi, G., Magri, F., *et al.* (2012). Genetic Correction of Human Induced Pluripotent Stem Cells from Patients with Spinal Muscular Atrophy. Science Translational Medicine *4*, 165ra162--165ra162. https://doi.org/10.1126/scitranslmed.3004108
Cunningham, M., Cho, J.-H., Leung, A., Savvidis, G., Ahn, S., Moon, M., Lee, Paula K.J., Han, Jason J., Azimi, N., Kim, K.-S., *et al.* (2014). hPSC-Derived Maturing GABAergic Interneurons Ameliorate Seizures and Abnormal Behavior in Epileptic Mice. Cell Stem Cell *15*, 559--573. https://doi.org/10.1016/j.stem.2014.10.006
Daley, George Q., Hyun, I., Apperley, Jane F., Barker, Roger A., Benvenisty, N., Bredenoord, Annelien L., Breuer, Christopher K., Caulfield, T., Cedars, Marcelle I., Frey-Vasconcells, J., *et al.* (2016). Setting Global Standards for Stem Cell Research and Clinical Translation: The 2016 ISSCR Guidelines. Stem Cell Reports *6*, 787--797. https://doi.org/10.1016/j.stemcr.2016.05.001
Dasen, J.S., and Jessell, T.M. (2009). Hox networks and the origins of motor neuron diversity. Current topics in developmental biology *88*, 169--200. https://doi.org/10.1016/s0070-2153(09)88006-x
Davis-Dusenbery, B.N., Williams, L.A., Klim, J.R., and Eggan, K. (2014). How to make spinal motor neurons. Development *141*, 491--501. https://doi.org/10.1242/dev.097410
Del Dosso, A., Urenda, J.P., Nguyen, T., and Quadrato, G. (2020). Upgrading the Physiological Relevance of Human Brain Organoids. Neuron *107*, 1014-1028. https://doi.org/10.1016/j.neuron.2020.08.029
Di Giorgio, F.P., Boulting, G.L., Bobrowicz, S., and Eggan, K.C. (2008). Human Embryonic Stem Cell-Derived Motor Neurons Are Sensitive to the Toxic Effect of Glial Cells Carrying an ALS-Causing Mutation. Cell Stem Cell *3*, 637--648. https://doi.org/10.1016/j.stem.2008.09.017
Di Giorgio, F.P., Carrasco, M.A., Siao, M.C., Maniatis, T., and Eggan, K. (2007). Non–cell autonomous effect of glia on motor neurons in an embryonic stem cell–based ALS model. Nature Neuroscience *10*, 608--614. https://doi.org/10.1038/nn1885

Dolan, M.J., Therrien, M., Jereb, S., Kamath, T., Atkenson, T., Marsh, S., Goeva, A., Lojek, N., Murphy, S., White, C.*, et al.* (2022). A resource for generating and manipulating human microglial states in vitro. bioRxiv [PREPRINT]. https://doi.org/10.1101/2022.05.02.490100
Douvaras, P., and Fossati, V. (2015). Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. Nature Protocols *10*, 1143--1154. https://doi.org/10.1038/nprot.2015.075
Douvaras, P., Sun, B., Wang, M., Kruglikov, I., Lallos, G., Zimmer, M., Terrenoire, C., Zhang, B., Gandy, S., Schadt, E.*, et al.* (2017). Directed Differentiation of Human Pluripotent Stem Cells to Microglia. Stem Cell Reports *8*, 1516--1524. https://doi.org/10.1016/j.stemcr.2017.04.023
Douvaras, P., Wang, J., Zimmer, M., Hanchuk, S., O'Bara, Melanie A., Sadiq, S., Sim, Fraser J., Goldman, J., and Fossati, V. (2014). Efficient Generation of Myelinating Oligodendrocytes from Primary Progressive Multiple Sclerosis Patients by Induced Pluripotent Stem Cells. Stem Cell Reports *3*, 250--259. https://doi.org/10.1016/j.stemcr.2014.06.012
Drager, N.M., Sattler, S.M., Huang, C.T., Teter, O.M., Leng, K., Hashemi, S.H., Hong, J., Aviles, G., Clelland, C.D., Zhan, L.*, et al.* (2022). A CRISPRi/a platform in human iPSC-derived microglia uncovers regulators of disease states. Nat Neurosci *25*, 1149-1162. https://doi.org/10.1038/s41593-022-01131-4
Draper, J.S., Smith, K., Gokhale, P., Moore, H.D., Maltby, E., Johnson, J., Meisner, L., Zwaka, T.P., Thomson, J.A., and Andrews, P.W. (2004). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nature Biotechnology *22*, 53--54. https://doi.org/10.1038/nbt922
Du, Z.-W., Chen, H., Liu, H., Lu, J., Qian, K., Huang, C.-L., Zhong, X., Fan, F., and Zhang, S.-C. (2015). Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. Nature Communications *6*, 6626. https://doi.org/10.1038/ncomms7626
Ehrlich, M., Mozafari, S., Glatza, M., Starost, L., Velychko, S., Hallmann, A.-L., Cui, Q.-L., Schambach, A., Kim, K.-P., Bachelin, C.*, et al.* (2017). Rapid and efficient generation of oligodendrocytes from human induced pluripotent stem cells using transcription factors. Proceedings of the National Academy of Sciences *114*, E2243--E2252. https://doi.org/10.1073/pnas.1614412114
Ellison, B. (2016). Stem Cell Research and Social Justice: Aligning Scientific Progress with Social Need. Current Stem Cell Reports *2*, 328--335. https://doi.org/10.1007/s40778-016-0063-3
Espuny-Camacho, I., Arranz, A.M., Fiers, M., Snellinx, A., Ando, K., Munck, S., Bonnefont, J., Lambot, L., Corthout, N., Omodho, L.*, et al.* (2017). Hallmarks of Alzheimer's Disease in Stem-Cell-Derived Human Neurons Transplanted into Mouse Brain. Neuron *93*, 1066--1081.e1068. https://doi.org/10.1016/j.neuron.2017.02.001
Espuny-Camacho, I., Michelsen, Kimmo A., Gall, D., Linaro, D., Hasche, A., Bonnefont, J., Bali, C., Orduz, D., Bilheu, A., Herpoel, A.*, et al.* (2013). Pyramidal Neurons Derived from Human Pluripotent Stem Cells Integrate Efficiently into Mouse Brain Circuits In Vivo. Neuron *77*, 440--456. https://doi.org/10.1016/j.neuron.2012.12.011
Fjodorova, M., Noakes, Z., and Li, M. (2015). How to make striatal projection neurons. Neurogenesis *2*, e1100227. https://doi.org/10.1080/23262133.2015.1100227
Freeman, T.B., Cicchetti, F., Hauser, R.A., Deacon, T.W., Li, X.-J., Hersch, S.M., Nauert, G.M., Sanberg, P.R., Kordower, J.H., Saporta, S.*, et al.* (2000). Transplanted fetal striatum in Huntington's disease: Phenotypic development and lack of pathology. Proceedings of the National Academy of Sciences *97*, 13877--13882. https://doi.org/10.1073/pnas.97.25.13877
Gage, Fred H., and Temple, S. (2013). Neural Stem Cells: Generating and Regenerating the Brain. Neuron *80*, 588--601. https://doi.org/10.1016/j.neuron.2013.10.037
García-León, J.A., Kumar, M., Boon, R., Chau, D., One, J., Wolfs, E., Eggermont, K., Berckmans, P., Gunhanlar, N., Vrij, F.d.*, et al.* (2018). SOX10 Single Transcription Factor-Based Fast and Efficient Generation of Oligodendrocytes from Human Pluripotent Stem Cells. Stem Cell Reports *10*, 655--672. https://doi.org/10.1016/j.stemcr.2017.12.014
Giacomelli, E., Vahsen, B.F., Calder, E.L., Xu, Y., Scaber, J., Gray, E., Dafinca, R., Talbot, K., and Studer, L. (2022). Human stem cell models of neurodegeneration: From basic science of amyotrophic lateral sclerosis to clinical translation. Cell Stem Cell *29*, 11-35. https://doi.org/10.1016/j.stem.2021.12.008
Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R.*, et al.* (2010). Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. Science *330*, 841--845. https://doi.org/10.1126/science.1194637
Goto, K., Imamura, K., Komatsu, K., Mitani, K., Aiba, K., Nakatsuji, N., Inoue, M., Kawata, A., Yamashita, H., Takahashi, R.*, et al.* (2017). Simple Derivation of Spinal Motor Neurons from ESCs/iPSCs Using Sendai Virus Vectors. Molecular Therapy Methods \& Clinical Development *4*, 115--125. https://doi.org/10.1016/j.omtm.2016.12.007

Grealish, S., Diguet, E., Kirkeby, A., Mattsson, B., Heuer, A., Bramoulle, Y., Van Camp, N., Perrier, Anselme L., Hantraye, P., Björklund, A., *et al.* (2014). Human ESC-Derived Dopamine Neurons Show Similar Preclinical Efficacy and Potency to Fetal Neurons when Grafted in a Rat Model of Parkinson's Disease. Cell Stem Cell *15*, 653--665. https://doi.org/10.1016/j.stem.2014.09.017
Haenseler, W., Sansom, S.N., Buchrieser, J., Newey, S.E., Moore, C.S., Nicholls, F.J., Chintawar, S., Schnell, C., Antel, J.P., Allen, N.D., *et al.* (2017). A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. Stem Cell Reports *8*, 1727--1742. https://doi.org/10.1016/j.stemcr.2017.05.017
Hall, C.E., Yao, Z., Choi, M., Tyzack, G.E., Serio, A., Luisier, R., Harley, J., Preza, E., Arber, C., Crisp, S.J., *et al.* (2017). Progressive Motor Neuron Pathology and the Role of Astrocytes in a Human Stem Cell Model of VCP-Related ALS. Cell Reports *19*, 1739--1749. https://doi.org/10.1016/j.celrep.2017.05.024
Hallett, Penelope J., Cooper, O., Sadi, D., Robertson, H., Mendez, I., and Isacson, O. (2014). Long-Term Health of Dopaminergic Neuron Transplants in Parkinson's Disease Patients. Cell Reports *7*, 1755--1761. https://doi.org/10.1016/j.celrep.2014.05.027
Han, X., Wang, M., Duan, S., Franco, P.J., Kenty, J.H.-R., Hedrick, P., Xia, Y., Allen, A., Ferreira, L.M.R., Strominger, J.L., *et al.* (2019). Generation of hypoimmunogenic human pluripotent stem cells. Proceedings of the National Academy of Sciences *116*, 10441--10446. https://doi.org/10.1073/pnas.1902566116
Hester, M.E., Murtha, M.J., Song, S., Rao, M., Miranda, C.J., Meyer, K., Tian, J., Boulting, G., Schaffer, D.V., Zhu, M.X., *et al.* (2011). Rapid and Efficient Generation of Functional Motor Neurons From Human Pluripotent Stem Cells Using Gene Delivered Transcription Factor Codes. Molecular Therapy *19*, 1905--1912. https://doi.org/10.1038/mt.2011.135
Hiragi, T., Andoh, M., Araki, T., Shirakawa, T., Ono, T., Koyama, R., and Ikegaya, Y. (2017). Differentiation of Human Induced Pluripotent Stem Cell (hiPSC)-Derived Neurons in Mouse Hippocampal Slice Cultures. Frontiers in Cellular Neuroscience *11*, 143. https://doi.org/10.3389/fncel.2017.00143
Holm, F., Ström, S., Inzunza, J., Baker, D., Strömberg, A.-M., Rozell, B., Feki, A., Bergström, R., and Hovatta, O. (2010). An effective serum- and xeno-free chemically defined freezing procedure for human embryonic and induced pluripotent stem cells. Human Reproduction (Oxford, England) *25*, 1271--1279. https://doi.org/10.1093/humrep/deq040
Hu, Y., Qu, Z.-y., Cao, S.-y., Li, Q., Ma, L., Krencik, R., Xu, M., and Liu, Y. (2016). Directed differentiation of basal forebrain cholinergic neurons from human pluripotent stem cells. Journal of Neuroscience Methods *266*, 42--49. https://doi.org/10.1016/j.jneumeth.2016.03.017
Ishida, Y., Kawakami, H., Kitajima, H., Nishiyama, A., Sasai, Y., Inoue, H., and Muguruma, K. (2016). Vulnerability of Purkinje Cells Generated from Spinocerebellar Ataxia Type 6 Patient-Derived iPSCs. Cell Reports *17*, 1482--1490. https://doi.org/10.1016/j.celrep.2016.10.026
Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E.G., Wieghofer, P., Heinrich, A., Riemke, P., Hölscher, C., *et al.* (2013). Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nature Neuroscience *16*, 273--280. https://doi.org/10.1038/nn.3318
Kikuchi, T., Morizane, A., Doi, D., Magotani, H., Onoe, H., Hayashi, T., Mizuma, H., Takara, S., Takahashi, R., Inoue, H., *et al.* (2017). Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. Nature *548*, 592--596. https://doi.org/10.1038/nature23664
Kim, T.W., Koo, S.Y., and Studer, L. (2020). Pluripotent Stem Cell Therapies for Parkinson Disease: Present Challenges and Future Opportunities. Front Cell Dev Biol *8*, 729. https://doi.org/10.3389/fcell.2020.00729
Kim, T.W., Piao, J., Koo, S.Y., Kriks, S., Chung, S.Y., Betel, D., Socci, N.D., Choi, S.J., Zabierowski, S., Dubose, B.N., *et al.* (2021). Biphasic Activation of WNT Signaling Facilitates the Derivation of Midbrain Dopamine Neurons from hESCs for Translational Use. Cell Stem Cell *28*, 343-355 e345. https://doi.org/10.1016/j.stem.2021.01.005
Kimbrel, E.A., and Lanza, R. (2015). Current status of pluripotent stem cells: moving the first therapies to the clinic. Nature Reviews Drug Discovery *14*, 681--692. https://doi.org/10.1038/nrd4738
Klim, J.R., Li, L., Wrighton, P.J., Piekarczyk, M.S., and Kiessling, L.L. (2010). A defined glycosaminoglycan-binding substratum for human pluripotent stem cells. Nature Methods *7*, 989--994. https://doi.org/10.1038/nmeth.1532
Klim, J.R., Pintacuda, G., Nash, L.A., Guerra San Juan, I., and Eggan, K. (2021). Connecting TDP-43 Pathology with Neuropathy. Trends Neurosci *44*, 424-440. https://doi.org/10.1016/j.tins.2021.02.008
Klim, J.R., Williams, L.A., Limone, F., Juan, I.G.S., Davis-Dusenbery, B.N., Mordes, D.A., Burberry, A., Steinbaugh, M.J., Gamage, K.K., Kirchner, R., *et al.* (2019). ALS-implicated protein TDP-43 sustains

levels of STMN2, a mediator of motor neuron growth and repair. Nature Neuroscience *22*, 167--179. https://doi.org/10.1038/s41593-018-0300-4
Krencik, R., Weick, J.P., Liu, Y., Zhang, Z.-J., and Zhang, S.-C. (2011). Specification of transplantable astroglial subtypes from human pluripotent stem cells. Nature biotechnology *29*, 528--534. https://doi.org/10.1038/nbt.1877
Krencik, R., and Zhang, S.-C. (2011). Directed differentiation of functional astroglial subtypes from human pluripotent stem cells. Nature Protocols *6*, 1710--1717. https://doi.org/10.1038/nprot.2011.405
Kriks, S., Shim, J.-W., Piao, J., Ganat, Y.M., Wakeman, D.R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A.*, et al.* (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature *480*, 547--551. https://doi.org/10.1038/nature10648
Lepore, A.C., Rauck, B., Dejea, C., Pardo, A.C., Rao, M.S., Rothstein, J.D., and Maragakis, N.J. (2008). Focal transplantation–based astrocyte replacement is neuroprotective in a model of motor neuron disease. Nature Neuroscience *11*, 1294--1301. https://doi.org/10.1038/nn.2210
Li, X., Tao, Y., Bradley, R., Du, Z., Tao, Y., Kong, L., Dong, Y., Jones, J., Yan, Y., Harder, C.R.K.*, et al.* (2018). Fast Generation of Functional Subtype Astrocytes from Human Pluripotent Stem Cells. Stem Cell Reports *11*, 998--1008. https://doi.org/10.1016/j.stemcr.2018.08.019
Limone, F., Mitchell, J.M., Juan, I.G.S., Smith, J.L.M., Raghunathan, K., Couto, A., Ghosh, S.D., Meyer, D., Mello, C.J., Nemesh, J.*, et al.* (2022). Efficient generation of lower induced Motor Neurons by coupling Ngn2 expression with developmental cues. bioRxiv [PREPRINT], 2022.2001.2012.476020. https://doi.org/10.1101/2022.01.12.476020
Limone, F., Mordes, D.A., Couto, A., Pietilainen, O., Joseph, B.J., Burberry, A., Mitchell, J.M., Ghosh, S., Meyer, D., Therrien, M.*, et al.* (2021). Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neurons to degeneration in ALS. bioRxiv [PREPRINT]. https://doi.org/10.1101/2021.07.12.452054
Lippmann, Ethan S., Williams, Clay E., Ruhl, David A., Estevez-Silva, Maria C., Chapman, Edwin R., Coon, Joshua J., and Ashton, Randolph S. (2015). Deterministic HOX Patterning in Human Pluripotent Stem Cell-Derived Neuroectoderm. Stem Cell Reports *4*, 632--644. https://doi.org/10.1016/j.stemcr.2015.02.018
Liu, Y., Liu, H., Sauvey, C., Yao, L., Zarnowska, E.D., and Zhang, S.-C. (2013a). Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells. Nature Protocols *8*, 1670--1679. https://doi.org/10.1038/nprot.2013.106
Liu, Y., Weick, J.P., Liu, H., Krencik, R., Zhang, X., Ma, L., Zhou, G.-m., Ayala, M., and Zhang, S.-C. (2013b). Medial ganglionic eminence–like cells derived from human embryonic stem cells correct learning and memory deficits. Nature Biotechnology *31*, 440--447. https://doi.org/10.1038/nbt.2565
Lu, J., Zhong, X., Liu, H., Hao, L., Huang, C.T.-L., Sherafat, M.A., Jones, J., Ayala, M., Li, L., and Zhang, S.-C. (2016). Generation of serotonin neurons from human pluripotent stem cells. Nature Biotechnology *34*, 89--94. https://doi.org/10.1038/nbt.3435
Ma, L., Hu, B., Liu, Y., Vermilyea, S.C., Liu, H., Gao, L., Sun, Y., Zhang, X., and Zhang, S.C. (2012). Human embryonic stem cell-derived GABA neurons correct locomotion deficits in quinolinic acid-lesioned mice. Cell Stem Cell *10*, 455-464. https://doi.org/10.1016/j.stem.2012.01.021
Mandai, M., Watanabe, A., Kurimoto, Y., Hirami, Y., Morinaga, C., Daimon, T., Fujihara, M., Akimaru, H., Sakai, N., Shibata, Y.*, et al.* (2017). Autologous Induced Stem-Cell–Derived Retinal Cells for Macular Degeneration. The New England Journal of Medicine *376*, 1038--1046. https://doi.org/10.1056/nejmoa1608368
Maroof, Asif M., Keros, S., Tyson, Jennifer A., Ying, S.-W., Ganat, Yosif M., Merkle, Florian T., Liu, B., Goulburn, A., Stanley, Edouard G., Elefanty, Andrew G.*, et al.* (2013). Directed Differentiation and Functional Maturation of Cortical Interneurons from Human Embryonic Stem Cells. Cell Stem Cell *12*, 559--572. https://doi.org/10.1016/j.stem.2013.04.008
Martinez, J.L., Zammit, M.D., West, N.R., Christian, B.T., and Bhattacharyya, A. (2021). Corrigendum: Basal Forebrain Cholinergic Neurons: Linking Down Syndrome and Alzheimer's Disease. Front Aging Neurosci *13*, 742233. https://doi.org/10.3389/fnagi.2021.742233
Marton, R.M., Miura, Y., Sloan, S.A., Li, Q., Revah, O., Levy, R.J., Huguenard, J.R., and Pasca, S.P. (2019). Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. Nat Neurosci *22*, 484-491. https://doi.org/10.1038/s41593-018-0316-9
Maury, Y., Côme, J., Piskorowski, R.A., Salah-Mohellibi, N., Chevaleyre, V., Peschanski, M., Martinat, C., and Nedelec, S. (2015). Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes. Nature Biotechnology *33*, 89--96. https://doi.org/10.1038/nbt.3049
Merkle, F.T., Ghosh, S., Genovese, G., Handsaker, R.E., Kashin, S., Meyer, D., Karczewski, K.J., O'Dushlaine, C., Pato, C., Pato, M.*, et al.* (2022). Whole-genome analysis of human embryonic stem

cells enables rational line selection based on genetic variation. Cell Stem Cell *29*, 472-486 e477. https://doi.org/10.1016/j.stem.2022.01.011
Merkle, F.T., Ghosh, S., Kamitaki, N., Mitchell, J., Avior, Y., Mello, C., Kashin, S., Mekhoubad, S., Ilic, D., Charlton, M.*, et al.* (2017). Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. Nature *545*, 229--233. https://doi.org/10.1038/nature22312
Mertens, J., Reid, D., Lau, S., Kim, Y., and Gage, F.H. (2018). Aging in a Dish: iPSC-Derived and Directly Induced Neurons for Studying Brain Aging and Age-Related Neurodegenerative Diseases. Annu Rev Genet *52*, 271-293. https://doi.org/10.1146/annurev-genet-120417-031534
Meyer, K., Ferraiuolo, L., Miranda, C.J., Likhite, S., McElroy, S., Renusch, S., Ditsworth, D., Lagier-Tourenne, C., Smith, R.A., Ravits, J.*, et al.* (2014). Direct conversion of patient fibroblasts demonstrates non-cell autonomous toxicity of astrocytes to motor neurons in familial and sporadic ALS. Proceedings of the National Academy of Sciences *111*, 829--832. https://doi.org/10.1073/pnas.1314085111
Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., Bakiasi, G., Tsai, L.-H., Aubourg, P., Ransohoff, R.M.*, et al.* (2016). Efficient derivation of microglia-like cells from human pluripotent stem cells. Nature medicine *22*, 1358--1367. https://doi.org/10.1038/nm.4189
Muguruma, K., Nishiyama, A., Kawakami, H., Hashimoto, K., and Sasai, Y. (2015). Self-Organization of Polarized Cerebellar Tissue in 3D Culture of Human Pluripotent Stem Cells. Cell Reports *10*, 537--550. https://doi.org/10.1016/j.celrep.2014.12.051
Nehme, R., Zuccaro, E., Ghosh, S.D., Li, C., Sherwood, J.L., Pietilainen, O., Barrett, L.E., Limone, F., Worringer, K.A., Kommineni, S.*, et al.* (2018). Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission. Cell Reports *23*, 2509--2523. https://doi.org/10.1016/j.celrep.2018.04.066
Nicholas, Cory R., Chen, J., Tang, Y., Southwell, Derek G., Chalmers, N., Vogt, D., Arnold, Christine M., Chen, Y.-Jiun J., Stanley, Edouard G., Elefanty, Andrew G.*, et al.* (2013). Functional Maturation of hPSC-Derived Forebrain Interneurons Requires an Extended Timeline and Mimics Human Neural Development. Cell Stem Cell *12*, 573--586. https://doi.org/10.1016/j.stem.2013.04.005
Nishiyama, Y., Iwanami, A., Kohyama, J., Itakura, G., Kawabata, S., Sugai, K., Nishimura, S., Kashiwagi, R., Yasutake, K., Isoda, M.*, et al.* (2016). Safe and efficient method for cryopreservation of human induced pluripotent stem cell-derived neural stem and progenitor cells by a programmed freezer with a magnetic field. Neuroscience Research *107*, 20--29. https://doi.org/10.1016/j.neures.2015.11.011
Pandya, H., Shen, M.J., Ichikawa, D.M., Sedlock, A.B., Choi, Y., Johnson, K.R., Kim, G., Brown, M.A., Elkhaloun, A.G., Maric, D.*, et al.* (2017). Differentiation of human and murine induced pluripotent stem cells to microglia-like cells. Nature neuroscience *20*, 753--759. https://doi.org/10.1038/nn.4534
Paull, D., Sevilla, A., Zhou, H., Hahn, A.K., Kim, H., Napolitano, C., Tsankov, A., Shang, L., Krumholz, K., Jagadeesan, P.*, et al.* (2015). Automated, high-throughput derivation, characterization and differentiation of induced pluripotent stem cells. Nature Methods *12*, 885--892. https://doi.org/10.1038/nmeth.3507
Pera, M.F. (2011). Stem cells: The dark side of induced pluripotency. Nature *471*, 46--47. https://doi.org/10.1038/471046a
Pfisterer, U., Kirkeby, A., Torper, O., Wood, J., Nelander, J., Dufour, A., Björklund, A., Lindvall, O., Jakobsson, J., and Parmar, M. (2011). Direct conversion of human fibroblasts to dopaminergic neurons. Proceedings of the National Academy of Sciences *108*, 10343--10348. https://doi.org/10.1073/pnas.1105135108
Politis, M., and Loane, C. (2011). Serotonergic Dysfunction in Parkinson's Disease and Its Relevance to Disability. TheScientificWorldJOURNAL *11*, 1726--1734. https://doi.org/10.1100/2011/172893
Qi, Y., Zhang, X.-J., Renier, N., Wu, Z., Atkin, T., Sun, Z., Ozair, M.Z., Tchieu, J., Zimmer, B., Fattahi, F.*, et al.* (2017). Combined small-molecule inhibition accelerates the derivation of functional cortical neurons from human pluripotent stem cells. Nature Biotechnology *35*, 154--163. https://doi.org/10.1038/nbt.3777
Qian, H., Kang, X., Hu, J., Zhang, D., Liang, Z., Meng, F., Zhang, X., Xue, Y., Maimon, R., Dowdy, S.F.*, et al.* (2020). Reversing a model of Parkinson's disease with in situ converted nigral neurons. Nature *582*, 550-556. https://doi.org/10.1038/s41586-020-2388-4
Ridley, R.M., Baker, J.A., Baker, H.F., and MacLean, C.J. (1994). Restoration of cognitive abilities by cholinergic grafts in cortex of monkeys with lesions of the basal nucleus of meynert. Neuroscience *63*, 653--666. https://doi.org/10.1016/0306-4522(94)90512-6
Riolobos, L., Hirata, R.K., Turtle, C.J., Wang, P.-R., Gornalusse, G.G., Zavajlevski, M., Riddell, S.R., and Russell, D.W. (2013). HLA Engineering of Human Pluripotent Stem Cells. Molecular Therapy *21*, 1232--1241. https://doi.org/10.1038/mt.2013.59

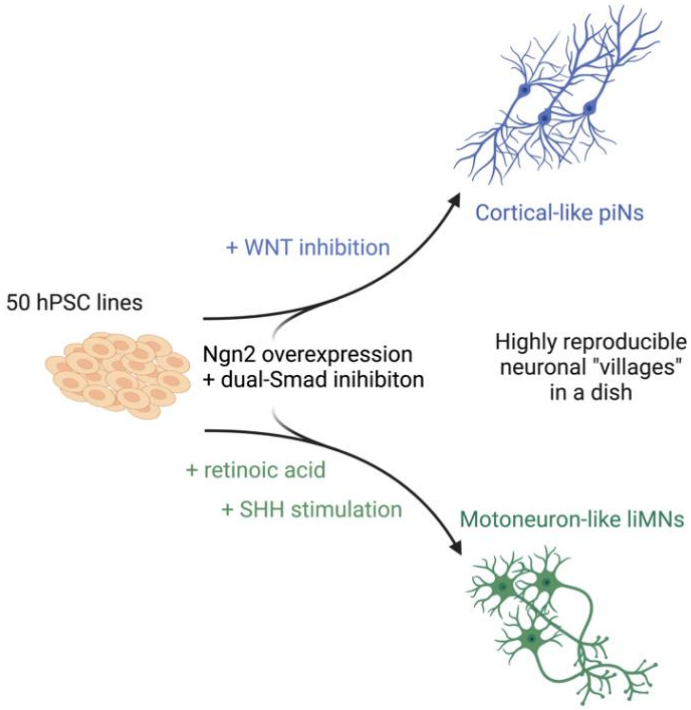
Sakaguchi, H., Kadoshima, T., Soen, M., Narii, N., Ishida, Y., Ohgushi, M., Takahashi, J., Eiraku, M., and Sasai, Y. (2015). Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. Nature Communications *6*, 8896. https://doi.org/10.1038/ncomms9896
Shaltouki, A., Peng, J., Liu, Q., Rao, M.S., and Zeng, X. (2013). Efficient Generation of Astrocytes from Human Pluripotent Stem Cells in Defined Conditions. Stem Cells *31*, 941--952. https://doi.org/10.1002/stem.1334
Silva, T.P., Bekman, E.P., Fernandes, T.G., Vaz, S.H., Rodrigues, C.A.V., Diogo, M.M., Cabral, J.M.S., and Carmo-Fonseca, M. (2020). Maturation of Human Pluripotent Stem Cell-Derived Cerebellar Neurons in the Absence of Co-culture. Frontiers in Bioengineering and Biotechnology *8*, 70. https://doi.org/10.3389/fbioe.2020.00070
Son, Esther Y., Ichida, Justin K., Wainger, Brian J., Toma, Jeremy S., Rafuse, Victor F., Woolf, Clifford J., and Eggan, K. (2011). Conversion of Mouse and Human Fibroblasts into Functional Spinal Motor Neurons. Cell Stem Cell *9*, 205--218. https://doi.org/10.1016/j.stem.2011.07.014
Song, J.-J., Oh, S.-M., Kwon, O.-C., Wulnansari, N., Lee, H.-S., Chang, M.-Y., Lee, E., Sun, W., Lee, S.-E., Chang, S.*, et al.* (2017). Cografting astrocytes improves cell therapeutic outcomes in a Parkinson's disease model. Journal of Clinical Investigation *128*, 463--482. https://doi.org/10.1172/jci93924
Sonn, I., Honda-Ozaki, F., Yoshimatsu, S., Morimoto, S., Watanabe, H., and Okano, H. (2022). Single transcription factor efficiently leads human induced pluripotent stem cells to functional microglia. Inflamm Regen *42*, 20. https://doi.org/10.1186/s41232-022-00201-1
Southwell, D.G., Nicholas, C.R., Basbaum, A.I., Stryker, M.P., Kriegstein, A.R., Rubenstein, J.L., and Alvarez-Buylla, A. (2014). Interneurons from Embryonic Development to Cell-Based Therapy. Science *344*, 1240622. https://doi.org/10.1126/science.1240622
Steinbeck, Julius A., and Studer, L. (2015). Moving Stem Cells to the Clinic: Potential and Limitations for Brain Repair. Neuron *86*, 187--206. https://doi.org/10.1016/j.neuron.2015.03.002
Sun, Alfred X., Yuan, Q., Tan, S., Xiao, Y., Wang, D., Khoo, Audrey Tze T., Sani, L., Tran, H.-D., Kim, P., Chiew, Yong S.*, et al.* (2016). Direct Induction and Functional Maturation of Forebrain GABAergic Neurons from Human Pluripotent Stem Cells. Cell Reports *16*, 1942--1953. https://doi.org/10.1016/j.celrep.2016.07.035
Svoboda, D.S., Barrasa, M.I., Shu, J., Rietjens, R., Zhang, S., Mitalipova, M., Berube, P., Fu, D., Shultz, L.D., Bell, G.W.*, et al.* (2019). Human iPSC-derived microglia assume a primary microglia-like state after transplantation into the neonatal mouse brain. Proc Natl Acad Sci U S A *116*, 25293-25303. https://doi.org/10.1073/pnas.1913541116
Takata, K., Kozaki, T., Lee, C.Z.W., Thion, M.S., Otsuka, M., Lim, S., Utami, K.H., Fidan, K., Park, D.S., Malleret, B.*, et al.* (2017). Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Tissue-Resident Macrophage Differentiation and Function. Immunity *47*, 183--198.e186. https://doi.org/10.1016/j.immuni.2017.06.017
Tao, Y., and Zhang, S.-C. (2016). Neural Subtype Specification from Human Pluripotent Stem Cells. Cell Stem Cell *19*, 573--586. https://doi.org/10.1016/j.stem.2016.10.015
Tchieu, J., Calder, E.L., Guttikonda, S.R., Gutzwiller, E.M., Aromolaran, K.A., Steinbeck, J.A., Goldstein, P.A., and Studer, L. (2019). NFIA is a gliogenic switch enabling rapid derivation of functional human astrocytes from pluripotent stem cells. Nat Biotechnol *37*, 267-275. https://doi.org/10.1038/s41587-019-0035-0
Tcw, J., Wang, M., Pimenova, A.A., Bowles, K.R., Hartley, B.J., Lacin, E., Machlovi, S.I., Abdelaal, R., Karch, C.M., Phatnani, H.*, et al.* (2017). An Efficient Platform for Astrocyte Differentiation from Human Induced Pluripotent Stem Cells. Stem Cell Reports *9*, 600-614. https://doi.org/10.1016/j.stemcr.2017.06.018
Thiruvalluvan, A., Czepiel, M., Kap, Y.A., Mantingh-Otter, I., Vainchtein, I., Kuipers, J., Bijlard, M., Baron, W., Giepmans, B., Brück, W.*, et al.* (2016). Survival and Functionality of Human Induced Pluripotent Stem Cell-Derived Oligodendrocytes in a Nonhuman Primate Model for Multiple Sclerosis. STEM CELLS Translational Medicine *5*, 1550--1561. https://doi.org/10.5966/sctm.2016-0024
Trounson, A., and DeWitt, N.D. (2016). Pluripotent stem cells progressing to the clinic. Nature Reviews Molecular Cell Biology *17*, 194--200. https://doi.org/10.1038/nrm.2016.10
Vadodaria, K.C., Mertens, J., Paquola, A., Bardy, C., Li, X., Jappelli, R., Fung, L., Marchetto, M.C., Hamm, M., Gorris, M.*, et al.* (2016). Generation of functional human serotonergic neurons from fibroblasts. Molecular Psychiatry *21*, 49--61. https://doi.org/10.1038/mp.2015.161
Victor, Matheus B., Richner, M., Hermanstyne, Tracey O., Ransdell, Joseph L., Sobieski, C., Deng, P.-Y., Klyachko, Vitaly A., Nerbonne, Jeanne M., and Yoo, Andrew S. (2014). Generation of Human

Striatal Neurons by MicroRNA-Dependent Direct Conversion of Fibroblasts. Neuron *84*, 311--323. https://doi.org/10.1016/j.neuron.2014.10.016
Wakeman, D.R., Hiller, B.M., Marmion, D.J., McMahon, C.W., Corbett, G.T., Mangan, K.P., Ma, J., Little, L.E., Xie, Z., Perez-Rosello, T.*, et al.* (2017). Cryopreservation Maintains Functionality of Human iPSC Dopamine Neurons and Rescues Parkinsonian Phenotypes In Vivo. Stem Cell Reports *9*, 149--161. https://doi.org/10.1016/j.stemcr.2017.04.033
Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., Maherali, N., Studer, L., Hochedlinger, K., Windrem, M.*, et al.* (2013). Human iPSC-Derived Oligodendrocyte Progenitor Cells Can Myelinate and Rescue a Mouse Model of Congenital Hypomyelination. Cell Stem Cell *12*, 252--264. https://doi.org/10.1016/j.stem.2012.12.002
Wang, S., Wang, B., Pan, N., Fu, L., Wang, C., Song, G., An, J., Liu, Z., Zhu, W., Guan, Y.*, et al.* (2015). Differentiation of human induced pluripotent stem cells to mature functional Purkinje neurons. Scientific Reports *5*, 9232. https://doi.org/10.1038/srep09232
Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.-H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A.*, et al.* (2010). Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. Cell Stem Cell *7*, 618--630. https://doi.org/10.1016/j.stem.2010.08.012
Watson, L.M., Wong, M.M.K., Vowles, J., Cowley, S.A., and Becker, E.B.E. (2018). A Simplified Method for Generating Purkinje Cells from Human-Induced Pluripotent Stem Cells. The Cerebellum *17*, 419--427. https://doi.org/10.1007/s12311-017-0913-2
Wichterle, H., Lieberam, I., Porter, J.A., and Jessell, T.M. (2002). Directed Differentiation of Embryonic Stem Cells into Motor Neurons. Cell *110*, 385--397. https://doi.org/10.1016/s0092-8674(02)00835-8
Windrem, M.S., Schanz, S.J., Zou, L., Chandler-Militello, D., Kuypers, N.J., Nedergaard, M., Lu, Y., Mariani, J.N., and Goldman, S.A. (2020). Human Glial Progenitor Cells Effectively Remyelinate the Demyelinated Adult Brain. Cell Reports *31*, 107658. https://doi.org/10.1016/j.celrep.2020.107658
Xu, R., Li, X., Boreland, A.J., Posyton, A., Kwan, K., Hart, R.P., and Jiang, P. (2020). Human iPSC-derived mature microglia retain their identity and functionally integrate in the chimeric mouse brain. Nat Commun *11*, 1577. https://doi.org/10.1038/s41467-020-15411-9
Xu, Z., Jiang, H., Zhong, P., Yan, Z., Chen, S., and Feng, J. (2016). Direct conversion of human fibroblasts to induced serotonergic neurons. Molecular Psychiatry *21*, 62--70. https://doi.org/10.1038/mp.2015.101
Yohn, D.C., Miles, G.B., Rafuse, V.F., and Brownstone, R.M. (2008). Transplanted Mouse Embryonic Stem-Cell-Derived Motoneurons Form Functional Motor Units and Reduce Muscle Atrophy. Journal of Neuroscience *28*, 12409--12418. https://doi.org/10.1523/jneurosci.1761-08.2008
Yu, Diana X., Di Giorgio, Francesco P., Yao, J., Marchetto, Maria C., Brennand, K., Wright, R., Mei, A., McHenry, L., Lisuk, D., Grasmick, Jaeson M.*, et al.* (2014). Modeling Hippocampal Neurogenesis Using Human Pluripotent Stem Cells. Stem Cell Reports *2*, 295--310. https://doi.org/10.1016/j.stemcr.2014.01.009
Yuan, F., Chen, X., Fang, K.-H., Wang, Y., Lin, M., Xu, S.-B., Huo, H.-Q., Xu, M., Ma, L., Chen, Y.*, et al.* (2018). Induction of human somatostatin and parvalbumin neurons by expressing a single transcription factor LIM homeobox 6. eLife *7*, e37382. https://doi.org/10.7554/elife.37382
Yuan, S.H., Martin, J., Elia, J., Flippin, J., Paramban, R.I., Hefferan, M.P., Vidal, J.G., Mu, Y., Killian, R.L., Israel, M.A.*, et al.* (2011). Cell-Surface Marker Signatures for the Isolation of Neural Stem Cells, Glia and Neurons Derived from Human Pluripotent Stem Cells. PLoS ONE *6*, e17540. https://doi.org/10.1371/journal.pone.0017540
Yue, C., and Jing, N. (2015). The promise of stem cells in the therapy of Alzheimer's disease. Translational Neurodegeneration *4*, 8. https://doi.org/10.1186/s40035-015-0029-x
Yun, S.-W., Leong, C., Zhai, D., Tan, Y.L., Lim, L., Bi, X., Lee, J.-J., Kim, H.J., Kang, N.-Y., Ng, S.H.*, et al.* (2012). Neural stem cell specific fluorescent chemical probe binding to FABP7. Proceedings of the National Academy of Sciences *109*, 10214--10217. https://doi.org/10.1073/pnas.1200817109
Zhang, S.-C., Wernig, M., Duncan, I.D., Brüstle, O., and Thomson, J.A. (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nature Biotechnology *19*, 1129--1133. https://doi.org/10.1038/nbt1201-1129
Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J.*, et al.* (2013). Rapid Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells. Neuron *78*, 785--798. https://doi.org/10.1016/j.neuron.2013.05.029
Zheng, W., Li, Q., Zhao, C., Da, Y., Zhang, H.-L., and Chen, Z. (2018). Differentiation of Glial Cells From hiPSCs: Potential Applications in Neurological Diseases and Cell Replacement Therapy. Frontiers in Cellular Neuroscience *12*, 239. https://doi.org/10.3389/fncel.2018.00239

Chapter 4:

Efficient generation of lower induced Motor Neurons by coupling *Ngn2* expression with developmental cues

In this chapter we describe a new protocol to differentiate motor neurons from human Pluripotent Stem Cells (hPSCs) for a big-scale, high-throughput study of neurodegenerative diseases with the aim to provide a platform for more reproducible modelling of ALS from large cohort of hiPSC lines.



Graphical abstract. Limone et al. induce neuralization of hPSCs into spinal MNs by small molecule patterning and TF overexpression. Multiplexed, pooled single-cell RNAsequencing showcases high reproducibility in dozens of cell lines. These MN villages resemble in vivo spinal MNs and produce disease-relevant MN populations.

This work is published on *Cell Reports,* 2022
https://doi.org/10.1016/j.celrep.2022.111896
Earlier versions of this chapter can be found on bioRxiv
https://doi.org/10.1101/2022.01.12.476020

Efficient generation of lower induced Motor Neurons by coupling *Ngn2* expression with developmental cues

Francesco Limone[1,2,3,], Jana M. Mitchell[1,2,9], Irune Guerra San Juan[1,2,6,8,9], Janell L. M. Smith[1], Kavya Raghunathan[1,2], Daniel Meyer[2,4], Sulagna Dia Ghosh[1,2,4], Alexander Couto[1], Joseph R. Klim[1,2], Brian J. Joseph[1,7], John Gold[1], Curtis J. Mello[2,4], James Nemesh[2,4], Brittany M. Smith[1], Matthijs Verhage[6,8], Steven A. McCarroll[2,4], Olli Pietiläinen[1,2,5], Ralda Nehme[1,2] and Kevin Eggan[1,10,*]*

[1]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA [2]Stanley Centre for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA [3]Leiden University medical Center, LUMC, 2333 ZA Leiden, The Netherlands [4]Department of Genetics, Harvard Medical School, Boston, MA 02115, USA [5]Neuroscience Center, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland [6]Department of Functional Genomics, Center for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, The Netherlands [7]Departments of Pathology and Cell Biology, Columbia University Irving Medical Centre, New York, NY 10032, USA [8]Human genetics, Amsterdam University Medical Center, 1081 HV Amsterdam, The Netherlands [9]These authors contributed equally [10]Lead Contact. *Correspondence: kevin.eggan@bmrn.com, francesco_limone@fas.harvard.edu

Human pluripotent stem cells (hPSCs) are a powerful tool for disease modelling of hard-to-access tissues (such as the brain). Current protocols either direct neuronal differentiation with small molecules or use transcription-factor-mediated programming. In this study, we couple overexpression of transcription factor Neurogenin2 (*Ngn2*) with small molecule patterning to differentiate hPSCs into lower induced Motor Neurons (liMoNes/liMNs). This approach induces canonical MN markers including motor neuron (MN) specific marker *Hb9/MNX1* activation in >95% of cells. liMNs resemble *bona fide* hPSC-derived MN, exhibit spontaneous electrical activity, express synaptic markers and can contact muscle cells *in vitro*. Pooled, multiplexed single-cell RNA sequencing on 50 hPSC-lines reveals reproducible populations of distinct subtypes of cervical and brachial MNs that resemble their *in vivo*, embryonic counterparts. Combining small molecule patterning with *Ngn2* overexpression facilitates high-yield, reproducible production of disease-relevant MN subtypes, which is fundamental in propelling our knowledge of MN biology and its disruption in disease.

INTRODUCTION

Many groups have recognised the ability of stem cells to differentiate into almost any cell type of the body. This unique capability can facilitate the understanding of basic biology of tissues that are hard to access and that are specifically highly evolved in humans, such as the Central Nervous System (CNS)[1]. Most neuronal differentiation schemes mimic developmental embryonic signals by small molecule patterning. The neuralisation of stem cells is achieved by manipulating bone morphogenic protein (BMP) and transforming growth factor β (TGFβ), commonly referred to as “dual-Smad inhibition”[2]. This study further showed that different combinations of small molecules used as patterning factors could push neuronal

progenitors towards distinct neuronal fates. From there, many have developed and refined differentiation protocols for specific neuronal subtypes. However, caveats still remain, such as: the incomplete neuralisation of cultures, underlining the need for additional neuralising factors[3]; the long time needed to generate mature cultures and the heterogeneity in differentiation efficiency amongst cell lines[4,5].

To overcome these limitations, others have employed different approaches such as the overexpression of a transcription factors (TFs)[6]. These TFs have been used to generate induced Neurons (iNs) from fibroblasts[7], and the combination with subtype-specific TFs was able to generate specific types of neurons[8]. These approaches have been transferred to stem cells with one of the more recent reports of *Neurogenin2* (*Ngn2*, *Neurog2*, *Atoh4*) being able to differentiate human Pluripotent Stem Cells (hPSCs) into glutamatergic neurons[9]. These advances allowed reproducible generation of neurons in a shorter time and fewer steps. This approach may, however, skip pivotal developmental steps part of neuronal specification so questions have been raised regarding the identity of the generated populations and the impact of the overexpression of TFs to downstream applications[10].

Previously, we have demonstrated that overexpression of Ngn2 coupled with small molecule patterning is able to enhance the regional specification of neurons to cortical-like patterned induced Neurons - *piNs*[11]. Additionally, small molecules have also been reported to enhance efficiency of MN programming[12,13]. These findings led us to hypothesize that combining Ngn2 expression with different patterning molecules could generate different neuronal cells.

We wanted to generate spinal Motor Neurons (MNs) for biological modelling of degenerative motoneuron diseases, such as Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA) that selectively affect these highly specialised neurons[14]. MNs reside in the spinal cord and are the only neurons to exit the nervous system and contact skeletal muscles to allow us to breathe and move through a specific synaptic contact, the Neuro-Muscular Junction (NMJ). Protocols to differentiate MNs are based on decades of developmental biology studies[15,16] and are extensively reviewed elsewhere[17,18]. Most protocols entail the neuralization inputs described above coupled with ventralising factors like Sonic Hedgehog and/or its agonists (Shh/SAG) and the caudalising effects of retinoids (retinoic acid – RA)[4,19-21] or, alternatively, the overexpression of a combination of transcription factors: *Ngn2, Isl1, Lhx3* (i.e. NILs)[12,22]. Both approaches have proven to be useful for investigating MN biology. However, on one hand directed differentiation produces cultures containing different cell types other than MNs with high line-to-line heterogeneity rendering disease modelling difficult. On the other hand, the overexpression of three TFs produces pure cultures but very specific subtypes of MNs limiting the scalability of these studies since several, specific combinations of TFs are needed to reproduce the diversity of MN subtypes *in vitro*.

Here, we report that the addition of patterning molecules during Ngn2-programming of hPSCs can lead to specification of regionally defined neuronal states. With time in culture, differentially patterned cells developed into morphologically distinct neurons that maintain regionally defined features according to developmental patterning mimicry. A reporter cell line for the MN-specific transcription factor *MNX1/Hb9* demonstrated that ~95% of the cells subjected to MN patterning activated this master regulator of MN development. This finding, in combination with the expression of pan-MN markers validated the cellular identity of SAG- and RA-patterned-Ngn2 cells as MN-like cells: the lower induced Motor Neurons (liMoNes/liMNs). liMNs expressed canonical markers and resembled bona fide hiPSC-derived MNs, they were electrophysiologically active and able to form synaptic contact with muscle cells *in vitro*. By leveraging newly developed analysis tools for single-cell RNA-sequencing (scRNAseq) technology that enable analysis of many cell lines cultured in the same dish simultaneously, we demonstrated that our protocol produced several subtypes of disease-relevant diaphragm- and limb-innervating MNs in a robust fashion, that is reproducible across 47 stem cell lines, which resemble primary MNs from the human spinal cord. This combinatorial approach addressed several shortcomings from previously published protocols and will facilitate the understanding of basic spinal MNs biology and its disruption in disease.

RESULTS

Ngn2-driven neuralization can be directed to different neuronal fates by small molecules patterning

Given that the combination of patterning molecules with Ngn2 expression could generate cortical excitatory neurons[11], we wondered whether the protocol could be repurposed with alternative patterning factors to generate other types of neurons. To test this hypothesis, we used an overexpression system in which a doxycycline inducible tetO-Ngn2-T2A-Puro/rtTA lentiviral system is used to infect hPSCs for strong overexpression of the neuralising factor Neurogenin2[9]. We started by substituting WNT inhibition, used to generate cortical cells (piNs)[11], with ventralising SAG and caudalising RA to induce a ventral-posterior fate and ultimately produce lower-induced Motor Neurons (liMNs) (Figures 1A-B).

To test if the patterning induced regionally specified neuronal states, we selected markers pivotal for early neuronal development that are divergent between cortex and spinal cord (Figures 1C). To this end we collected RNA and performed RT-qPCR at day 4, a stage described as Neuronal Progenitor Cell (NPC)-like[11], to assess the expression of these markers. While rostro-dorsalising WNT inhibition induced the expression of master regulators of cortical development *EMX1*, *FOXG1*, *OTX1* and *OTX2* (Figure 1D), the caudal-ventral patterning induced the expression of posterior markers *HOXB4* and *HOXC6*, of cholinergic

master regulator *ISL1*[23] and of *MNX1* (Hb9), expressed by spinal motor neurons in the nervous system[24] (Figure 1E). Importantly, caudal-ventral patterning reduced the expression of *OTX1* and *OTX2*, transcription factors that regulate the schism between the cortex and posterior regions of the CNS[25]. In line with previous studies, dual-Smad inhibition in combination with Ngn2 resulted in loss of pluripotency markers, *OCT4* and *SOX2*, and acquisition of pan-neuronal markers, *PAX6* and *TUBB3* (Figures S1A-B)[11].

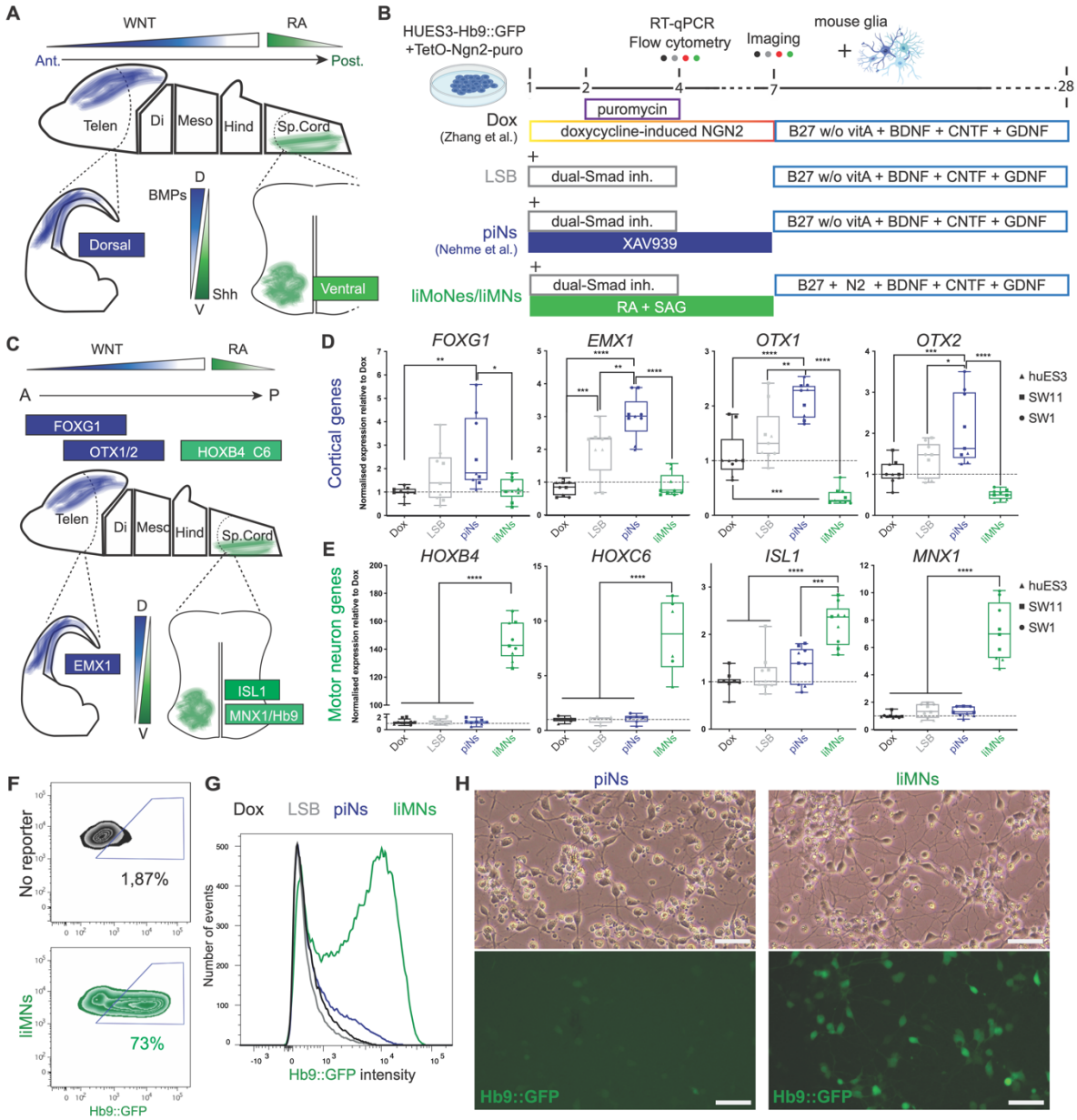


Figure 1 Ngn2-driven neuralization can be directed to different neuronal fates by small molecules patterning. (A) Diagram of known developmental cues used to design patterning strategy. **(B)** Differentiation schemes used for comparison of divergent Ngn2-driven trajectories: Dox – original Ngn2 overexpression from Zhang et al. 2013; LSB – Ngn2 overexpression coupled with neuralising dual-Smad inhibition (LDN193189, SB431542); piNs – cortical-like patterned induced Neurons (Nehme et al. 2018); liMoNes/liMNs – lower induced Motor Neurons generated by Ngn2-overexpression and ventro-caudal patterning (Retinoic Acid and Smoothened Agonist). **(C)** Genes selected as master regulators of anterior-dorsal, cortical development and ventro-caudal, spinal cord development. **(D)** RT-qPCR quantification for induction of cortical genes after rostro-dorsalising WNT inhibition at day 4 (three cell lines in *n*=3 technical replicates each, p-values from one-way ANOVA). **(E)** RT-qPCR quantification demonstrating induction of spinal genes after caudal-ventralising induction of SAG and RA at day 4 (three cell lines in *n*=3 technical replicates each, one-way ANOVA). **(F)** Flow cytometry quantification of Hb9::GFP positive cells at day 4. **(G)** Hb9::GFP intensity at day 4 of differentiation demonstrating higher total intensity of the Hb9::GFP signal in liMNs **(H)** Hb9::GFP expression day 7 post-induction in piNs and liMNs, the majority of liMNs express the reporter (scale bar 50 μm).

To further confirm the regional specification of NPCs, we took advantage of a reporter line that expresses GFP under the murine, MN-specific, Hb9 promoter[26,27] inserted into human embryonic stem cell line used to validate differentiation protocols[19,28-30]. Flow cytometry analysis confirmed that by day 4 after induction, more than 70% of cells treated with RA and SAG were GFP positive (Figures 1F). Strikingly, not only was the percentage of GFP^+ cells higher, but the intensity of GFP signal also increased (Figure 1G and S1C), in agreement with higher levels of *MNX1*/Hb9 RNA. By day 7, cells subjected to RA and SAG showed strong *Hb9::GFP* expression whereas only a fewer, dimmer GFP positive cells were visible in the other conditions (Figures 1H and S1D). Taken together, these data suggests that differential patterning coupled with Ngn2-overexpression leads to the specification of different neuronal fates, including MN.

Neuronal fates induced by patterned Ngn2 expression maintained throughout differentiation

We then proceeded to confirm that regional specification was maintained long-term after neurogenesis. For this purpose, we extended *in vitro* culturing by replating cells in neuronally supportive conditions (Figure 2A). First, we analysed cell morphology by microscopy. Patterning produced neurons with strikingly different morphology; with piNs showing small, polarised cell bodies and MN-patterned cells showing a wider soma with a multipolar shape with one extended axon-like structure (Figure 2B and S2A-B), strikingly reminiscent of the morphology of cortical pyramidal neurons and spinal, ventral-horn motor neurons *in vivo,* respectively[31].

To confirm that the regional identity specified by patterning was maintained, we collected RNA at day 30 of differentiation and investigated the expression of genes known to be specifically expressed in either glutamatergic neurons of the cortex or cholinergic MNs of the spinal cord (Figure 2C). We confirmed that caudalisation repressed cortical genes *SATB2* and *TBR1* (Figure 2D). Expression of posterior markers *HOXB4* and *HOXC6* was sustained in caudalised cells and suppressed in piNs (Figure 2E). Moreover, mature ventralised cells expressed the MN-specific TF, *MNX1*/*Hb9* and higher transcript levels of the main component of the cholinergic machinery, Choline Acetyltransferase (*CHAT*) (Figure 2E), while maintaining expression of pan-neuronal markers (Figure S2C). According to this polarised gene expression, expression of the *Hb9::GFP* reporter was also maintained through-out differentiation only in RA- and SAG-patterned cells, reaching a peak of ~95% at day 7 (Figure 2F-G and S2E), and was then slightly downregulated as seen in early development of MNs of the spinal cord *in vivo*[32]. To further ensure their MN identity and overcome some of the

limitations of the reporter, we combined the Hb9::GFP reporter with staining for Islet1 and SMI32, the triad recognised as the human pan-Motor Neuron staining[19] and confirmed that 80% of the cells co-expressed at least two of these markers (Figure 2 H-I and S2D). The data so far confirmed that coupling of Ngn2 overexpression with patterning factors can produce regionally specified neurons and we define the ventralised and caudalised cultures as lower-induced Motor Neurons: *liMoNes/liMNs*.

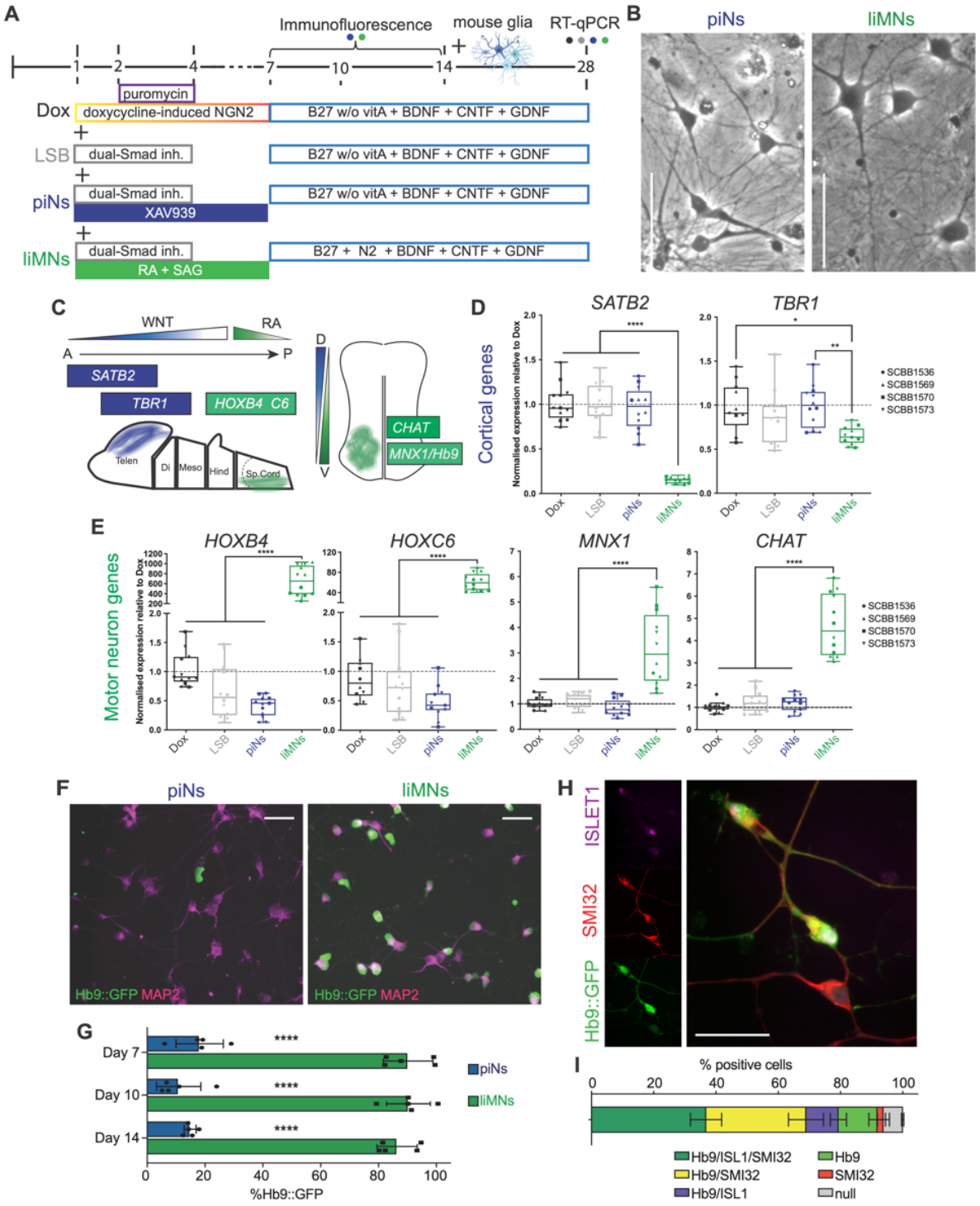


Figure 2 Patterned Ngn2-induced neuronal fate is maintained throughout the differentiation (A) Differentiation schemes for neuronal maturation after one-week of patterning: Dox – original Zhang et al. 2013; LSB – Ngn2 with dual-Smad inhibition; piNs – cortical-like piNs (Nehme et al. 2018); liMNs – lower induced Motor Neurons. **(B)** Brightfield image at day 30 of piNs and liMNs (scale bar 100 μm). **(C)** Diagram of genes specifically expressed in either anterior-dorsal cortical neurons or ventro-caudal, spinal cord motor neurons. **(D)** RT-qPCR quantification for induction of cortical genes at day 30 (four cell lines in *n*=3 technical replicates, one-way ANOVA). **(E)** RT-qPCR quantification for spinal cord genes at day 28 (four cell lines in *n*=3 technical replicates, one-way ANOVA). **(F)** Hb9::GFP reporter expression at day 14 post-induction in piNs and liMNs (scale bar 50 μm). **(G)** Quantification of Hb9::GFP reporter expression at day 7, 10 and 14 post-induction in piNs (blue) and liMNs (green) by immunofluorescence (*n*=5, p-values from t-test at each time point). **(H)** IF analysis for pan-MN SMI-32, Islet1 and Hb9::GFP reporter expression at day 7 post-induction (scale bar 50 μm). **(I)** Quantification H (*n*=3 replicates).

liMNs reproducibly express canonical pan-Motor Neuron markers and resemble bona fide hPSC-MN

Given that neuralisation by Ngn2 overexpression can be directed to different neuronal fates and maintained during *in vitro* culture, we wanted to confirm the expression of key motor neuron markers at the protein level. As early as day 14, liMNs expressed the ventral horn motor neuron specific marker Stathmin2 (STMN2) (Figure 3E)[33,34]. By day 30, liMNs expressed Cholinergic Acetyltransferase (ChAT) (Figure 3B) and limb-innervating marker Foxp1 (Figure 3C)[35]. Moreover, liMNs showed reactivity for antibodies against the transcription factor Islet1 along with SMI-32, that recognises spinal MN-enriched neurofilament heavy chain (Figure 3D). Indeed, 60-90% of cells express at least one of these markers (figure 3E), while MN markers were robustly and reproducibly expressed by 80-90% of cells by different cell lines (Figure 3F).

We next wanted to confirm that liMNs resembled cells defined by the scientific community as bona fide hiPSC-derived motor neurons. We thus differentiated MNs following a conventional, widely used method using just small molecule patterning factors (*2D MN*)[36]. Briefly, stem cells were subjected to neuralising dual-Smad inhibition followed by DAPT and SU5402 while caudalised and ventralised with RA and SAG. Differentiated neurons were separated from the mixed cultures by sorting for cell surface marker N-CAM 14 days post-neuronal induction[36], and then cultured in neuronal differentiation media, under similar conditions to liMNs for 14 more days (Figure 3G, Figure S3A-B). We then compared the morphologies of the conventional 2D MNs and liMNs by imaging. We found that liMNs were morphologically similar to 2D MN, with large multipolar cell bodies, and very distinct from cortical cells (Figure 3H). Moreover, liMNs and 2D MN expressed similar patterns of pan-MN staining (Figure S3C-D). Remarkably, RT-qPCR analysis revealed that liMNs expressed comparable levels of other motor-neuron markers and even higher transcript levels of limb-innervating motor neurons marker *HOXC6* (Figure 3I). These results confirmed that liMNs resemble one kind of bona fide hiPSC-derived motor neurons defined by the broader scientific community.

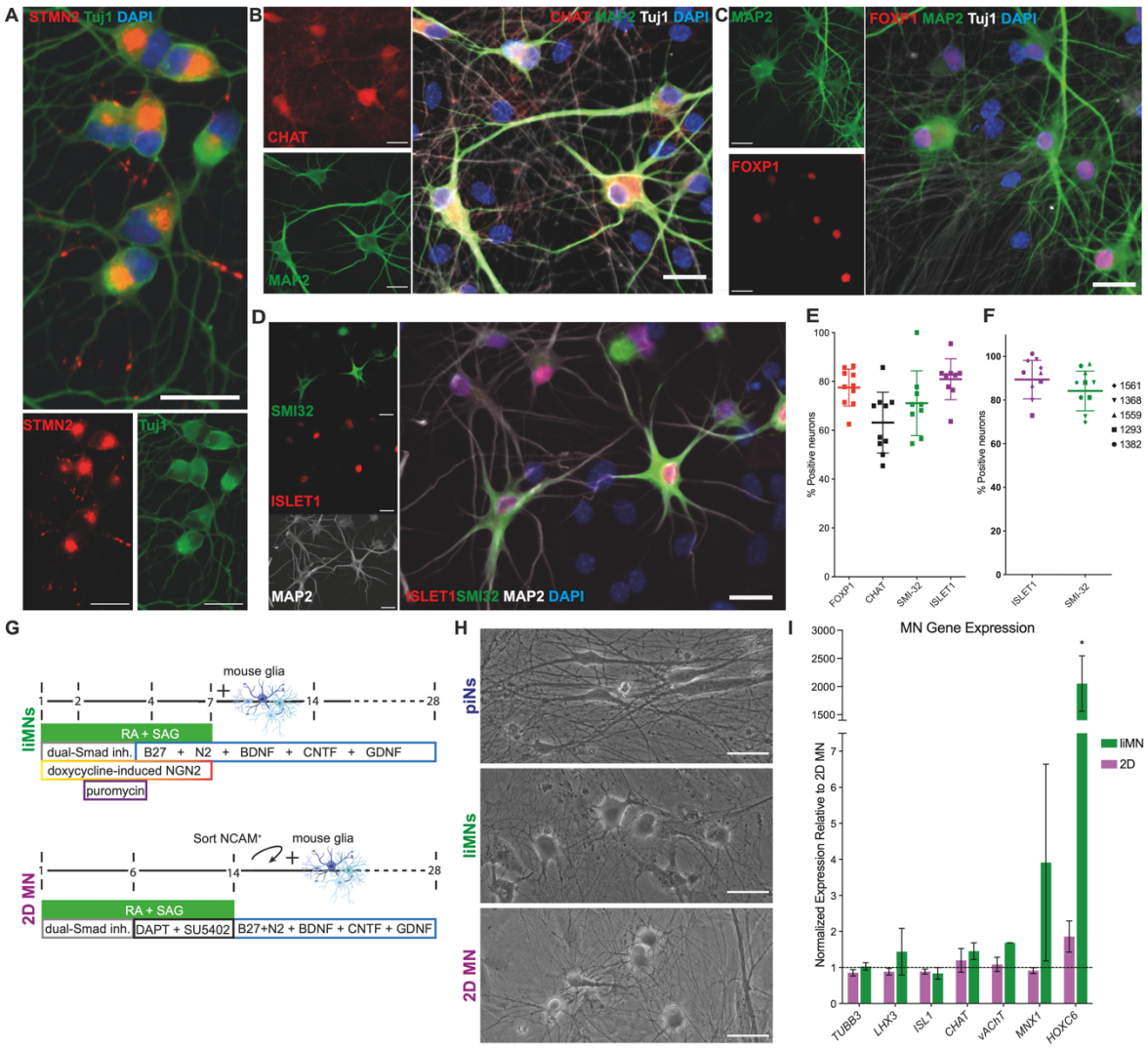


Figure 3 liMoNes reproducibly express canonical Motor Neuron markers (A) Immunofluorescent staining for spinal MN-specific marker Stathmin2 (STMN2) and neuronal cytoskeletal proteinTUBB3 (Tuj1) in day14 liMNs cultures (scale bar 100 μm). **(B)** Immunofluorescent staining for cholinergic marker Chat and neuronal cytoskeletal proteins MAP2 and TUBB3 (Tuj1) at day 30 (glial co-cultures - scale bar 30 μm). **(C)** Immunofluorescent staining for limb-innervating MN marker FOXP1 and neuronal MAP2 and TUBB3 (Tuj1) at day 30 (glial co-cultures - scale bar 30 μm). **(D)** Immunofluorescent staining for MN-enriched SMI-32, cholinergic transcription factor Islet1 and neuronal MAP2 at day 30 (glial co-cultures - scale bar 30 μm). **(E)** Quantification for cells in B-D (n=10). **(F)** Quantification of expression of selected markers in five independently differentiated lines (five cell lines, n=2 each). **(G)** Differentiation schemes implemented to compare liMNs with bona fide MN derived from pluripotent cells by conventional small molecule induction (2D MN, in purple). **(H)** Morphology of neuronal cells produced: piNs, liMNs and 2D-MN (scale bar 50 μm). **(I)** RT-qPCR quantification of MN markers between liMNs (green) and 2D-MN (purple) (n=3).

liMNs form active synaptic networks and contact muscle cells *in vitro*

We next set out to assess liMNs functional properties and ability to form synapses. liMNs expressed both pre- and post-synaptic molecules Synaptophysin and PSD-95 (Figure 4A, Figure S4A-B) and displayed abundant staining for Synapsin and axonal AnkyrinG, similarly to piNs (Figure S4C). Multielectrode arrays (MEAs) analyses showed that cultures have a steady increase in spiking rates over time (Figure S4D-E). Treating cells with potassium-gated channel opener Retigabine, a potential therapeutic agent for ALS[37,38], silenced cultures underlining the usefulness of liMNs as model for therapeutic strategies in neurodegenerative diseases (Figure 4B).

MNs are the only neurons to connect with muscles through a highly specific synapse: the NMJ. To test the ability of liMNs to form NMJ-like structures we established co-cultures with murine muscle cells in compartmentalised microfluidic devices where neurons grown in one chamber can extend axons through groves that connect to muscle cells (Figure 4C). Staining showed that liMNs extended neurites to the second chamber, contact muscle cells and form structures expressing pre-synaptic protein Synapsin (Figure 4D^{i}-D^{ii} and S5A-E), a sign of an early development of contact.

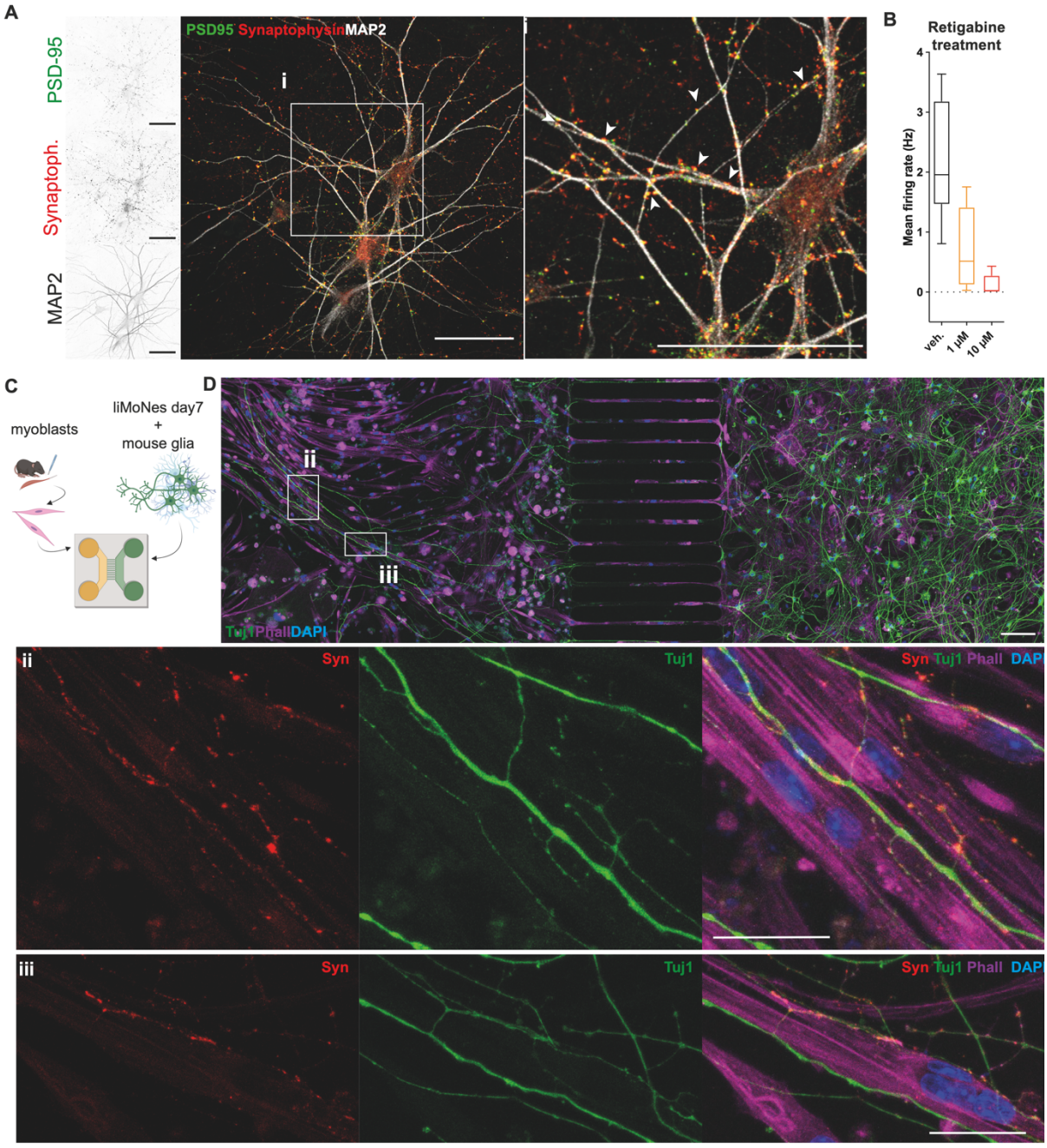


Figure 4 liMoNes can form active synaptic structures *in vitro* (A) Day50 liMNs express pre- and post-synaptic density proteins (scale bar 50 μm). **(B)** Mean number of spikes in day50 cultures treated with raising concentrations of Retigabine (n=6). **(C)** Diagram of co-culture experiments of liMNs and primary murine myoblasts in microfluidic devices. **(D)** Immunofluorescence of co-culture of liMNs and primary murine myoblasts showing glia-liMNs co-cultures (right), where neurons extend axons through the channels (middle), contacting primary muscle cells (left). (D^{i}-D^{ii}) Insets of (D) showing liMNs forming synaptic-like contacts with muscles cells (scale bar 50 μm).

scRNA-seq confirms expression of MN-specific genes and reproducibility of the protocol

After confirming the MN-like properties of liMNs, we set out to further characterise their molecular identity and reproducibility by single cell RNA sequencing. We coupled sequencing with two newly developed technologies: *Census-seq* and *Dropulation*[39,40] to enable the characterization of lines from many different donors in a single experiment. These methods utilise the intrinsic variability of single nucleotide polymorphisms (SNPs) within a population as a barcode to assign identities in a mixed culture - a "village" - of multiple donors, similarly to pooled CRISPR-Cas9 barcoded screens[41-43]. More precisely, *Census-seq* allows population-scale, quantitative identity assignment from a mixed group of donors[39], *Dropulation* can assign identities at a single cell level in a "village" for scRNAseq studies[40]. With this aim in mind, we produced liMNs "villages": 50 embryonic stem cell lines, previously subjected to whole-genome sequencing, were separately differentiated into liMNs. At day 7 post-induction, postmitotic cells were pooled in equal numbers to make up "villages" containing all donors in one dish. Using genotypes from WGS we were able to reassign the donor identities in a mixed village (Figure 5A).

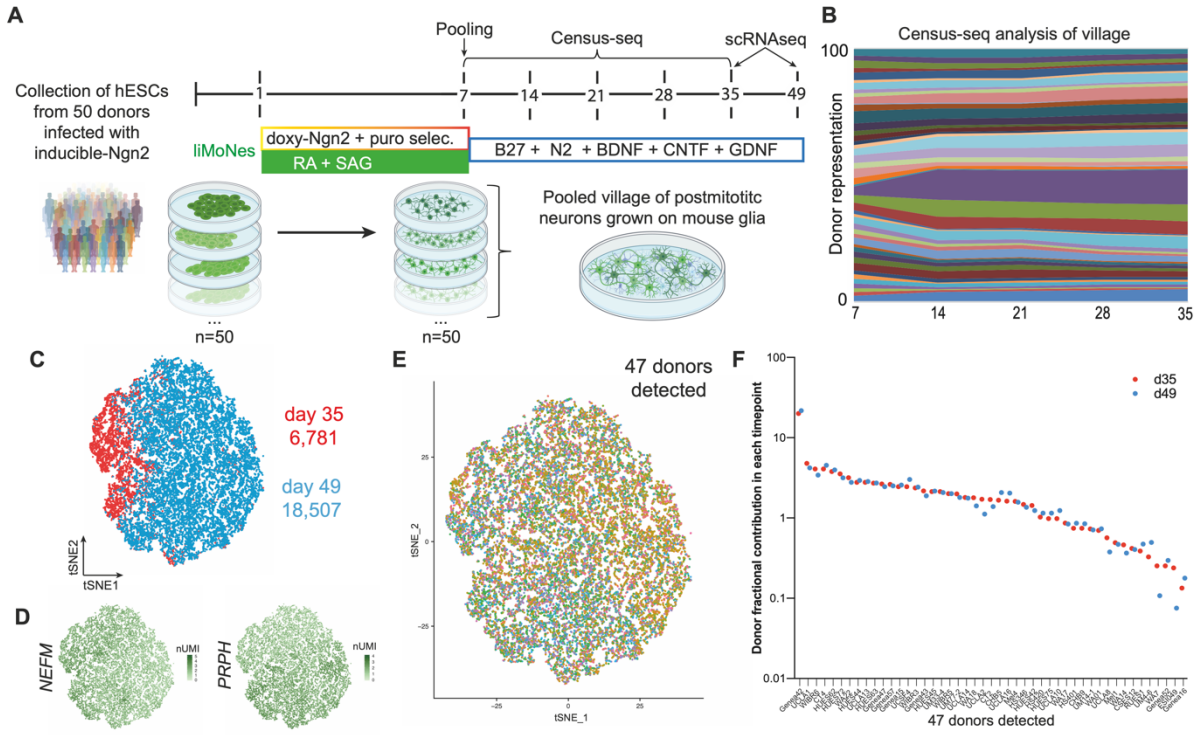


Figure 5 scRNA-seq confirms expression of MN-specific genes and reproducibility of the protocol (A) Pooling strategy and village construction for *Census-seq* and *Dropulation* analysis. **(B)** Sandplot of *Census-seq* analysis showing balanced representation of 47 detected donors throughout several days post-induction. **(C)** *t*-SNE projection of scRNAseq analysis of 25,288 cells of two timepoints of mature liMNs differentiation. **(D)** *t*-SNE projection with expression of markers for neurons of the peripheral nervous system. **(E)** *t*-SNE projection of 25,288 cells depicting donor's identity of each cell from 47 donors detected by *Dropulation* analysis. **(F)** Fraction representation of 47 donors in the two timepoints of mature liMNs differentiation.

To ensure that the donor composition remained balanced, cells were harvested once a week to collect genomic DNA for low-coverage sequencing. *Census-seq* analyses showed that we could detect 47 of the 50 donors originally pooled and confirmed that donor distribution remained consistent for four weeks (Figure 5B). Neurons were harvested at day 35 and day 49 for scRNA-seq and *Dropulation* analysis. Libraries generated from 25,288 cells demonstrated strong expression of neuronal markers, especially of the peripheral nervous system (PNS), *NEFM* and *PRPH* (Figure 5C-D and S6A). liMNs did not express cycling cells markers (Figure S6B), nor markers of ventral, spinal interneuronal pools V1, V2a, V2b, V3 nor mid-dorsal spinal interneurons V0 (Figure S6C-E). liMNs expressed MN-enriched *STMN2*, *NEFH*, *ISL1* and *MNX1* (Figure S6F)[19,33,34,44] and low but detectable expression of cholinergic genes *ACHE*, *SLC5A7* (Cht1), *SLC18A3* (vAChT) (Figure S6G). Finally, we detected expression of *AGRN* and *NRG1*, expressed by MNs to form NMJs (Figure S6H).

Using the newly devised *Dropulation* analytical pipeline, we assigned donor identity to barcoded droplets. Initial *t*-SNE clustering showed an even distribution of each donor (Figure 5E) and we confirmed that the contribution of each donor remained constant at both timepoints (Figure 5F) underlying the robustness and reproducibility of the protocol. We therefore confirmed that our protocol can reproducibly generate MN-like cells from many cell lines.

Cell villages confirm polarization generated by differential patterning of Ngn2 differentiation

To unbiasedly confirm that differential patterning strategies could generate different neuronal fates we then compared single cell libraries from liMNs to libraries similarly generated from piNs (Figure S7A). *t*-SNE clustering showed a clear separation of piNs and liMNs (Figure 6A). All cells expressed neuronal markers (Figure 6B and S7B) but piNs expressed higher levels of genes of dorsal, cortical and glutamatergic cells (Figure 6C and S7C), whereas liMNs expressed higher levels of genes of ventral, spinal and cholinergic cells (Figure 6D and S7D), confirming that the two different patterning strategies preferentially upregulate genes connected to these distinct cellular identities in a strongly polarised manner (Figure 6E). Interestingly, HOX genes, mostly expressed in the midbrain and in the spinal cord and known markers of caudalisation, were highly expressed in liMNs and barely detected in piNs (Figure 6F-G). We assigned donor identity to barcoded droplets with *Dropulation* and showed an even distribution of each donor across the different clusters (Figure S7E-F) underlying the robustness and reproducibility of these protocols.

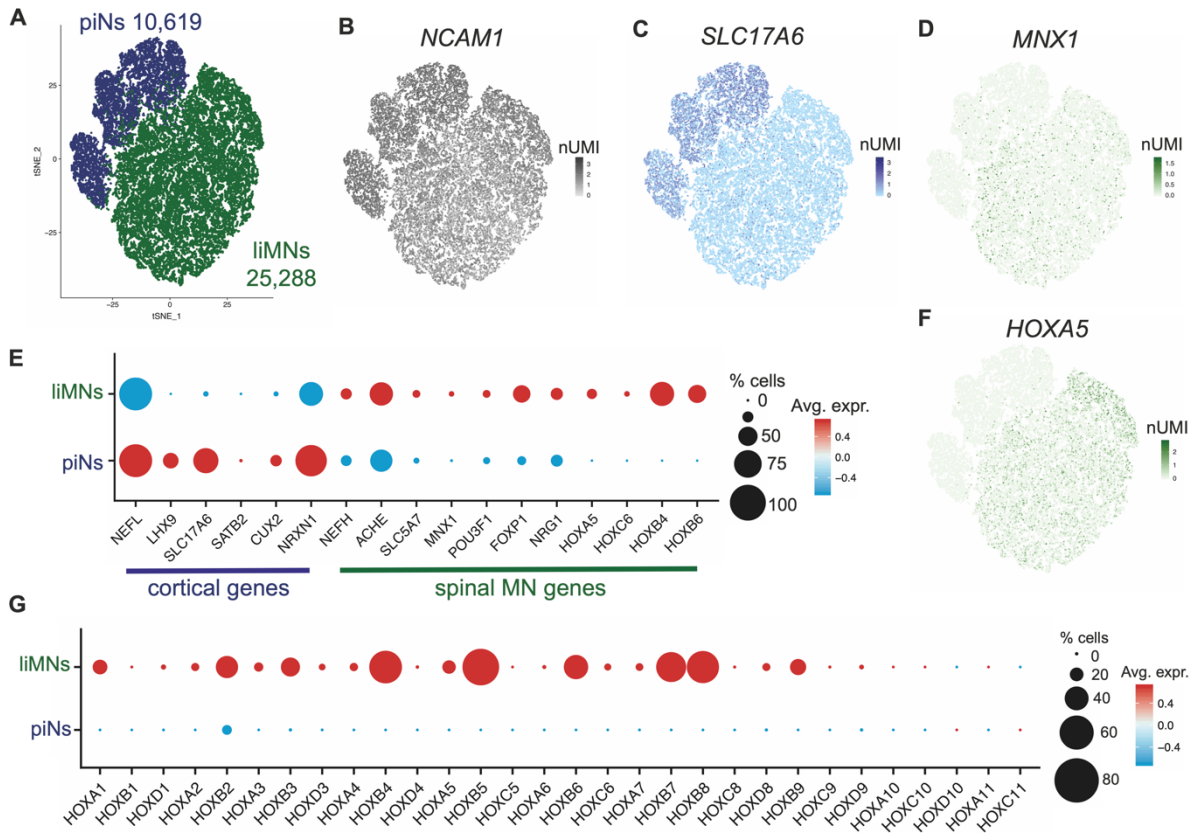


Figure 6 Confirmed divergent neuronal fate of piNs and liMNs (A) *t*-SNE projection of scRNAseq analysis of 25,288 cells of two timepoints of piNs and liMNs differentiation. **(B)** *t*-SNE projection with expression of neuronal marker. **(C)** *t*-SNE projection with expression of cortical-enriched marker. **(D)** *t*-SNE projection with expression of MN-specific marker. **(E)** Dotplot for differential gene expression of markers specific to either cortical excitatory neurons or spinal MNs. **(F)** *t*-SNE projection with expression of brachial MN-specific HOX gene expression. **(G)** Dotplot for gene expression of all retinoid-dependent HOX genes in piNs and liMNs.

Ventro-caudal patterning of Ngn2 can produce different MN subtypes

In vivo motor neurons are classified in subtypes (a.k.a. pools or columns) according to their position along the cord and the anatomical part of the body they innervate. Four groups lie in spinal cord areas developmentally regulated by retinoids: 1. Medial Motor Column (MMC), along the entire spine, connects to axial musculature to maintain posture, 2. cervical Spinal Accessory Column (SAC) innervates head and neck, 3. Phrenic Motor Column (PMC), also cervical, innervates the diaphragm, 4. Lateral Motor Column (LMC), at brachial level on the cervico-thoracic boundary, connects to forelimbs and is divided in ventral-innervating, medial or dorsal-innervating, lateral LMC (Figure 7A)[16]. Remarkably, we were able to find markers specific to these pools in our dataset: a small group of *PHOX2B*-expressing SAC-like cells, wide expression of PMC-enriched *ALCAM* and *POU3F1* (*SCIP*) and markers of both lateral- and medial-LMCs: *FOXP1* and *LHX1* (Figure S8A-D).

We wondered if the discrete expression of these markers shaped subgroups with different transcriptomic profiles. We decided to unbiasedly identify subclusters and found four groups: liMNs 0,1,2,3 (Figure 7B). Intriguingly, markers of MN pools segregated within the

groups demarcating an *ALCAM*$^{+}$ group, an *LHX1*$^{+}$ and a *FOXP1*$^{+}$ groups, and a small *PHOX2B*$^{+}$ group (Figure 7C and S8E-G). No expression of MMC markers was found (Figure S8H) consistent with reports identifying this population as less responsive to certain patterning factors[19,45]. Differential genes expression analysis for genes specifically expressed in each subgroup unbiasedly confirmed regional specification consistent with the markers described above (Figure S8I, Table S1). We observed two additional features: expression of markers of anterior digit-innervating MNs *FIGN* and *CPNE4* in a small percentage of cells (Figure S8J)[46,47]; and expression of *HOX* genes activated in response to retinoids[48] and specifically in cervical/brachial MNs[49] (Figure S8K-L). Taking advantage of the *Dropulation* technology, we investigated the distribution of donors within each subcluster and surprisingly found that each of the 47 donors distributed evenly within clusters highlighting the robustness and reproducibility of the protocol (Figure 7D and S9A-B).

To ensure that liMNs resembled cervico-brachial MNs, we integrated our data with a recently published scRNA-seq dataset generated from human embryonic spinal cord[44], and visualised the resulting dataset using UMAP (Uniform Manifold Approximation and Projection). First, we confirmed we could identify neurons and progenitors of different spinal lineages matching the cell types identified in Rayon et al. (Figure S10A-D). In the integrated analysis, liMoNes clustered closely to embryonic post-mitotic MNs (MNs) (Figure 7E), while they clustered separately from both sensory neurons and dorsal interneurons (Figure 7F) and partially closer to ventral interneurons (Figure 7G and S10E), further validating the MN-like fate of liMNs. We then isolated MN-like cells from the integrated dataset and analysed them separately from the rest of the spinal cord, liMNs and primary MN clustered separately from progenitor cells (pMNs) (Figure 7H). Consistent with *HOX* expression, liMNs clustered more closely to MN of brachial origin, consistent with the more caudal position of samples in the primary human dataset and therefore low expression of more hindbrain markers (Figure S10F-G), and from mid-to-late stages of development (Figure 7I). Taken together, our integrative analyses with human embryonic spinal cord cells not only confirms the MN identity of liMoNes, but also demonstrates that are composed of a plethora of motor neuron subtypes that intrinsically recapitulates pools and columns identified in the cervical and brachial spinal cord and that these subtypes can be robustly generated in a myriad of cells lines.

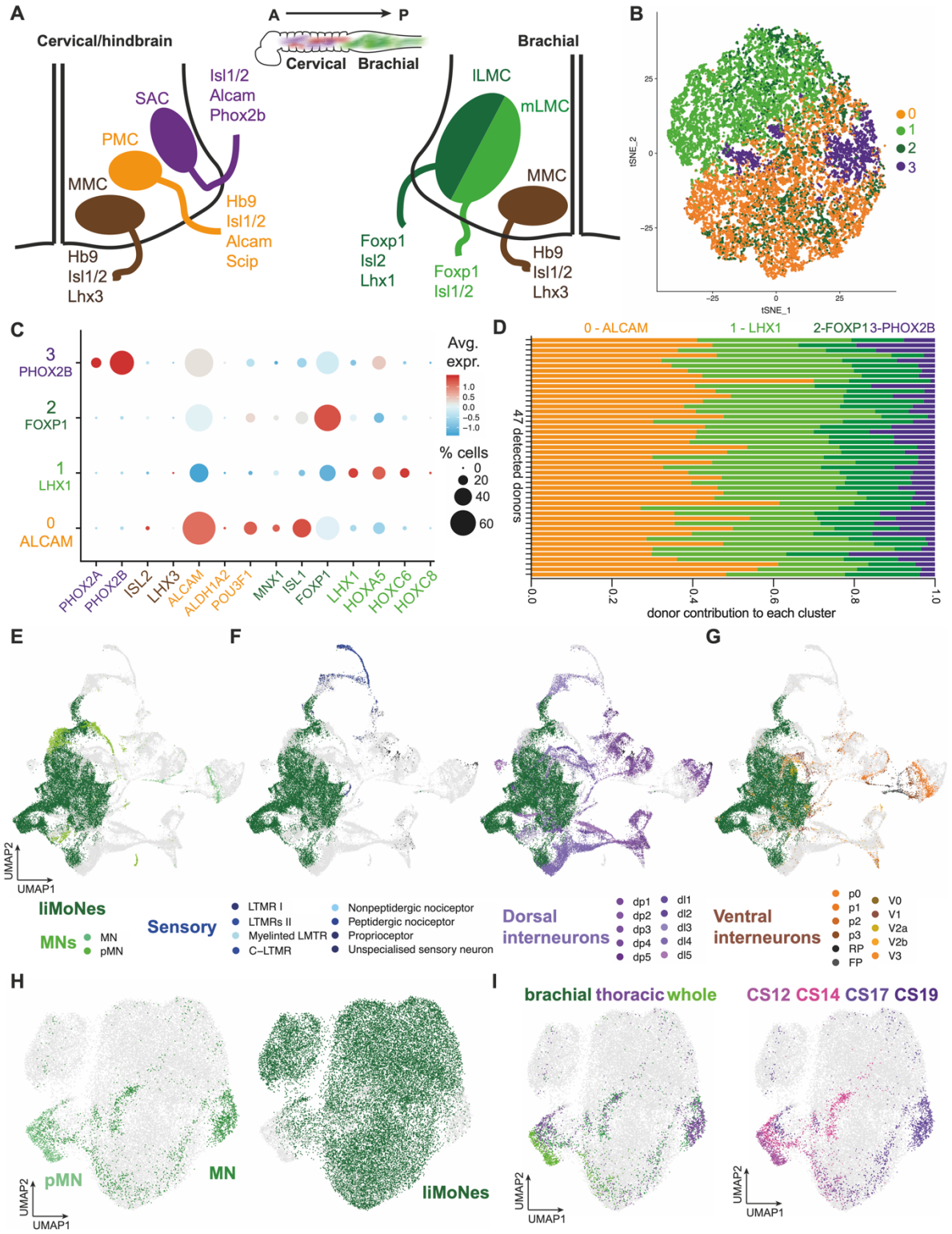


Figure 7 Ventro-caudal patterning of NGN2 can produce different MN subtypes. (A) Diagram of known pools of MN subtypes along mammalian spinal cord. **(B)** *t*-SNE projection of four, unbiasedly identified subclusters in the 25,288 cells analysed. **(C)** Dotplot for differential gene expression of MN subtype-specific markers in the four cervico-brachial MN groups. **(D)** Fraction of each donor's share between the identified subclusters as calculated by *Dropulation*. **(E)** *t*-SNE projection of integrated datasets: liMoNes and MNs and pMNs (progenitors) from human embryonic spinal cord Rayon et al. 2021. **(F)** *t*-SNE projection of integrated datasets: liMoNes, sensory neurons and dorsal interneurons from Rayon et al. 2021. **(G)** *t*-SNE projection of integrated datasets: liMoNes and ventral interneurons from Rayon et al. 2021. **(H)** *t*-SNE projection of integrated datasets with MNs only. **(I)** *t*-SNE projection of integrated datasets with MNs only with regionality and timepoints (Carnegie Stage) from Rayon et al. highlighted.

DISCUSSION

In this study we describe a rapid and efficient protocol to generate human MN-like cells from hPSCs by combining the overexpression of neuralising factor Ngn2[9] and ventralising and caudalising small molecules patterning in human ESC/iPSCs[28,50]. We demonstrated that different patterning molecules can direct Ngn2-driven neuralisation into the specification of distinct neuronal fates that are maintained during *in vitro* culture. In particular, we show that ventral-caudal patterning induces expression of the MN-specific TF *MNX1*/Hb9 in >90% of differentiated cells bypassing the previously used sorting methods to isolate MN from mixed cultures and in a shorter period of time. The ventro-caudalised cells expressed pan-MN markers as identified *in vivo* and resembled bona fide hPSC-derived MN giving them a lower motor neuron identity - hence lower induced Motor Neuron (liMoNes/liMNs). liMNs generated electrophysiologically active cultures capable to form early contact points with muscle cells *in vitro*. By leveraging newly developed single-cell RNA-sequencing analyses tools, we demonstrated that this protocol could successfully generate a previously reported hard-to-produce neuronal cell type by a straightforward one-step programming. Additionally, we showed that the differentiation scheme is highly scalable and reproducible across 47 cell lines, and that the generated cultures contain a diverse population of disease-relevant MN subtypes that closely in part resemble their human, embryonic, *in vivo* counterpart.

The protocol described here enabled us to overcome some of the main issues reported in previously published differentiation schemes based on small molecules patterning. Specifically, we showed how with a single step induction, we were able to generate in only seven days, a pure population of post-mitotic neurons in which virtually all cells expressed the MN-specific marker *MNX1*/Hb9, whereas most protocols reported at least two weeks of differentiation to achieve partial expression of this reporter[17]. Moreover, we demonstrated how the enforced expression of one transcription factor can achieve complete neuralisation of cells to avoid the heterogeneous generation of other cell types on a cell-line-to-cell-line dependent manner[4] and how this method could be replicated in dozens of pluripotent lines. This single-step, 7-day induction protocol would allow the generation of defined motor neuron cultures for *in vitro* modelling studies and avoid time-consuming and expensive cell-sorting step to select relevant cell types from mixed ventral-caudal populations[36]. Intriguingly, very few reports showed NMJ-like structures *in vitro* from human MNs[51-56] and so far only one has established a system that allows it in culture conditions that resemble human physiology[57]. The combination of our highly pure, accelerated protocol and this report could allow further understanding of NMJs in a physiologically relevant, human context.

Our study is among the first reports to highlight the malleability of Ngn2-based reprogramming and its ability to be directed to differential states by small molecules patterning mimicking embryonic development. We have thoroughly demonstrated in a previous report

that patterning can direct Ngn2 towards a cortical-like state[11], but this is the first side-by-side, systematic comparison of the ability of this programming method to diverge into different neuronal fates. Others have reported that overexpression of Ngn2 alone is able to produce an admixture of different neuronal subtypes of both the central and peripheral nervous system[10], confirming that Ngn2-driven neuralisation yields several neuronal subtypes. Here, we expand on this biology showing that small molecule patterning can direct the multipotent neuralising ability of Nuerogenin2 to populations of regionally specified neurons in a robust, reproducible manner.

Many molecular studies have shown how retinoids can specifically act as epigenetic modulators, open chromatin domains in neural progenitor cells consistent with spinal cord identity and aid posteriorisation in MN differentiation systems[13]. Moreover, transcriptomic and epigenomic studies along NIL-based MN differentiation have shown that Ngn2 acts independently of the Isl1-Lhx3 heterodimers, upregulating neuralising factors that in turn open sites of chromatin that allow further specification into MN-fates[58]. Intriguingly, others have reported that overexpression of Ngn2 in fibroblasts coupled with patterning factors could generate small populations of cholinergic neurons, hinting at the malleability of this system[59]. We speculate that the addition of patterning molecules to Ngn2-programming permits the opening of chromatin at sites of MN-specific genes usually achieved by the overexpression of other TFs forming a permissive epigenetic landscape that allows specification into motor neuron identity. Other groups have reported that in other TF-based differentiation systems, addition of RA can upregulate sets of genes that the TFs alone could not achieve[60], confirming that combinatorial approaches might aid specification into desired cell types.

The use of only one transcription factor combined with small quantities of inexpensive patterning molecules renders this protocol amenable to large-scale, high-throughput studies compared to previous studies[12]. The combinatorial use of multiple TFs often induces the generation of extremely specified subtypes of MNs[22,30] that, even though pure and well-defined, limit the ability of hPSC to differentiate into the intrinsic admixture of MNs generated by retinoids/Shh and only elicits the transcriptomic programs of restricted pools[30]. Moreover, others have demonstrated how combinations of multiple transcription factors might take longer time to develop hPSC into neurons when compared to Ngn2 alone and that the timing of overexpression could interfere with the subtypes of neurons generated[60]. Here we propose that a short pulse of Ngn2 overexpression coupled with patterning molecules not only reduces the number of TF needed to direct the specification of neuralisation but also allows intrinsic developmental processes to take place and generate myriad MN subtypes seen in spinal cord development, as shown by the similarities with our cells and primary samples. Given the differential susceptibility of subtypes of MNs to degenerate in certain diseases like ALS[61],

having both resistant and susceptible populations of MNs reproducibly generated in one dish could help to further understand the dynamic process of neurodegeneration.

Limitations of this study

One of the strengths of this protocol is its high reproducibility and accelerated nature. However, the method still lacks pivotal positional, geographical, sequentially timed signals that generate the milieu of motoneurons in the spinal cord. This method thus produces MNs that do not exactly reproduce transcriptomic profiles of columns *in vivo,* for example the incomplete co-expression of HB9 and Islet1 in young neurons that is only achieved later into the differentiation, the discrepancy between liMNs identities at protein and RNA-levels, or the incomplete overlap of certain markers between liMNs and their *in vivo* counterparts. Considering different concentrations and timing of patterning molecules and also exploring RA-independent ways of generating MNs[45,62], would be an important follow-up to this study. Furthermore, our microfluidic system did not show clustering of postsynaptic acetylcholine receptors (AchR) on muscle cells. Formation of mature NMJ contacts has been a primary limitation of in vitro hPSC-derived MNs and muscle co-cultures as observed *in vivo*[63]. Towards optimization of this model, a recent study has shown that supplementation of agrin and laminin increased clustering of AchR in in vitro human co-cultures system[57], adapting this system to our protocol might provide essential steps for the further maturation of these synaptic structures for liMNs. These discrepancies are shortcomings of accelerated systems like ours, nonetheless, our approach provides a platform for the study of the biology of different MN subtypes and their functionality in health and disease in a scalable, highly reproducible manner never achieved before.

REFERENCES

1. Penney, J., Ralvenius, W.T., and Tsai, L.H. (2020). Modeling Alzheimer's disease with iPSC-derived brain cells. Mol Psychiatry *25*, 148-167. 10.1038/s41380-019-0468-3.
2. Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol *27*, 275-280. 10.1038/nbt.1529.
3. Chambers, S.M., Qi, Y., Mica, Y., Lee, G., Zhang, X.J., Niu, L., Bilsland, J., Cao, L., Stevens, E., Whiting, P., et al. (2012). Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors. Nat Biotechnol *30*, 715-720. 10.1038/nbt.2249.
4. Boulting, G.L., Kiskinis, E., Croft, G.F., Amoroso, M.W., Oakley, D.H., Wainger, B.J., Williams, D.J., Kahler, D.J., Yamaki, M., Davidow, L., et al. (2011). A functionally characterized test set of human induced pluripotent stem cells. Nat Biotechnol *29*, 279-286. 10.1038/nbt.1783.
5. Kampmann, M. (2020). CRISPR-based functional genomics for neurological disease. Nat Rev Neurol *16*, 465-480. 10.1038/s41582-020-0373-z.
6. Mertens, J., Marchetto, M.C., Bardy, C., and Gage, F.H. (2016). Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience. Nat Rev Neurosci *17*, 424-437. 10.1038/nrn.2016.46.

7. Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Sudhof, T.C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature *463*, 1035-1041. 10.1038/nature08797.
8. Son, E.Y., Ichida, J.K., Wainger, B.J., Toma, J.S., Rafuse, V.F., Woolf, C.J., and Eggan, K. (2011). Conversion of mouse and human fibroblasts into functional spinal motor neurons. Cell Stem Cell *9*, 205-218. 10.1016/j.stem.2011.07.014.
9. Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J., et al. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. Neuron *78*, 785-798. 10.1016/j.neuron.2013.05.029.
10. Lin, H.C., He, Z., Ebert, S., Schornig, M., Santel, M., Nikolova, M.T., Weigert, A., Hevers, W., Kasri, N.N., Taverna, E., et al. (2021). NGN2 induces diverse neuron types from human pluripotency. Stem Cell Reports. 10.1016/j.stemcr.2021.07.006.
11. Nehme, R., Zuccaro, E., Ghosh, S.D., Li, C., Sherwood, J.L., Pietilainen, O., Barrett, L.E., Limone, F., Worringer, K.A., Kommineni, S., et al. (2018). Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission. Cell Rep *23*, 2509-2523. 10.1016/j.celrep.2018.04.066.
12. Hester, M.E., Murtha, M.J., Song, S., Rao, M., Miranda, C.J., Meyer, K., Tian, J., Boulting, G., Schaffer, D.V., Zhu, M.X., et al. (2011). Rapid and efficient generation of functional motor neurons from human pluripotent stem cells using gene delivered transcription factor codes. Mol Ther *19*, 1905-1912. 10.1038/mt.2011.135.
13. Mazzoni, E.O., Mahony, S., Peljto, M., Patel, T., Thornton, S.R., McCuine, S., Reeder, C., Boyer, L.A., Young, R.A., Gifford, D.K., and Wichterle, H. (2013). Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals. Nat Neurosci *16*, 1191-1198. 10.1038/nn.3490.
14. Han, S.S., Williams, L.A., and Eggan, K.C. (2011). Constructing and deconstructing stem cell models of neurological disease. Neuron *70*, 626-644. 10.1016/j.neuron.2011.05.003.
15. Jessell, T.M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet *1*, 20-29. 10.1038/35049541.
16. Stifani, N. (2014). Motor neurons and the generation of spinal motor neuron diversity. Front Cell Neurosci *8*, 293. 10.3389/fncel.2014.00293.
17. Sances, S., Bruijn, L.I., Chandran, S., Eggan, K., Ho, R., Klim, J.R., Livesey, M.R., Lowry, E., Macklis, J.D., Rushton, D., et al. (2016). Modeling ALS with motor neurons derived from human induced pluripotent stem cells. Nat Neurosci *19*, 542-553. 10.1038/nn.4273.
18. Davis-Dusenbery, B.N., Williams, L.A., Klim, J.R., and Eggan, K. (2014). How to make spinal motor neurons. Development *141*, 491-501. 10.1242/dev.097410.
19. Amoroso, M.W., Croft, G.F., Williams, D.J., O'Keeffe, S., Carrasco, M.A., Davis, A.R., Roybon, L., Oakley, D.H., Maniatis, T., Henderson, C.E., and Wichterle, H. (2013). Accelerated high-yield generation of limb-innervating motor neurons from human stem cells. J Neurosci *33*, 574-586. 10.1523/JNEUROSCI.0906-12.2013.
20. Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., et al. (2008). Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science *321*, 1218-1221. 10.1126/science.1158799.
21. Kiskinis, E., Sandoe, J., Williams, L.A., Boulting, G.L., Moccia, R., Wainger, B.J., Han, S., Peng, T., Thams, S., Mikkilineni, S., et al. (2014). Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. Cell Stem Cell *14*, 781-795. 10.1016/j.stem.2014.03.004.
22. De Santis, R., Garone, M.G., Pagani, F., de Turris, V., Di Angelantonio, S., and Rosa, A. (2018). Direct conversion of human pluripotent stem cells into cranial motor neurons using a piggyBac vector. Stem Cell Res *29*, 189-196. 10.1016/j.scr.2018.04.012.
23. Pfaff, S.L., Mendelsohn, M., Stewart, C.L., Edlund, T., and Jessell, T.M. (1996). Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell *84*, 309-320. 10.1016/s0092-8674(00)80985-x.
24. Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T.M., and Sockanathan, S. (1999). Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron *23*, 659-674. 10.1016/s0896-6273(01)80026-x.
25. Muhr, J., Graziano, E., Wilson, S., Jessell, T.M., and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. Neuron *23*, 689-702. 10.1016/s0896-6273(01)80028-3.
26. Di Giorgio, F.P., Carrasco, M.A., Siao, M.C., Maniatis, T., and Eggan, K. (2007). Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. Nat Neurosci *10*, 608-614. 10.1038/nn1885.
27. Wichterle, H., Lieberam, I., Porter, J.A., and Jessell, T.M. (2002). Directed differentiation of embryonic stem cells into motor neurons. Cell *110*, 385-397. 10.1016/s0092-8674(02)00835-8.

28. Di Giorgio, F.P., Boulting, G.L., Bobrowicz, S., and Eggan, K.C. (2008). Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. Cell Stem Cell *3*, 637-648. 10.1016/j.stem.2008.09.017.
29. Machado, C.B., Kanning, K.C., Kreis, P., Stevenson, D., Crossley, M., Nowak, M., Iacovino, M., Kyba, M., Chambers, D., Blanc, E., and Lieberam, I. (2014). Reconstruction of phrenic neuron identity in embryonic stem cell-derived motor neurons. Development *141*, 784-794. 10.1242/dev.097188.
30. Mazzoni, E.O., Mahony, S., Closser, M., Morrison, C.A., Nedelec, S., Williams, D.J., An, D., Gifford, D.K., and Wichterle, H. (2013). Synergistic binding of transcription factors to cell-specific enhancers programs motor neuron identity. Nat Neurosci *16*, 1219-1227. 10.1038/nn.3467.
31. Abdel-Maguid, T.E., and Bowsher, D. (1984). Classification of neurons by dendritic branching pattern. A categorisation based on Golgi impregnation of spinal and cranial somatic and visceral afferent and efferent cells in the adult human. J Anat *138 (Pt 4)*, 689-702.
32. Thaler, J.P., Koo, S.J., Kania, A., Lettieri, K., Andrews, S., Cox, C., Jessell, T.M., and Pfaff, S.L. (2004). A postmitotic role for Isl-class LIM homeodomain proteins in the assignment of visceral spinal motor neuron identity. Neuron *41*, 337-350. 10.1016/s0896-6273(04)00011-x.
33. Guerra San Juan, I., Nash, L.A., Smith, K.S., Leyton-Jaimes, M.F., Qian, M., Klim, J.R., Limone, F., Dorr, A.B., Couto, A., Pintacuda, G., et al. (2022). Loss of mouse Stmn2 function causes motor neuropathy. Neuron. 10.1016/j.neuron.2022.02.011.
34. Yadav, A., Matson, K.J.E., Li, L., Hua, I., Gaur, P., Alkaslasi, M., Hasan, S., Galuta, A., A., D., Ameri, S., et al. (2022). The Human Motoneuron Expression Signature is Defined by ALS-Related Genes. bioRxiv [PREPRINT]. https://doi.org/10.1101/2022.03.25.485808.
35. Dasen, J.S., De Camilli, A., Wang, B., Tucker, P.W., and Jessell, T.M. (2008). Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. Cell *134*, 304-316. 10.1016/j.cell.2008.06.019.
36. Klim, J.R., Williams, L.A., Limone, F., Guerra San Juan, I., Davis-Dusenbery, B.N., Mordes, D.A., Burberry, A., Steinbaugh, M.J., Gamage, K.K., Kirchner, R., et al. (2019). ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair. Nat Neurosci *22*, 167-179. 10.1038/s41593-018-0300-4.
37. Wainger, B.J., Kiskinis, E., Mellin, C., Wiskow, O., Han, S.S., Sandoe, J., Perez, N.P., Williams, L.A., Lee, S., Boulting, G., et al. (2014). Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. Cell Rep *7*, 1-11. 10.1016/j.celrep.2014.03.019.
38. Wainger, B.J., Macklin, E.A., Vucic, S., McIlduff, C.E., Paganoni, S., Maragakis, N.J., Bedlack, R., Goyal, N.A., Rutkove, S.B., Lange, D.J., et al. (2021). Effect of Ezogabine on Cortical and Spinal Motor Neuron Excitability in Amyotrophic Lateral Sclerosis: A Randomized Clinical Trial. JAMA Neurol *78*, 186-196. 10.1001/jamaneurol.2020.4300.
39. Mitchell, J.M., Nemesh, J., Ghosh, S., Handsaker, R.E., Mello, C.J., Meyer, D., Raghunathan, K., de Rivera, H., Tegtmeyer, M., Hawes, D., et al. (2020). Mapping genetic effects on cellular phenotypes with “cell villages”. bioRxiv [PREPRINT]. https://doi.org/10.1101/2020.06.29.174383.
40. Wells, M.F., Nemesh, J., Ghosh, S., Mitchell, J.M., Mello, C.J., Meyer, D., Raghunathan, K., Tegtmeyer, M., Hawes, D., Neumann, A., et al. (2021). Natural variation in gene expression and Zika virus susceptibility revealed by villages of neural progenitor cells. bioRxiv [PREPRINT]. https://doi.org/10.1101/2021.11.08.467815
41. Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelson, T., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G., and Zhang, F. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. Science *343*, 84-87. 10.1126/science.1247005.
42. Wang, T., Wei, J.J., Sabatini, D.M., and Lander, E.S. (2014). Genetic screens in human cells using the CRISPR-Cas9 system. Science *343*, 80-84. 10.1126/science.1246981.
43. Ashoti, A., Limone, F., van Kranenburg, M., Alemany, A., Baak, M., Vivie, J., Piccioni, F., Dijkers, P.F., Creyghton, M., Eggan, K., and Geijsen, N. (2022). Considerations and practical implications of performing a phenotypic CRISPR/Cas survival screen. PLoS One *17*, e0263262. 10.1371/journal.pone.0263262.
44. Rayon, T., Maizels, R.J., Barrington, C., and Briscoe, J. (2021). Single-cell transcriptome profiling of the human developing spinal cord reveals a conserved genetic programme with human-specific features. Development *148*. 10.1242/dev.199711.
45. Patani, R., Hollins, A.J., Wishart, T.M., Puddifoot, C.A., Alvarez, S., de Lera, A.R., Wyllie, D.J., Compston, D.A., Pedersen, R.A., Gillingwater, T.H., et al. (2011). Retinoid-independent motor neurogenesis from human embryonic stem cells reveals a medial columnar ground state. Nat Commun *2*, 214. 10.1038/ncomms1216.
46. Mendelsohn, A.I., Dasen, J.S., and Jessell, T.M. (2017). Divergent Hox Coding and Evasion of Retinoid Signaling Specifies Motor Neurons Innervating Digit Muscles. Neuron *93*, 792-805 e794. 10.1016/j.neuron.2017.01.017.

47. Blum, J.A., Klemm, S., Shadrach, J.L., Guttenplan, K.A., Nakayama, L., Kathiria, A., Hoang, P.T., Gautier, O., Kaltschmidt, J.A., Greenleaf, W.J., and Gitler, A.D. (2021). Single-cell transcriptomic analysis of the adult mouse spinal cord reveals molecular diversity of autonomic and skeletal motor neurons. Nat Neurosci *24*, 572-583. 10.1038/s41593-020-00795-0.
48. Philippidou, P., and Dasen, J.S. (2013). Hox genes: choreographers in neural development, architects of circuit organization. Neuron *80*, 12-34. 10.1016/j.neuron.2013.09.020.
49. Dasen, J.S., Tice, B.C., Brenner-Morton, S., and Jessell, T.M. (2005). A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. Cell *123*, 477-491. 10.1016/j.cell.2005.09.009.
50. Li, X.J., Du, Z.W., Zarnowska, E.D., Pankratz, M., Hansen, L.O., Pearce, R.A., and Zhang, S.C. (2005). Specification of motoneurons from human embryonic stem cells. Nat Biotechnol *23*, 215-221. 10.1038/nbt1063.
51. Rigamonti, A., Repetti, G.G., Sun, C., Price, F.D., Reny, D.C., Rapino, F., Weisinger, K., Benkler, C., Peterson, Q.P., Davidow, L.S., et al. (2016). Large-Scale Production of Mature Neurons from Human Pluripotent Stem Cells in a Three-Dimensional Suspension Culture System. Stem Cell Reports *6*, 993-1008. 10.1016/j.stemcr.2016.05.010.
52. Demestre, M., Orth, M., Fohr, K.J., Achberger, K., Ludolph, A.C., Liebau, S., and Boeckers, T.M. (2015). Formation and characterisation of neuromuscular junctions between hiPSC derived motoneurons and myotubes. Stem Cell Res *15*, 328-336. 10.1016/j.scr.2015.07.005.
53. Guo, X., Gonzalez, M., Stancescu, M., Vandenburgh, H.H., and Hickman, J.J. (2011). Neuromuscular junction formation between human stem cell-derived motoneurons and human skeletal muscle in a defined system. Biomaterials *32*, 9602-9611. 10.1016/j.biomaterials.2011.09.014.
54. Lin, C.Y., Yoshida, M., Li, L.T., Ikenaka, A., Oshima, S., Nakagawa, K., Sakurai, H., Matsui, E., Nakahata, T., and Saito, M.K. (2019). iPSC-derived functional human neuromuscular junctions model the pathophysiology of neuromuscular diseases. JCI Insight *4*. 10.1172/jci.insight.124299.
55. Puttonen, K.A., Ruponen, M., Naumenko, N., Hovatta, O.H., Tavi, P., and Koistinaho, J. (2015). Generation of Functional Neuromuscular Junctions from Human Pluripotent Stem Cell Lines. Front Cell Neurosci *9*, 473. 10.3389/fncel.2015.00473.
56. Umbach, J.A., Adams, K.L., Gundersen, C.B., and Novitch, B.G. (2012). Functional neuromuscular junctions formed by embryonic stem cell-derived motor neurons. PLoS One *7*, e36049. 10.1371/journal.pone.0036049.
57. Stoklund Dittlau, K., Krasnow, E.N., Fumagalli, L., Vandoorne, T., Baatsen, P., Kerstens, A., Giacomazzi, G., Pavie, B., Rossaert, E., Beckers, J., et al. (2021). Human motor units in microfluidic devices are impaired by FUS mutations and improved by HDAC6 inhibition. Stem Cell Reports *16*, 2213-2227. 10.1016/j.stemcr.2021.03.029.
58. Velasco, S., Ibrahim, M.M., Kakumanu, A., Garipler, G., Aydin, B., Al-Sayegh, M.A., Hirsekorn, A., Abdul-Rahman, F., Satija, R., Ohler, U., et al. (2017). A Multi-step Transcriptional and Chromatin State Cascade Underlies Motor Neuron Programming from Embryonic Stem Cells. Cell Stem Cell *20*, 205-217 e208. 10.1016/j.stem.2016.11.006.
59. Liu, M.L., Zang, T., Zou, Y., Chang, J.C., Gibson, J.R., Huber, K.M., and Zhang, C.L. (2013). Small molecules enable neurogenin 2 to efficiently convert human fibroblasts into cholinergic neurons. Nat Commun *4*, 2183. 10.1038/ncomms3183.
60. Nickolls, A.R., Lee, M.M., Espinoza, D.F., Szczot, M., Lam, R.M., Wang, Q., Beers, J., Zou, J., Nguyen, M.Q., Solinski, H.J., et al. (2020). Transcriptional Programming of Human Mechanosensory Neuron Subtypes from Pluripotent Stem Cells. Cell Rep *30*, 932-946 e937. 10.1016/j.celrep.2019.12.062.
61. Nijssen, J., Comley, L.H., and Hedlund, E. (2017). Motor neuron vulnerability and resistance in amyotrophic lateral sclerosis. Acta Neuropathol *133*, 863-885. 10.1007/s00401-017-1708-8.
62. Maury, Y., Come, J., Piskorowski, R.A., Salah-Mohellibi, N., Chevaleyre, V., Peschanski, M., Martinat, C., and Nedelec, S. (2015). Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes. Nat Biotechnol *33*, 89-96. 10.1038/nbt.3049.
63. Jones, R.A., Harrison, C., Eaton, S.L., Llavero Hurtado, M., Graham, L.C., Alkhammash, L., Oladiran, O.A., Gale, A., Lamont, D.J., Simpson, H., et al. (2017). Cellular and Molecular Anatomy of the Human Neuromuscular Junction. Cell Rep *21*, 2348-2356. 10.1016/j.celrep.2017.11.008.
64. Fukuda, A., Hazelbaker, D.Z., Motosugi, N., Hao, J., Limone, F., Beccard, A., Mazzucato, P., Messana, A., Okada, C., San Juan, I.G., et al. (2021). De novo DNA methyltransferases DNMT3A and DNMT3B are essential for XIST silencing for erosion of dosage compensation in pluripotent stem cells. Stem Cell Reports *16*, 2138-2148. 10.1016/j.stemcr.2021.07.015.
65. Mordes, D.A., Prudencio, M., Goodman, L.D., Klim, J.R., Moccia, R., Limone, F., Pietilainen, O., Chowdhary, K., Dickson, D.W., Rademakers, R., et al. (2018). Dipeptide repeat proteins activate a

heat shock response found in C9ORF72-ALS/FTLD patients. Acta Neuropathol Commun *6*, 55. 10.1186/s40478-018-0555-8.
66. Limone, F., Mordes, D.A., Couto, A., Pietilainen, O., Joseph, B.J., Burberry, A., Mitchell, J.M., Ghosh, S., Meyer, D., Therrien, M., et al. (2021). Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neurons to degeneration in ALS. bioRxiv [PREPRINT]. doi.org/10.1101/2021.07.12.452054.
67. Shahini, A., Vydiam, K., Choudhury, D., Rajabian, N., Nguyen, T., Lei, P., and Andreadis, S.T. (2018). Efficient and high yield isolation of myoblasts from skeletal muscle. Stem Cell Res *30*, 122-129. 10.1016/j.scr.2018.05.017.
68. Hao, J., Wells, M.F., Niu, G., Guerra San Juan, I., Limone, F., Fukuda, A., Lyeton-Jaimes, M.F., Joseph, B.J., Qian, M., Mordes, D.A., et al. (2021). Loss of TBK1 activity leads to TDP-43 proteinopathy through lysosomal dysfunction in human motor neurons. bioRxiv [PREPRINT]. doi.org/10.1101/2021.10.11.464011.
69. Merkle, F.T., Ghosh, S., Kamitaki, N., Mitchell, J., Avior, Y., Mello, C., Kashin, S., Mekhoubad, S., Ilic, D., Charlton, M., et al. (2017). Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. Nature *545*, 229-233. 10.1038/nature22312.
70. Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell *161*, 1202-1214. 10.1016/j.cell.2015.05.002.
71. Limone, F., Mitchell, J.M., Guerra San Juan, I., Smith, J.L.M., Raghunathan, K., Couto, A., Ghosh, S., Meyer, D., Mello, C.J., Nemesh, J., et al. (2022). Efficient generation of lower induced Motor Neurons by coupling Ngn2 expression with developmental cues. bioRxiv [PREPRINT]. doi.org/10.1101/2022.01.12.476020.
72. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., 3rd, Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. Cell *177*, 1888-1902 e1821. 10.1016/j.cell.2019.05.031.
73. Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W.M., 3rd, Zheng, S., Butler, A., Lee, M.J., Wilk, A.J., Darby, C., Zager, M., et al. (2021). Integrated analysis of multimodal single-cell data. Cell *184*, 3573-3587 e3529. 10.1016/j.cell.2021.04.048.
74. Hafemeister, C., and Satija, R. (2019). Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. Genome Biol *20*, 296. 10.1186/s13059-019-1874-1.

ACKNOWLEDGMENTS

We would like to thank members of the Eggan, McCarroll, Nehme and Pietiläinen groups for insights and useful discussions and Lee Rubin and members of the Rubin lab for support in the final steps of this project. We are grateful to the Harvard Stem Cell and Regenerative Biology Department (HSCRB) for the use of shared equipment, microscopy core and FACS facility. We are also grateful to the Stanley Center at the Broad Institute of Harvard and MIT for support with bioinformatics and cloud space. We thank BioRender.com for useful diagrams and images used in figures throughout this publication. This work was supported by U01MH115727 to S.A.M., R.N. and K.E. as well as support from Stanley Center for Psychiatric Research at Broad Institute of MIT and Harvard and Harvard Stem Cell and Regenerative Biology Department at Harvard University.

AUTHOR CONTRIBUTION

Conception and study design F.L., R.N. Data analysis and interpretation F.L., O.P., R.N. Manuscript writing and editing F.L., O.P., R.N., M.V. “Villages” design and multi-cell line experiments J.M., J.L.M.S., K.R. Immunofluorescence analysis and co-culture studies F.L., I.G.S.J., A.C., B.M.S. Conventional motor neuron experiments F.L., B.M.S., I.G.S.J., J.R.K. Electrophysiology analysis B.J.J., J.G, F.L. Bioinformatics and scRNA-seq F.L., S.M., O.P., S.D.G., D.M., C.J.M., J.M.

DECLARATION OF INTERESTS

K.E. is cofounder of Q-State Biosciences, Quralis, Enclear Therapies, and is group vice president at BioMarin Pharmaceutical.

INCLUSION AND DIVERSITY

We worked to ensure diversity in experimental samples through the selection of the cell lines. We worked to ensure diversity in experimental samples through the selection of the genomic datasets. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paperself-identifies as a member of the LGBTQIA+ community.

STAR METHODS

LEAD CONTACT

Kevin Eggan (kevin.eggan@bmrn.com).

MATERIALS AVAILABILITY

This study did not generate new unique reagents.

DATA AND CODE AVAILABILITY

All codes and algorithms necessary for re-analysis of the single-cell RNA-sequencing data are publicly available and can be found in other publications[39,40]. Raw sequencing data and count matrices have been deposited in GEO and DUOS and can be requested using the ID GSE219112 and DUOS-000121 (https://duos.broadinstitute.org/). This paper does not report original code. Further information requests can be directed to Kevin Eggan (kevin.eggan@bmrn.com) or Francesco Limone (francesco_limone@fas.harvard.edu).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

NGN2-based differentiations

Stem cells were grown in mTeSR1 (Stem Cell Technologies, 05850) and grown on Matrigel (Corning) coated pates at 37ºC and 5% CO_2. hPSCs were infected with TetO-Ngn2-Puro, TetO-GFP and rtTA lentiviral constructs[9] produced by Alstem in mTeSR medium with 1 μM RoCK inhibitor Y-27632 for 24 hours. hPSs were then passaged and differentiation was started when cells reached 70-80% confluency. For the first four days of differentiation cells were grown in induction medium: DMEM/F12 (Life Technologies, 11320-033), N2 supplement (0.5%v/v, Gibco), 1X GlutaMAX (Gibco), 0.1mM Non-essential amino acid (Gibco), 0,5% glucose, doxycycline hyclate (2 μg/mL). Small molecules added: day 1 – DOX: none; LSB: 10 μM SB431543 (Custom Synthesis), 200 nM LDN193189 (Custom Synthesis); piNs: 10 μM SB431543 (Custom Synthesis), 200 nM LDN193189 (Custom Synthesis), 4 μM XAV939 (Stemgent, 04-00046); liMNs: 10 μM SB431543 (Custom Synthesis), 200 nM LDN193189 (Custom Synthesis), 2 μM retinoic acid (Sigma) and 2 μM Smoothened agonist (Custom Synthesis). Day 2 to 4 - DOX: puromycin (5 μg/mL); LSB: puromycin (5 μg/mL), 10 μM SB431543 (Custom Synthesis), 100 nM LDN193189 (Custom Synthesis); piNs: puromycin (5 μg/mL), 10 μM SB431543 (Custom Synthesis), 100 nM LDN193189 (Custom Synthesis), 2 μM XAV939 (Stemgent, 04-00046); liMNs: puromycin (5 μg/mL), 10 μM SB431543 (Custom Synthesis), 100 nM LDN193189 (Custom Synthesis), 1 μM retinoic acid (Sigma) and 1 μM Smoothened agonist (Custom Synthesis). On day 4 cells were dissociated using Accutase (Gibco) and replated in a 1:2 dilution to ensure puromycin selection of uninfected cells. For day 4 to 7, DOX, LSB and piNs cells were grown in neuronally supportive medium supplemented with small molecules as described above: Neurobasal (Life Technologies 21103049) supplemented with B27 supplement w/o vitA (2%v/v, Gibco), 1X GlutaMAX (Gibco), 0.1mM Non-essential amino acid (Gibco), 0,5% glucose with the addition of 10 ng/ml of BDNF, CNTF and GDNF (R&D Systems). For day 7 to 10, liMNs were grown with small molecules as described above in neuronally supportive medium: Neurobasal (Life Technologies 21103049) supplemented with B27 supplement (2%v/v, Gibco), N2 supplement (0.5%v/v, Gibco), 1X GlutaMAX (Gibco), 0.1mM Non-essential amino acid (Gibco), 0,5% glucose with the addition of 10 ng/ml of BDNF, CNTF and GDNF (R&D Systems). On day 7, cells were dissociated using accutase and replated on glial co-cultures as described previously[26] in medium described above. From this time onwards, half-media change was performed every 2-3 days in neuronally supportive media described above with the only addition of 10 ng/ml of BDNF, CNTF and GDNF (R&D Systems). For most experiments, neurons were co-cultured with murine glial cells (50,000 cells/cm^2) derived from postnatal brains (P0-2) as previously described[26], neurons were mixed with glia when replating day 7 cells 30,00 cells/cm^2.

2D MN differentiation

Stem cells were grown in mTeSR1 (Stem Cell Technologies, 05850) and grown on Matrigel (Corning) coated pates at 37ºC and 5% CO_2. Stem cells were differentiated to bona fide 2D Motor Neurons as previously described[36,64,65]. This protocol based on the principle of neuralization by dual-Smad inhibition

followed by the inhibition of NOTCH/FGF pathway both under the patterning capability of retinoids and Sonic Hedgehog. Briefly, once 90-95% confluent, stem cell medium was switched to differentiation medium: 1:1 mix of Neurobasal (Life Technologies 21103049) and DMEM/F12 (Life Technologies, 11320-033) supplemented with B27 supplement (2%v/v, Gibco), N2 supplement (0.5%v/v, Gibco), 1X GlutaMAX (Gibco), 0.1mM Non-essential amino acid (Gibco). For the first six days, differentiation medium was supplemented with 10 μM SB431543 (Custom Synthesis), 100nM LDN193189 (Custom Synthesis), 1 μM retinoic acid (Sigma) and 1 μM Smoothened agonist (Custom Synthesis). For the second week, differentiation medium was supplemented with: 5 μM DAPT (Custom Synthesis), 4 μM SU-5402 (Custom Synthesis), 1 μM retinoic acid (Sigma) and 1 μM Smoothened agonist (Custom Synthesis). To isolate neurons from mixed cultures we utilised an immune-panning based method previously described[36,66]. At day 14, monolayers were dissociated with Accutase (Gibco) for 1 hour at 37ºC. After gentle, repeated pipetting, cells were collected, spun down and resuspended in sorting buffer and filtered. Single cell suspensions were incubated with antibody against NCAM (BD Bioscience, 557919, 1:200) for 25 minutes, washed and NCAM$^+$ cells were sorted with an BD FACS Aria II cell sorter. Sorted 2D MN were plated on mouse glial cultures in motor neuron medium (Neurobasal (Life Technologies 21103049) supplemented with B27 supplement (2%v/v, Gibco), N2 supplement (0.5%v/v, Gibco), 1X GlutaMAX (Gibco), 0.1mM Non-essential amino acid (Gibco), 0,5% glucose) with the addition of 10 ng/ml of BDNF, CNTF and GDNF (R&D Systems). Neurons were co-cultured with murine glial cells (150,000 cells/cm^2) derived from postnatal brains (P0-2) as previously described[26].

Co-culture of Ngn2 motor neurons and mouse myoblasts in microfluidic devices

Mouse myoblasts from hindlimb skeletal muscles of young adult mice and mouse glia form neonatal mouse brains were isolated and cultured as previously described[26,67]. Microfluidic device chips (XC450, XONA Microfluidics) were designated a motor neuron compartment and a muscle compartment. The motor neuron compartment was coated with 0.1 mg/ml poly-L-ornithine (Sigma-Aldrich) in 50 mM Borate buffer, pH = 8.5 and 5 μg/ml laminin (Invitrogen), while the muscle compartment was coated with Matrigel (Corning). Day 7 Ngn2 motor neurons and mouse glia were seeded at a concentration of 100,000 neurons-200,000 glia/device. Myoblasts were seeded at a concentration of 150,000 device. Motor neurons were seeded in the motor neuron media described above with the addition of 10 ng/ml of BDNF, CNTF and GDNF (R&D Systems). For seeding and culturing the first 2 days, myoblasts were maintained in Myoblast media (DMEM/F12, 20% Foetal Bovine Serum and 10% heat-inactivated Horse Serum, and 10 ng/ml bFGF), after that, differentiation was initiated by adding myoblast differentiation media (DMEM high glucose, 5% heat-inactivated Horse Serum). Myoblast were sustained in differentiation medium for 3 days and then switched to motor neuron medium with the addition of 10 ng/ml of BDNF, CNTF and GDNF (R&D Systems) and while medium in motor neuron compared contained no neurotrophic factors to start recruitment of motor neuron axons to the muscle compartment by generation of a volumetric gradient (50 μl difference in volume between the compartments) in the device. Volumetric gradient was kept for every medium change, done every other day. Co-cultures were fixed at day 21 post-seeding for visualization of motor axon-muscle synaptic contacts.

METHOD DETAILS

FACS analyses

We used an *Hb9:*:GFP reporter stem cell line previously described infected with the Ngn2 lentiviral constructs as described above[26]. Briefly, cells were differentiated in 24 well plates and subjected to different patterning molecules. At each time point, cells were dissociated with Accutase (Gibco) as previously described, each replicate was frozen in Cryostor® CS10 (STEMCELL Technologies). After all samples were collected, cells were thawed in separated tubes are resuspended in sorting buffer as described by others[36], The BD FACS Aria II cell sorted was used to quantify the percentage of *Hb9*::GFP$^+$ cells in each sample after using DAPI signal to determine cell viability.

Immunofluorescence assays

Cells were washed once with PBS, fixed with 4% PFA for 20 minutes, washed again in PBS and blocked for one hour in 0.1% Triton in PBS with 10% donkey serum. Fixed cells were then washed and incubated overnight with primary antibodies at 4ºC. Primary antibody solution was washed and cells were subsequently incubated with secondary antibodies (1:2000, Alexa Fluor, Life Technologies) at room temperature for 1 hour, washed with PBS and stained with DAPI. Primary antibodies used: Tuj1 (R&D, MAB1195), Islet1 (Abcam, ab178400), MAP2 (Abcam, ab5392), Synapsin (Millipore, AB1543), SMI-32 (BioLegend, 801702), Chat (Millipore, AB144P), Foxp1 (Abcam, ab16645), AnkyrinG (Millipore, MABN466), Synaptophysin (Synaptic Systems, 101 004), PSD-95 (Abcam, ab2723), STMN2 (Novus NBP49461). Images were analysed using FIJI.

RNA extraction and RT-qPCR analyses

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.

Western blots

For WB analyses, cells were lysed in RIPA buffer with protease inhibitors (Roche). After protein quantification by BCA assay (ThermoFisher), ten micrograms of protein 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 (1:1000 dilution). 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). Primary antibodies used: Tuj1 (R&D, MAB1195), Synapsin (Millipore, AB1543), PSD-95 (Neuromab, 75-028), GAPDH (Millipore, MAB374).

Multi Electrode Array analysis

Electrophysiological recordings were obtained by Axion Biosystems Multi-Electrode Array (MEA) plate system (Axion Biosystems, 12 wells or 48 wells formats) that recorded extracellular spike potential. On day 7 of differentiation, cells were detached and counted and mixed with murine glia as described above. MEA plates were previously coated with Matrigel (Corning) and cells were seeded in Neurobasal medium supplemented with ROCK inhibitor for 24 hours. Recordings were performed every 2-3 days and medium was changed after recordings. Analysis was performed with AxIS (Axion Biosystems – Neuronal Metric Tool) as described by others[11,68].

QUANTIFICATION AND STATISTICAL ANALYSIS

Stem cell lines, villages, single-cell RNA-sequencing, Census-seq and Dropulation

Methods for Census-seq and Dropulation are described elsewhere[39,40], brief description below:

Human pluripotent cell lines and village generation[39]

Human ESC lines used in this study were part of a collection previously described [69]. These lines were exome sequenced and whole genome sequenced after minimal passaging and cultured as described. Individual lines were cultured and differentiated into neurons as described. At day 6 after doxycycline induction, when cells are postmitotic, cultures were dissociated with Accutase (Gibco) and resuspended in mTeSR medium with 1 μM RoCK inhibitor Y-27632. To generate balanced "villages", cell suspensions were counted using a Scepter 2.0 Handheld Cell Counter (Millipore Sigma) with 60 μM Scepter Cell Counting Sensor (Millipore Sigma), 0.5M viable cells from each donor cell line were mixed. At this timepoint 0.5M cells were harvested for Census-seq analysis and ensure balanced representation, the rest was plated for subsequent experiments.

DNA isolation and library preparation[39]

Every seven days, pellets were harvested from separate wells of the "liMNs village" after dissociation with Accutase (Gibco). Pellets were lysed and DNA precipitated and DNA was used to generate libraries using TruSeq Nano DNA Library Prep Kit (Illumina), libraries were sequenced using NextSeq 500 Sequencing System (Illumina). Generated libraries were aligned to human genome using BWA, reference genome was selected to match the genomes used to generate VCF files containing the whole-genome sequenced genotypes of each donor cell line. To exclude confounding mouse DNA from glia, a multi-organism reference was used, reads competitively aligned to both genomes and only high quality (MQ≥10) were used for assignment.

Census-seq analysis[39]

The algorithms used to assign donor contribution to villages are extensively described elsewhere and their validation is outside the scope of this publication. However, briefly the aim of Census-seq algorithms is to accurately detect and precisely quantify the contribution of donors in a mixed DNA sample to monitor population dynamics over time and/or conditions. This can be achieved systematically and inexpensively by lightly sequencing genomic DNA, the algorithms attempt to determine the donors' mixture by determining the ratio of alleles present at every SNP. The gradient-descent algorithm can then use this data to identify the donor-mix that maximizes the likelihood of any observed sequence data. Once the best ratio is identified, the algorithms compare the computed "most likely donor mix" to a VCF file that contains whole genome-sequencing data from all stem cell lines in the collection. These VCF files contain a filtered and refined matrix with alternate alleles at each variant for every donor's genotype. Census-seq can use this data to find a vector of donor-specific contribution (to the mix) that can explain the allele counts detected at each site in the sequencing data provided. For each site, the allele frequency is inferred using the VCF reference files and its proportion of donor in the pool of DNA can then be calculated over the total counts for that specific site. The algorithms are

then able to sum the proportion of each donor's representation at every specific site and calculate total representation of each genotype, a.k.a. donor, in the pooled DNA, providing us an estimate of the ratio of donors in the village.

Dropulation: scRNA-sequencing and donor assignment[40]

For single-cell analyses, cells were harvested and prepared with 10X Chromium Single Cell 3' Reagents V3 and sequenced on a NovaSeq 6000 (Illumina) using a S2 flow cell at 2 x 100bp. Raw sequence files were then aligned and prepared following previous Drop-seq workflow[70]. Human reads were aligned to GRCh18 and filtered for high quality mapped reads (MQ≥10). In order to identify donor identity of each droplet, variants were filtered through several quality controls as described previously be included in the VCF files[40,71], to summarise the goal is to only use sites that unambiguously and unequivocally can be detected as A/T or G/C. Once both the sequenced single-cell libraries and VCF reference files are filtered and QC'ed, the Dropulation algorithm is run. Dropulation analyses each droplet, hence a cell, independently and for each cell generates a number representing the likely provenance of each droplet from one donor. Each variant site is assigned a probability score for a given allele in the sequenced unique molecular identifier (UMI) calculated as the probability of the base observed compared to expected based, and 1 – probability that those reads disagree with the base sequenced. Donor identity is then assigned as the computed diploid likelihood at each UMI summed up across all sites.

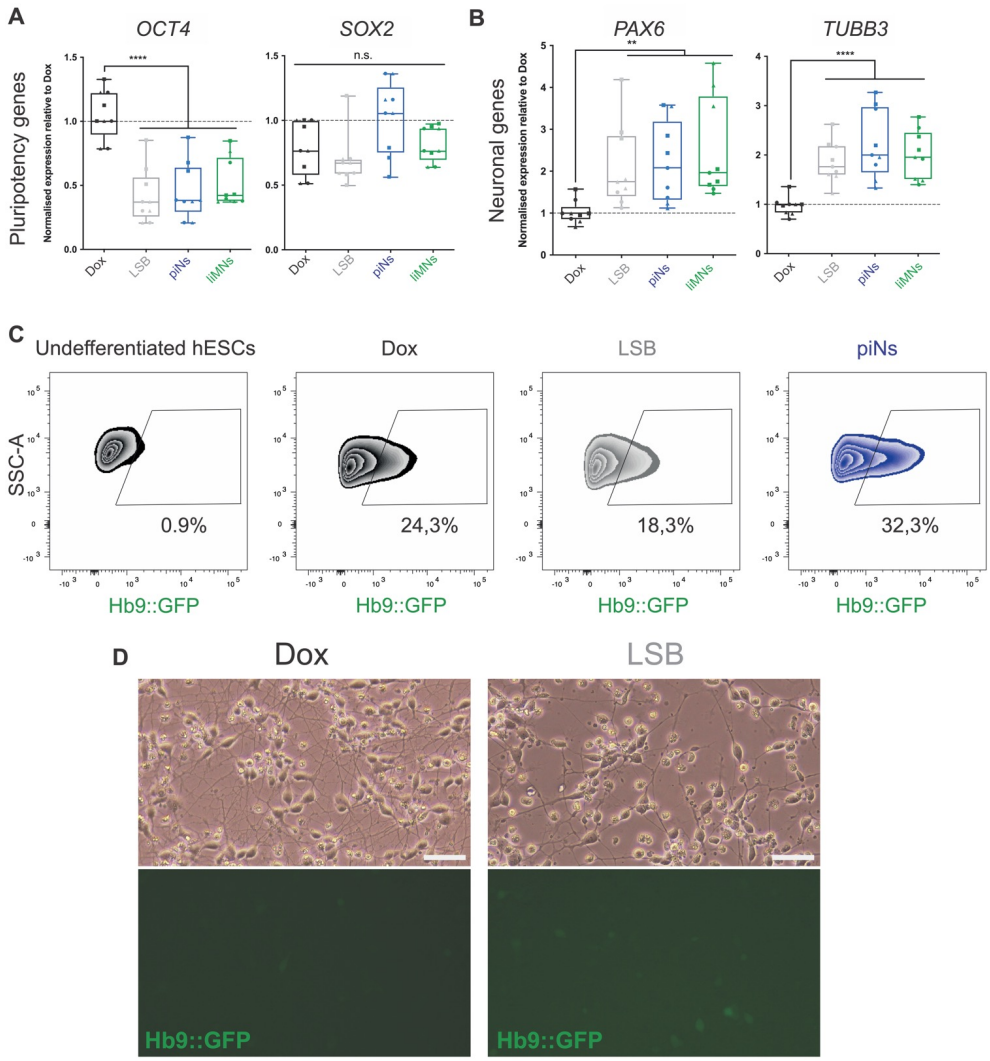
This probability-based analysis allows to increase confidence in donor detection per barcode by increase the numbers of individuals in the VCF files: more individuals, more UMIs with site variants, more confident scores, higher quality donor assignments. After assigning a "likelihood score", sites where only few donors have detected reads are ignored and scores are adjusted to allow only high confidence variant sites to be included. This second computer score is then added to the original likelihood as a weighted average score, this mixed coefficient defines the proportion of the population that presents each genotype and in adds to 1. Based on this mixed coefficient that takes into account reads mapped to each donors and the confidence to which each site can be used for this assignment, Dropulation then contains algorithms able to detect "doublets", barcoded droplets with genetic DNAs assigned to two different donors, to avoid analysing barcodes with admixed identity but also to avoid excluding barcoded droplets with unclear donor assignment based on the coefficient previously calculated[40].

Once scores are calculated, the algorithm assigns donors to single droplets. Then runs the double detection and cells that are likely doublets are filtered out. After that, donor identities are confirmed only if p-value<0.05. These cells are then validated by crossing proportions of each donors as known inputs in the village and excluding any unexpected identity. Donors composing less then 0.2% of the libraries are excluded from the experiment[40].

More details on the preparation of libraries and donor identification can be found in published work[40].

scRNAseq analysis of villages and integrated datasets

Matrices from neuronal villages were built from 12 separate runs of 10X Chromium Single Cell 3' Reagents V3 as described above. Any barcode with less than 400 genes and combined UMI matrices were used for downstream analysis using Seurat (v3.0.2)[72]. After that, barcodes were further filtered by number of genes detected 1500<nFeature_RNA<7000 and percent of mitochondrial and ribosomal genes to reduced the number of dying cells/debris: percent.mito<20, 3<percent.RPS<15, 5<percent.RPS<10. The matrix was then processed via the Seurat pipeline: log-normalized by a factor of 10,000, followed by regressing out UMI counts, mitochondrial and ribosomal genes, scaled for gene expression. After quality filtering, barcodes were used to compute SNN graphs and *t*-SNE projections using numbers of Principal Components based on ElbowPlot analysis. SNN-graphed *t*-SNE projection was used to determine minimum number of clusters obtain at resolution=0.2 (FindClusters) as described previously[66]. Integration with Rayon et al. 2021 was performed using matrices and metadata available at https://github.com/briscoelab/human_single_cell. Only barcodes with available metadata concerning their cellular identity from Rayon et al. were selected to use identities assigned by peer review publication[44]. The available barcodes were then loaded into Seurat v4.0.1[73]. Integration with libraries previously generated from villages of liMNs was achieved using SCTransform on a merged object running the PreSCTIntegration() function according to the sctransform integration pipeline[74]. Analysis of MN alone was conducted as described above by comparing liMNs generated in this study with barcodes identified as "pMN" and "MN" by Rayon et al.

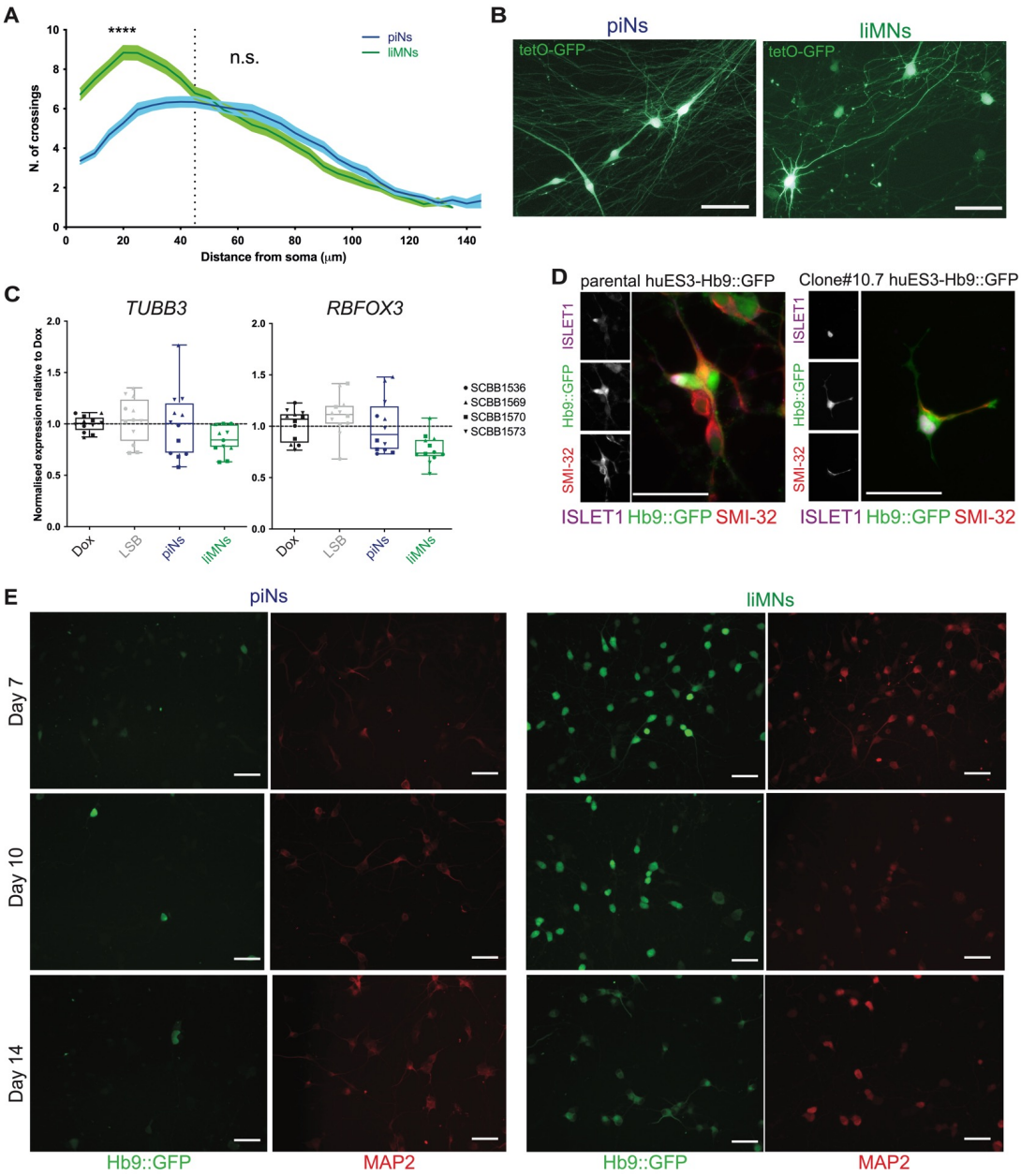


Supplementary Figure 1. Ngn2 neuronal patterning can be directed to different neuronal fates by small molecules patterning

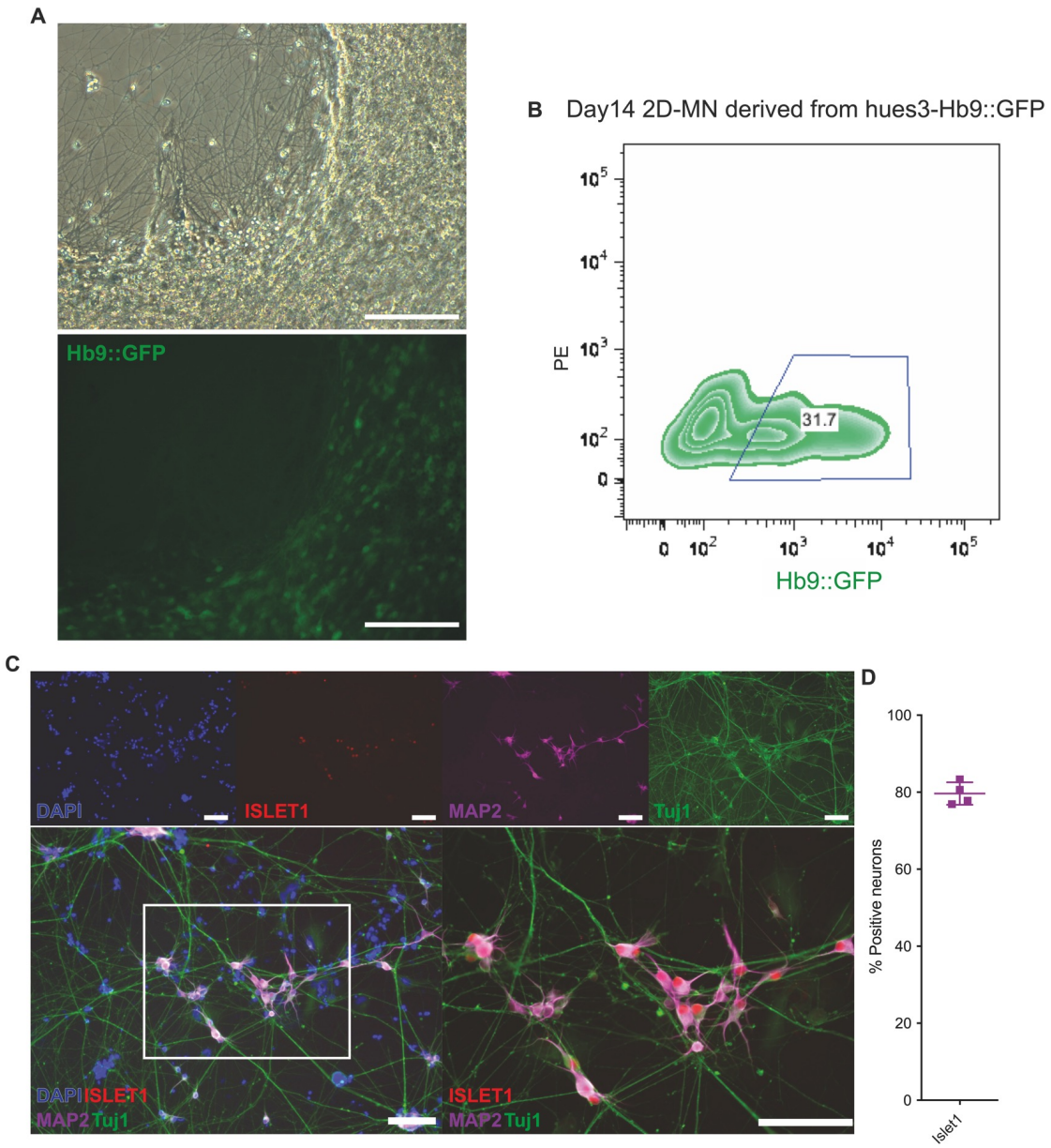
(A-B) RT-qPCR quantification of pluripotency genes and genes involved in pan-neuronal development (p-values from one-way ANOVA).

(C) Flow cytometry quantification of Hb9::GFP positive cells by day 4 for the other conditions.

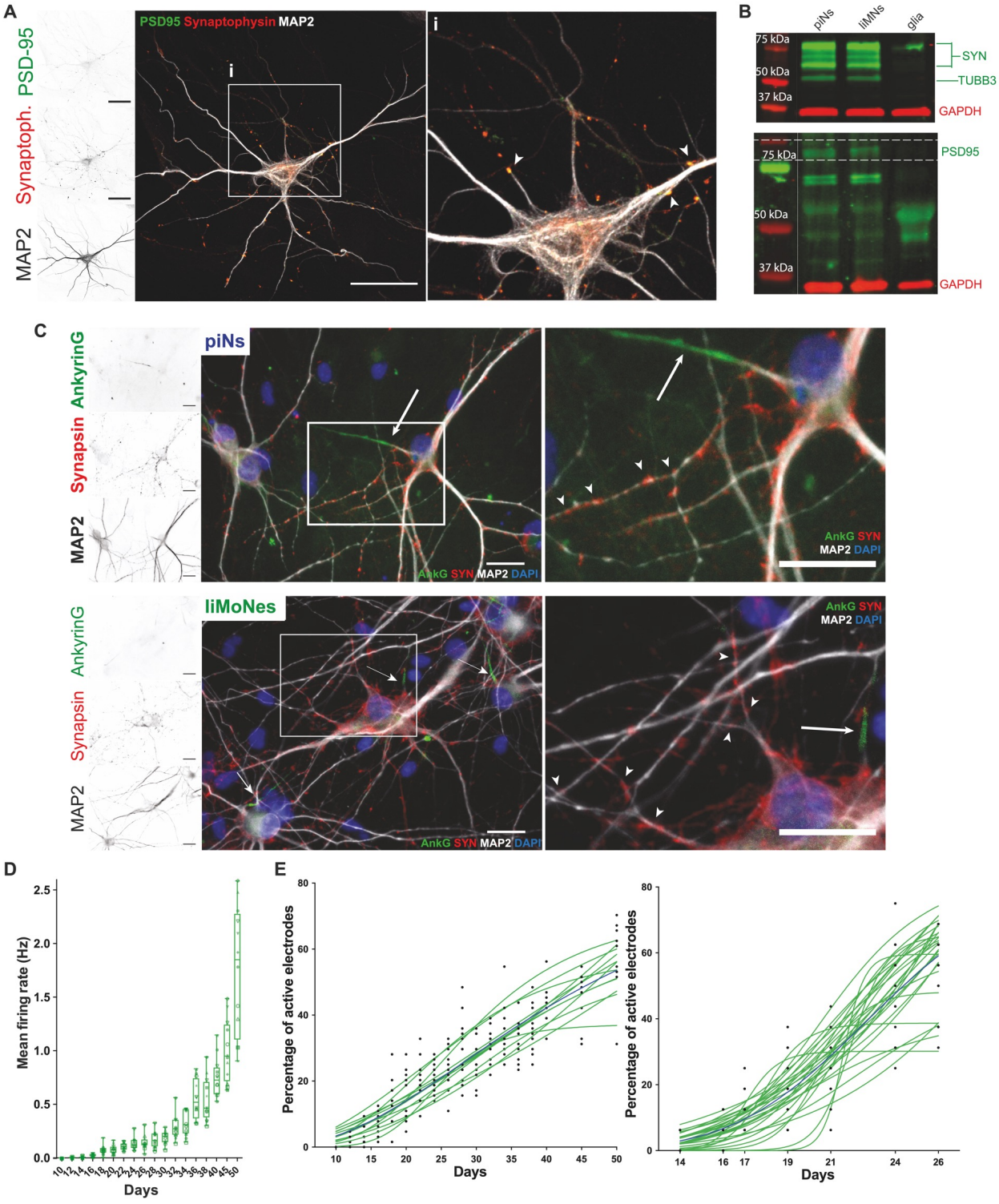
(D) Hb9::GFP expression at day 7 post-induction in original Ngn2-induced Dox and LSB conditions (scale bar 50 μm).



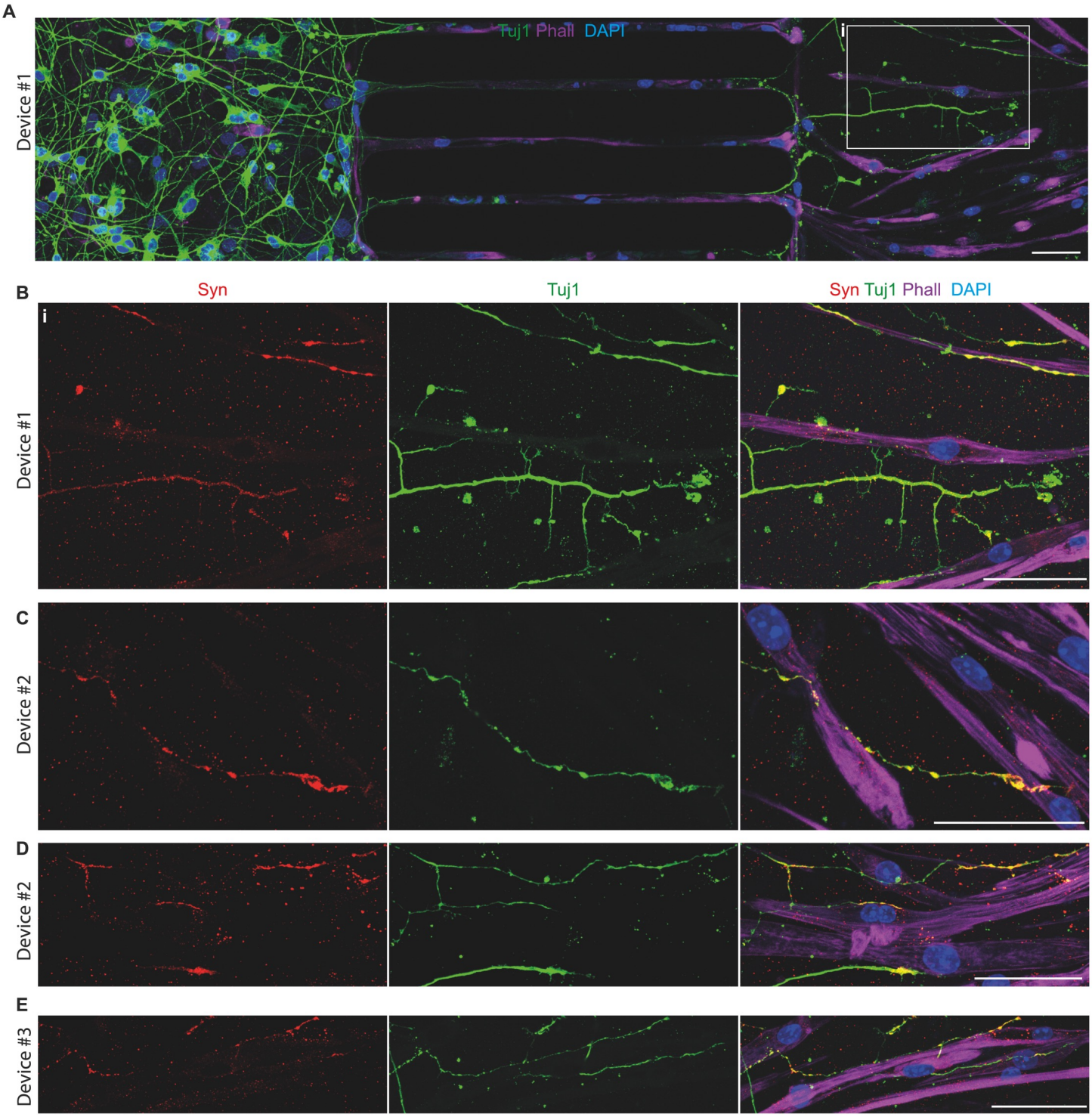
Supplementary Figure 2. Patterned Ngn2-induced neuronal fate is maintained throughout the differentiation.
(A) Quantification of arborization of piNs and liMNs.
(B) Viral tetO-GFP imaging at day 30 in piNs and liMNs, showing different cell morphology (scale bar 50 μm).
(C) RT-qPCR quantification of pan-neuronal markers (p-values from one-way ANOVA).
(D) IF analysis for pan-MN SMI-32, Islet1 and Hb9::GFP reporter expression at day 7 post-induction in two clones of the same reporter (scale bar 50 μm).
(E) Images of Hb9::GFP expression at day 7, 10 and 14 post-induction in piNs and liMNs by immunofluorescence.



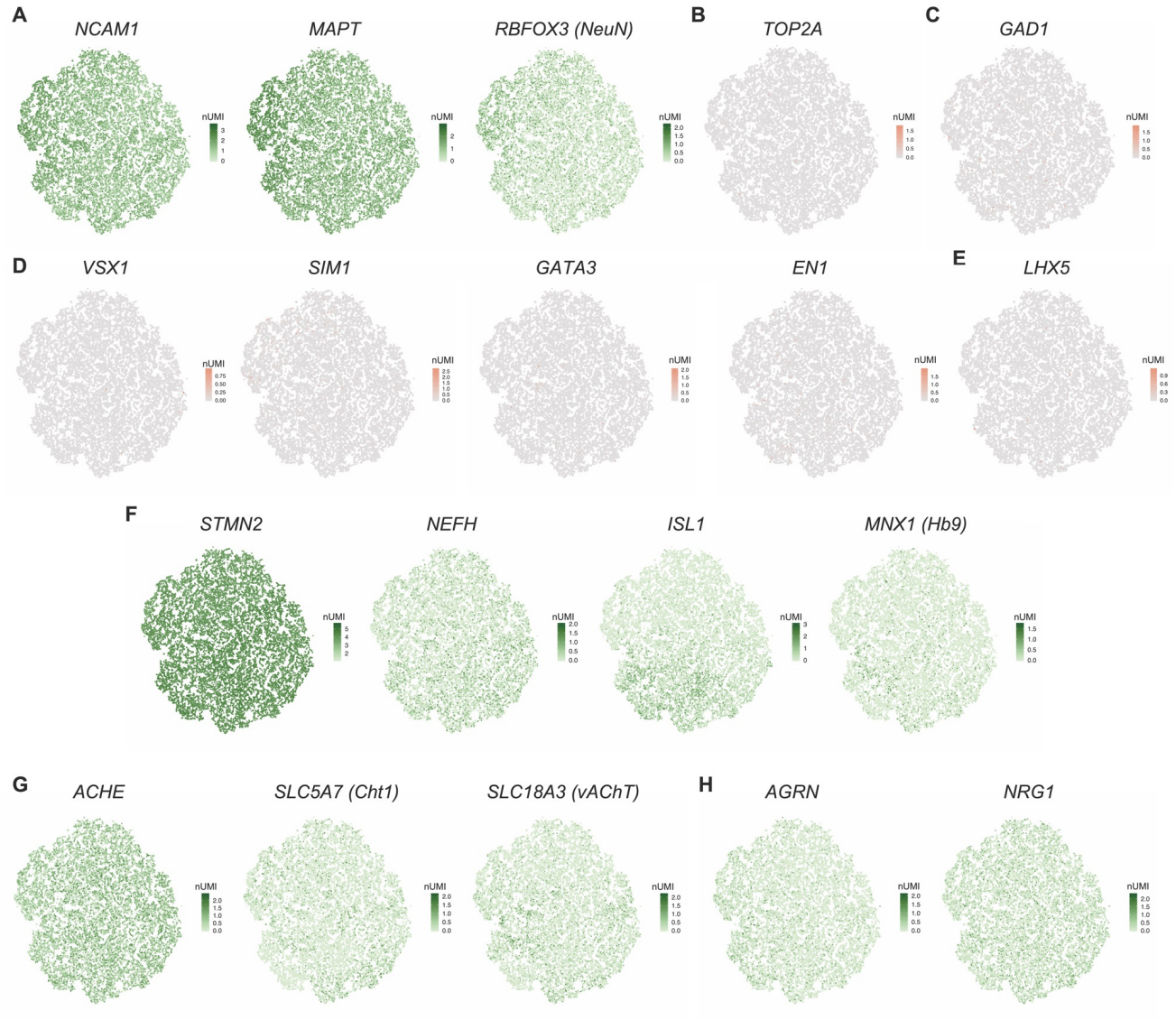
Supplementary Figure 3. Bona fide, hPSC-derived 2D MN express similar MN markers as liMNs.
(A) Hb9::GFP expression day 14 of differentiation in 2D-MN (scale bar 50 μm).
(B) Flow cytometry quantification of Hb9::GFP positive cells in day 14 2D-MN.
(C) Immunofluorescence analysis for cholinergic transcription factor Islet1 and neuronal cytoskeletal proteins MAP2 and TUBB3 (Tuj1) in sorted 2D MN (scale bar 50 μm).
(D) Quantification for cells in A.



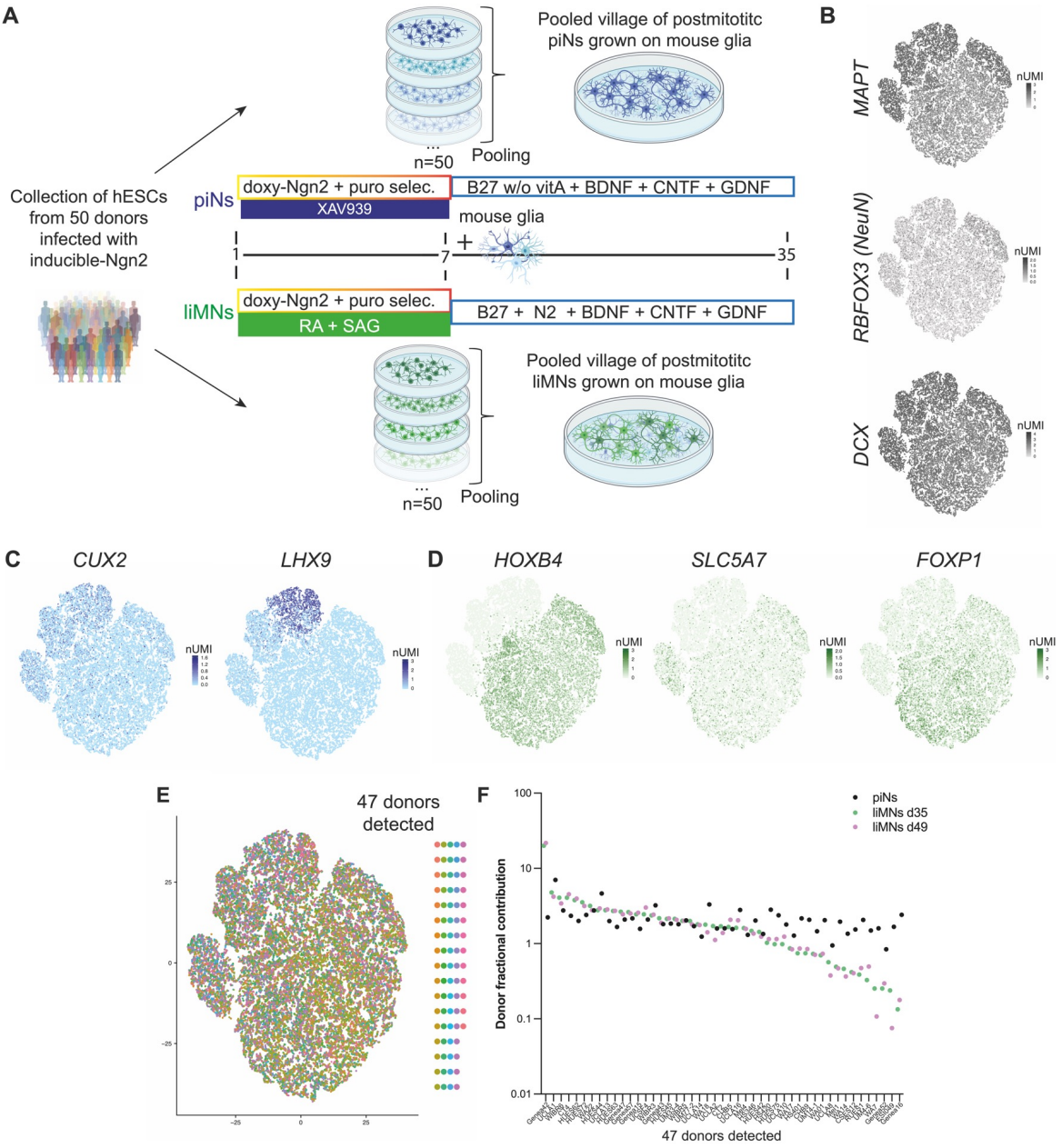
Supplementary Figure 4. liMNs can form active synaptic contacts comparable to previously characterised piNs.
(A) Day 50 liMNs express pre- and post-synaptic density proteins (glial co-cultures - scale bar 50 μm).
(B) Western blot analysis shows expression of pre- and post-synaptic density molecules in both cell types.
(C) Immunofluorescence for proteins involved in the formation of functional axons and synaptic structure in piNs and liMNs (glial co-cultures - scale bar 50 μm).
(C-D) Network activity of liMNs. (C) Mean number of spikes in 10-s period in liMNs co-cultured with murine cortical glial preparations. (D) Proportion of active electrodes detecting spontaneous activity throughout the differentiation (days). Data fit by sigmoidal function (green), median sigmoidal in black.



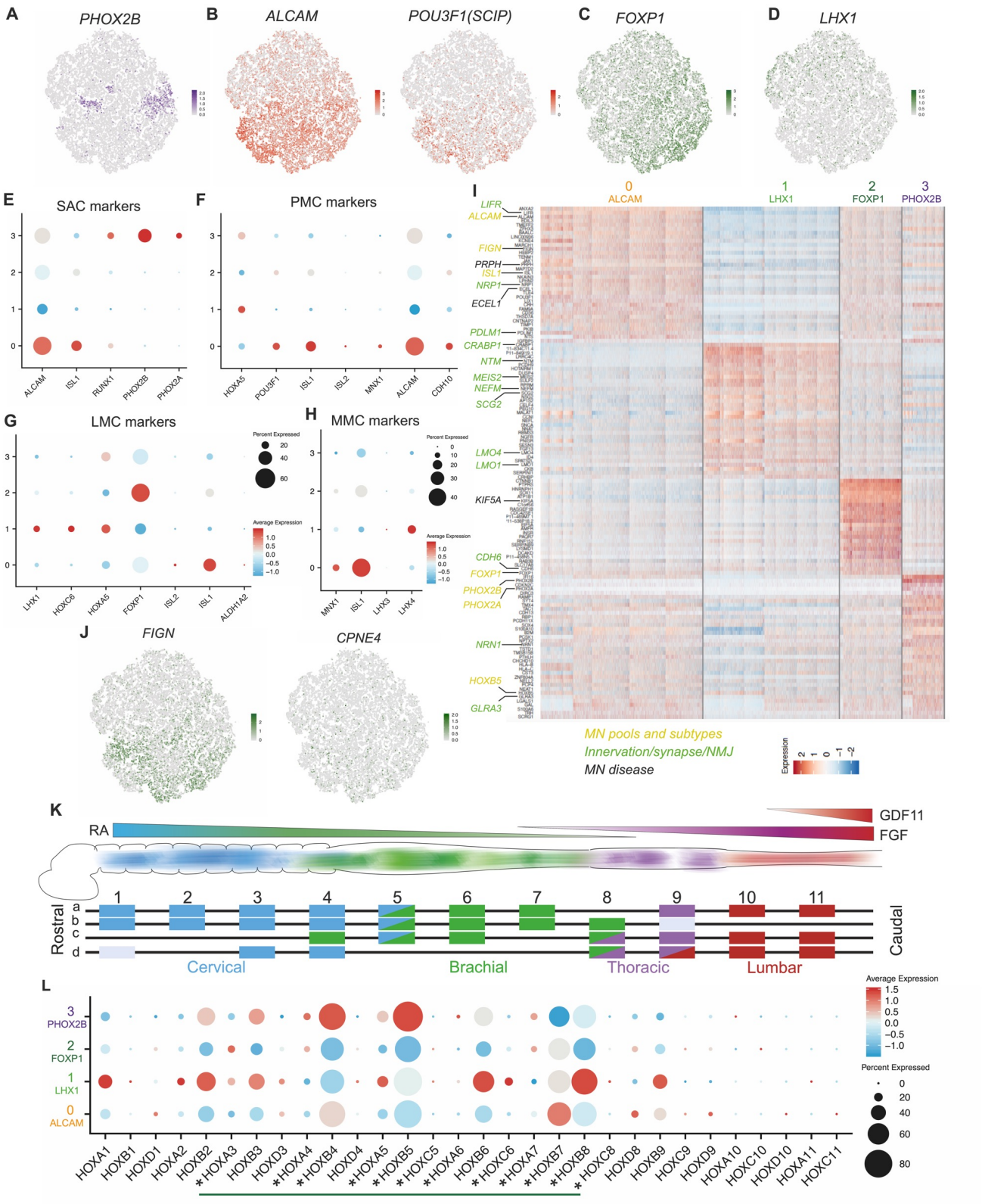
Supplementary Figure 5. liMoNes can form NMJ-like structures *in vitro*.
Immunofluorescence of co-cultures of liMNs and primary murine myoblasts from three devices from separate rounds of differentiation.
(A) Representative image of neurons extending axons through the channels (middle), contacting primary muscle cells (rigth).
(B) Insets of (A) showing liMNs forming synaptic-like contacts with muscles cells.
(C-E) Representative images from separate devices showing liMNs forming synaptic-like contacts with muscles cells (*n*=10 devices).



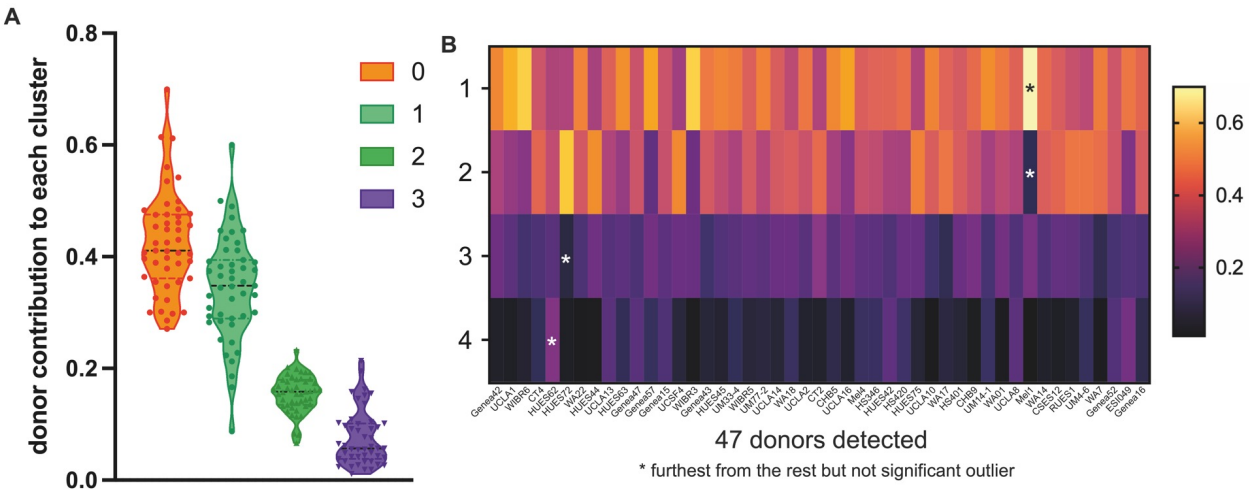
Supplementary Figure 6. scRNAseq confirms expression of MN markers.
(A) *t*-SNE projection with expression of markers specific for post-mitotic neurons.
(B) *t*-SNE projection with expression of cycling cells markers.
(C) *t*-SNE projection with expression of inhibitory neurons.
(D) *t*-SNE projection with expression of spinal ventral inhibitory V1, V2a, V2b, V3.
(E) *t*-SNE projection with expression of markers specific for mid-spinal neurons V0.
(F) *t*-SNE projection with expression of MN-specific markers.
(G) *t*-SNE projection with expression of genes involved in cholinergic machinery.
(H) *t*-SNE projection with expression of genes involved NMJ formation.



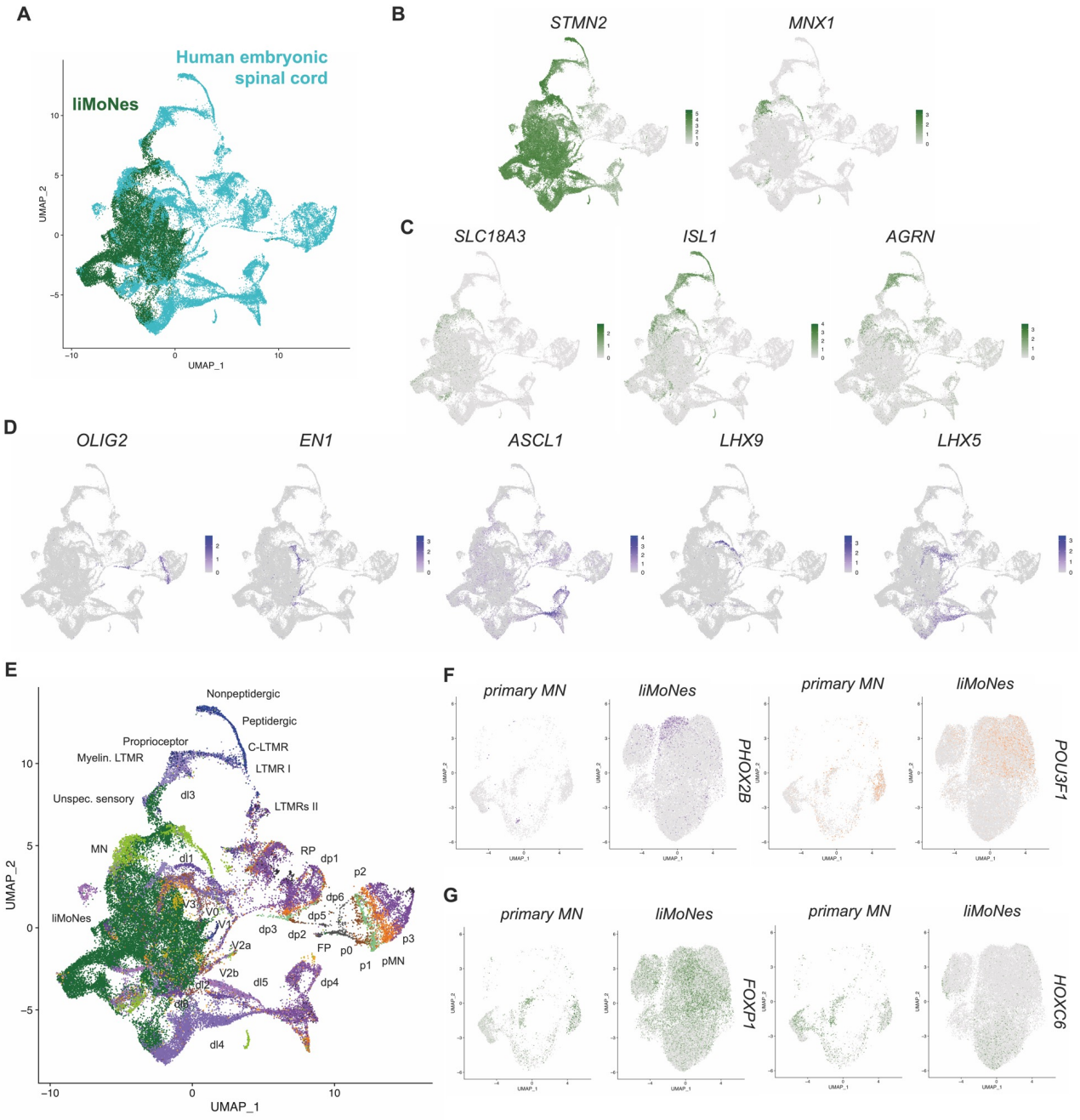
Supplementary Figure 7. piNs and liMNs transcriptomic differences.
(A) Pooling strategy and village construction for sequencing analysis of piNs and liMNs.
(B) *t*-SNE projection with expression of neuronal markers.
(C) *t*-SNE projection with expression of cortical-enriched markers.
(D) *t*-SNE projection with expression of MN-specific markers.
(E) *t*-SNE projection of cells depicting donor's identity of each cell from 47 donors detected by *Dropulation* analysis.
(F) Fraction representation of 47 donors in the two timepoints for liMNs and day 35 piNs.



Supplementary Figure 8. Sub-columnar localization of liMNs.
(A) *t*-SNE projection with expression of markers specific for SAC subtypes.
(B) *t*-SNE projection with expression of markers specific for PMC subtypes.
(C) *t*-SNE projection with expression of markers specific for mLMC subtypes.
(D) *t*-SNE projection with expression of markers specific for lLMC subtypes.
(E) Dotplot for expression of markers specific for SAC – Spinal Accessory Column.
(F) Dotplot for expression of markers specific for PMC – Phrenic Motor Column.
(G) Dotplot for expression of markers specific for LMC – Lateral Motor Column.
(H) Dotplot for expression of markers specific for MMC – Median Motor Column.
(I) Heatmap of genes differentially expressed in each subtype, highlighted genes in volved in subtype specific MN biology.
(J) *t*-SNE projection with expression of markers expressed by digit-innervating motor neurons.
(K) Schematic of spinal cord HOX genes expression.
(L) Dotplot for expression of all HOX genes detected in the four subclusters. Retinoid dependent Hox activation in vertebrates (green line) and specifically expressed in ventral spinal cord MNs (asterisks).



Supplementary Figure 9. Donor composition of iiMNs.
(A) Violin plot showing distribution of each donor in the villages.
(B) Heatmap depicting donors composition of each subgroup and highlighting the absence of outliers by Grubb's test alpha=0.05.



Supplementary Figure 10. Comparison of liMoNes and human embryonic spinal cord from Rayon et al. 2021.
(A) *t*-SNE projection of integrated datasets: liMoNes and human embryonic spinal cord Rayon et al. 2021.
(B) *t*-SNE projection with expression of MN-specific markers.
(C) *t*-SNE projection with expression of genes involved in cholinergic machinery.
(D) *t*-SNE projection with expression of markers specific to spinal, ventral inhibitory neurons, mid- and dorsal spinal neurons and progenitor cells.
(E) *t*-SNE projection of integrated datasets: liMoNes and human embryonic spinal cord with cell types identified by Rayon et al. 2021.
(F-G) *t*-SNE projection with expression of markers specific to hindbrain/cervical (F) and brachial (G) MN pools in primary MNs from Rayon et al. 2021 (left side of panel) and liMoNes (right side of panel).

Chapter 5:

Future directions: building stem cell models to study ALS-driven changes *in vitro*

This section will summarise ongoing efforts to use the models established in chapter 4 in conjunction with changes identified in chapter 3 to the nomination of proteostatic modulators that might function as neuroprotective targets. Moreover, this section will present preliminary data on the characterization of co-culture systems of different brain cell types derived from human iPSCs described in chapter 5 that could be use to study changes identified in sporadic ALS brain samples described in chapter 3.

With this section we hope to provide new, more complex *in vitro* systems to model degeneration and multicellular interactions disrupted in ALS.

This work has been generated thanks to the effort of several talented scientists:

Limone F., Couto A., Smith J.L.M., Guerra San Juan I., Joseph B. J., Therrien M., Smith K., Burberry A., Mordes D., Pietiläinen O., Kadiu I., Eggan K.

Work partially funded by UCB Pharma, Harvard Department of Stem Cell and Regenerative Biology, Broad Institute of MIT and Harvard and Harvard Stem Cell Institute.

Identification of potentially protective targets in response to proteostatic stress in human iPSC-derived neurons

SUMMARY

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterised by a progressive loss of motor function that results from the degeneration of motor neurons in the cortex and the spinal cord. As of today, only three drugs have been approved for use as therapeutics for ALS but, although efficient, they extent lifespan by only a few months. Many groups are tirelessly working to identify new candidates for therapies and eventually a cure but many have recognised the new for the field to build more robust, specifically human models that could enhance the translationability of laboratory findings. With this work, we aim to deeply characterise an *in vitro* system to robustly model proteostatic stress in human neurons derived from induced Pluripotent Stem Cells (iPSCs) by RNA sequencing. We used this system to, first of all, characterise canonical mechanisms activated by proteostatic stress, secondly, to identify specifically neuronal changes driven by proteostatic stress such as dysregulation of ALS-related genes and synaptic biology. By comparing our findings with published single-nucleus RNA-seq studies of ALS patient cortices, we confirmed that these alterations are not only disease-relevant but specific to excitatory neurons degenerating in the disease. Finally, we nominate a proteosomal regulatory subunit, PSMD12, as a possible neuroprotective target in proteostatic stress responses. Manipulation of PSMD12 in our system resulted in alterations of protein influx that, even though initially beneficial, might not be therapeutically translatable. The system we describe in this work provides a platform to further dissect mechanisms disrupted in stressed neurons that might reveal therapeutical targets useful for ALS but also for other neurodegenerative diseases.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterised by the loss of neurons that control movements, motor neurons (MNs), in both the cortex and the spinal cord. This rapidly progressive disease is fatal and respiratory failure occurs 2-5 years from onset[1]. Although genetic studies have advanced our knowledge of the disease, only ~10% of cases are inherited and familial (fALS), whereas 90-95% of diagnoses are sporadic (sALS), occurring without family history nor known genetic cause[1,2]. Several studies used bulk RNA-sequencing (RNA-seq) analysis of ALS post-mortem tissue to investigate disrupted mechanisms and identified differences between familial[3] and sporadic cases[4,5] and highlighted shared profiles independent of disease onset[6]. Besides the uncertainties in underlying mechanisms, one common feature to all ALS cases is the pathological accumulation of

ubiquitinated protein aggregates in neurons of patients[7]. These aggregates are composed of aggregated TAR DNA-binding protein-43 (TDP-43), DNA/RNA binding protein found insoluble in at least 90% of cases[8]. TDP-43 has an important role in RNA biology and metabolism and crucial studies have shown its importance in neuronal biology[9], specifically in a human context[10]. The convergence of mechanisms that lead to disease is still unknown but the loss of MNs and the fact that one of the few therapies approved for treatment is a drug that can modulate synaptic function[11] puts neurons and their proteostatic balance at the centre of interest in research and drug development.

Given the identification of highly ubiquitinated aggregates in neurons, many groups have focused on understanding the role of proteostasis in ALS. The ubiquitin-proteasome system, the unfolded protein response (UPR), stress of the endoplasmic reticulum (ER), autophagy and other pathways involved in protein folding and degradation are a large component of degenerative mechanisms in ALS[12], especially if we take into account the number of genes linked to fALS that are involved in these pathways: *UBQLN2*, *SQSTM1*/p62, *OPTN*, *VCP*, *CHMP2B*, *VAPB*, *TBK1*, *FIG4*, *GRN*, *C9orf72*[13]. Moreover, Dipeptide Repeats (DRPs) derived from *C9orf72* hexanucleotide RNA foci can form neuronal aggregates that sequester proteasome subunits compromising neuronal proteostasis[14]. This is just part of the evidence that points at a pivotal role of protein homeostasis, specifically in neurons, in ALS[12].

Two questions remain key in the field: why is protein homeostasis specifically disrupted in human neurons? What mechanisms are triggered by this imbalance? We therefore decided to expand our knowledge of human neuronal biology in a proteostatically disrupted context. First, we further characterised a previously established human iPSC-derived *in vitro* system of neurons subjected to proteostatic stress through proteasome inhibition[10,15]. Our meta-analysis identified upregulation of several pathways involved in proteostasis in neurons under this disease-relevant stress. We highlight novel biology by showing the downregulation of synaptic genes in stressed neurons, further confirming the importance of neuronal excitability in ALS. To gain more insights into primary disease, we compared this analysis with previously published single-nucleus RNA-seq study on motorcortices of sporadic ALS patients[15] and confirmed that ALS-triggered alterations in proteostasis are indeed accompanied by loss of synaptic transcripts in ALS patients and that these changes are specific to excitatory neurons of the motorcortex. Finally, we leverage this comparative analysis to nominate genes that could be neuroprotective under proteostatic stress. We focus on PSMD12, a regulatory subunit of the proteasome, because of its pro-survival role against proteasome inhibitors and because it was consistently upregulated both *in vitro* and in patients' cells. Knock-down of *PSMD12* in stressed human neurons triggered a complex regulation of its RNA and protein resulting in a general inhibition of the proteasome in favour of an increase in the production of autophagic machinery, a different route to protein degradation. Our data suggests that

manipulation of proteosomal biology could be beneficial for neuronal survival by diverting protein degradation towards other degradative systems, however, these mechanisms are also disrupted in ALS.

RESULTS

Modelling proteostatic stress in human neurons *in vitro*

We decided to start by further dissecting data presented in snRNAseq study, specifically we took a deeper dive into the comparison of RNA-sequencing experiments of human iPSC-derived neurons under proteostatic stress and the single-nucleus RNA sequencing of sporadic ALS motorcortices[15]. At the moment, *in vitro* modelling of sporadic ALS is complex and requires high numbers of lines and high-throughput methods and needs further standardization[16]. We therefore implemented transient proteasome inhibition as a model to induce ALS-related phenotypes such as TDP-43 nuclear-to-cytoplasmic translocation[10]. This model would allow us to: 1) identify differentially regulated by neurons under stress conditions, 2) focus on specific pathways or molecular processes that are altered in patients when comparing to the data derived from primary human control and patient samples.

In brief, to recapitulate proteostatic stress we generated neuronal cells from human embryonic stem cell line engineered with a GFP-reporter under the motor neuron specific gene Hb9, as previously described[10,15,17]. We then induced proteostatic stress through the use of known a proteasome inhibitor MG132. Neurons were cultured and once mature subjected to two different concentrations of MG132 (0.15 and 0.5 μM) for 48 hours and collected to extract RNA for sequencing studies (Fig. 1A). Treatment had a strong effect on cells with 97% variance in the dataset explained by treatment regardless of the concentration (Fig. 1B), 3% of the variance explained by different dosage and negligible residual variance which highlight the strength of this system. Treatments were so potent that transcriptomic changes driven by the two doses were almost overlapping (Fig.1C). For this reason, we decided to first treat the two doses as one and investigate broad changes driven by proteasome inhibition in human neurons. Differential gene expression analysis identified over 11,000 genes as being either upregulated or downregulated after treatment (Fig. 1D), underlying the substantial effect on this drug on neurons, and as a positive control we show that most proteasome subunits were upregulated upon treatment (Fig. S1).

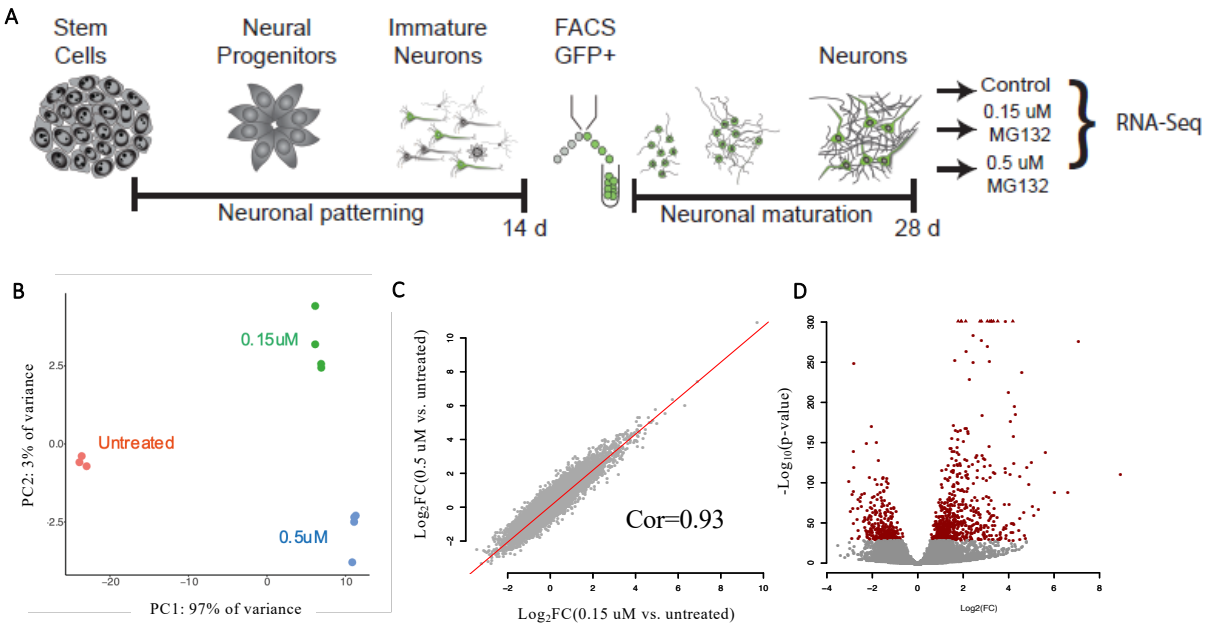


Fig. 1 | Proteasome inhibition in hiPSC-derived neurons drives strong transcriptomic changes. (A) Workflow of sequencing experiment. (B) Principle Component Analysis of sequenced samples. (C) Correlation analysis of different treatments (D) Differential gene expression analysis between treated and untreated samples.

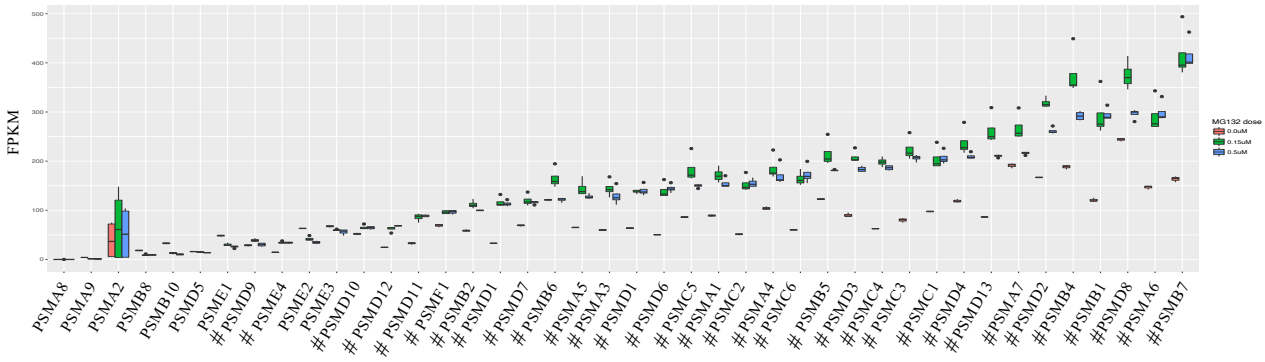


Fig. S1 | Proteasome subunits are significantly upregulated after treatment. # statistically significant

Proteostatic stress responses in human neurons alters ALS-related genes

We started by focusing on genes upregulated after treatment. Around 5464 genes were identified as being significantly upregulated by at least two-folds (adjusted p-value<0.05) between treated and untreated neurons. Gene Ontology analysis of these genes confirmed that protein folding responses, the proteasome and ubiquitin-binding processes were upregulated but also apoptotic processes and vesicle trafficking were induced by proteasome inhibition in human neurons (Fig. 2A-C and Fig. S2). Protein-protein interaction network analysis confirmed the involvement of proteasome components, chaperonins, ubiquitin ligases, proteins involved in autophagy and protein folding in the endoplasmic reticulum (ER) and control of translation, RNA biology and splicing (Fig. 2D). Intriguingly, we noticed that several genes connected to familial forms of ALS or identified as genetic risk factors and modifiers of the disease were similarly upregulated after treatment (Fig. 2E). This evidence underlines that several processes altered by ALS are directly connected to proteostatic stress. As previously shown (ref), it is well established that proteasome inhibition in human neurons results in nuclear translocation of TDP-43, histopathological hallmark of ALS[8] (Fig. 2F-G).

Proteostatic stress induces synaptic dysfunction in human neurons

We then carried out similar analyses for genes downregulated upon proteasome inhibition. (4413 genes, LFC>2, adj p-value<0.05). Intriguingly, genes downregulated upon treatment were connected to synaptic biology and modulation of electrical impulses through neuronal cultures (Fig. S3). Gene Ontology analysis of the downregulated genes confirmed that neuronal projections, axon guidance and synaptic processes were downregulated and specifically expression of genes involved in glutamatergic synapse and both pre- and post-synaptic machinery were decrease by proteasome inhibition in human neurons (Fig. 3A-C and Fig. S3) as confirmed by protein-protein interaction network (Fig. 3D). These observations are extremely relevant given that diagnostic tools for ALS include electrophysiological analysis for hyperexcitability in the motor circuit of patients[18,19] and one of the three approved drugs for ALS modulates synaptic function[11,20]. Even though quite similar, differences in between treatments include the striking downregulation of tubulins and cytoskeletal proteins in the higher dose treatment, also relevant to disruptions of cytoskeletal dynamics in ALS[2]. Taken together, this analysis suggests that proteostatic stress in human iPSC-derived neurons triggers upregulation of stress responses connected to protein degradation and downregulation of synaptic function likely to decrease metabolic need connected to electrophysiological activity.

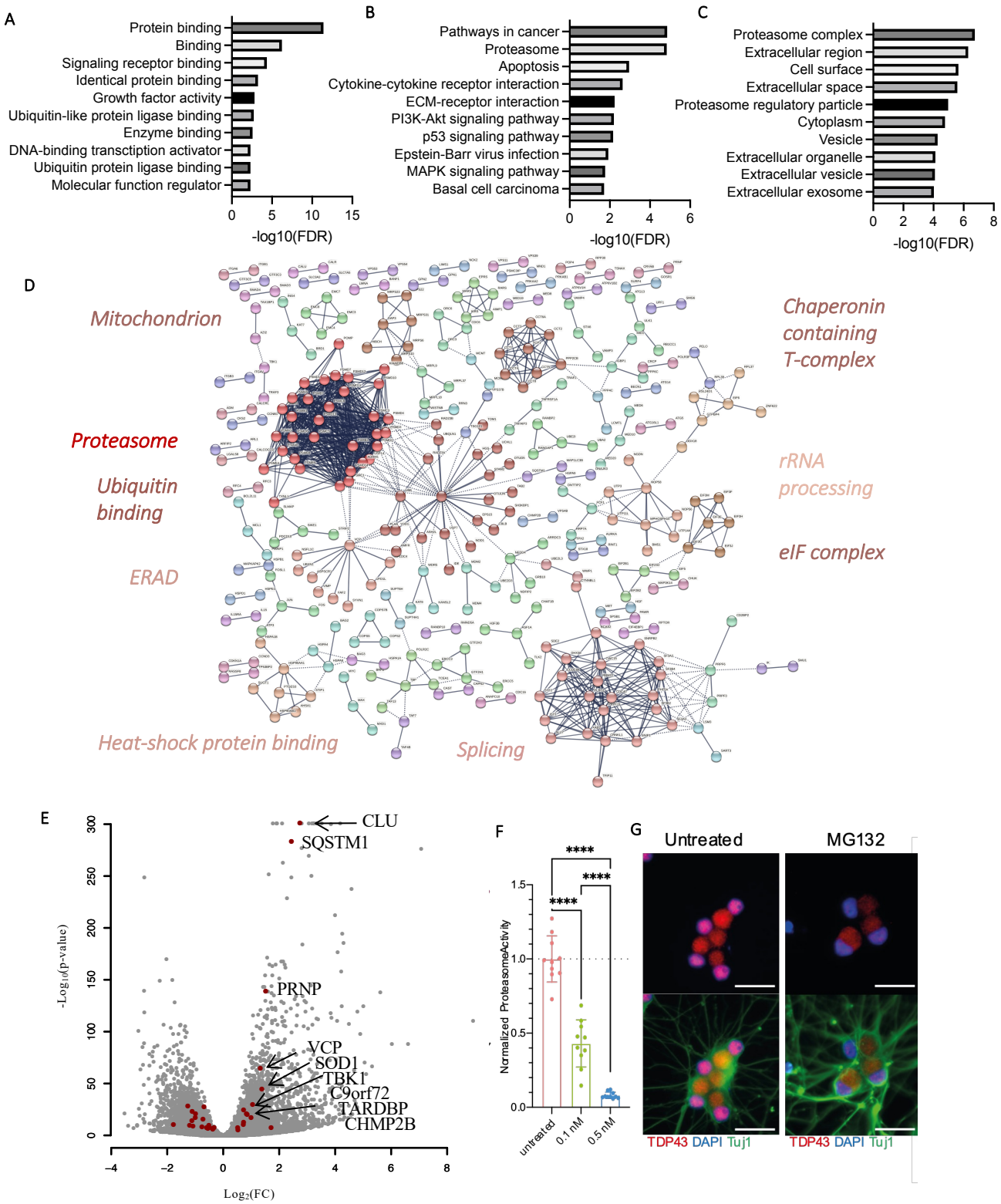


Fig. 2 | Genes upregulated by proteasome inhibition are connected to proteostatic stress and neurodegenerative diseases. **(A-C)** Gene Ontology Analysis of upregulated genes for Molecular Function (A), KEGG pathways (B) and Cellular Components (C). (D) Protein-protein interaction network of genes upregulated after proteasome inhibition. (E) Genes misregualted after treatment are connected to neurodegeneration and ALS. (F) Proteasome activity after treatment. (G) TDP-43 mislocalisation after treatment.

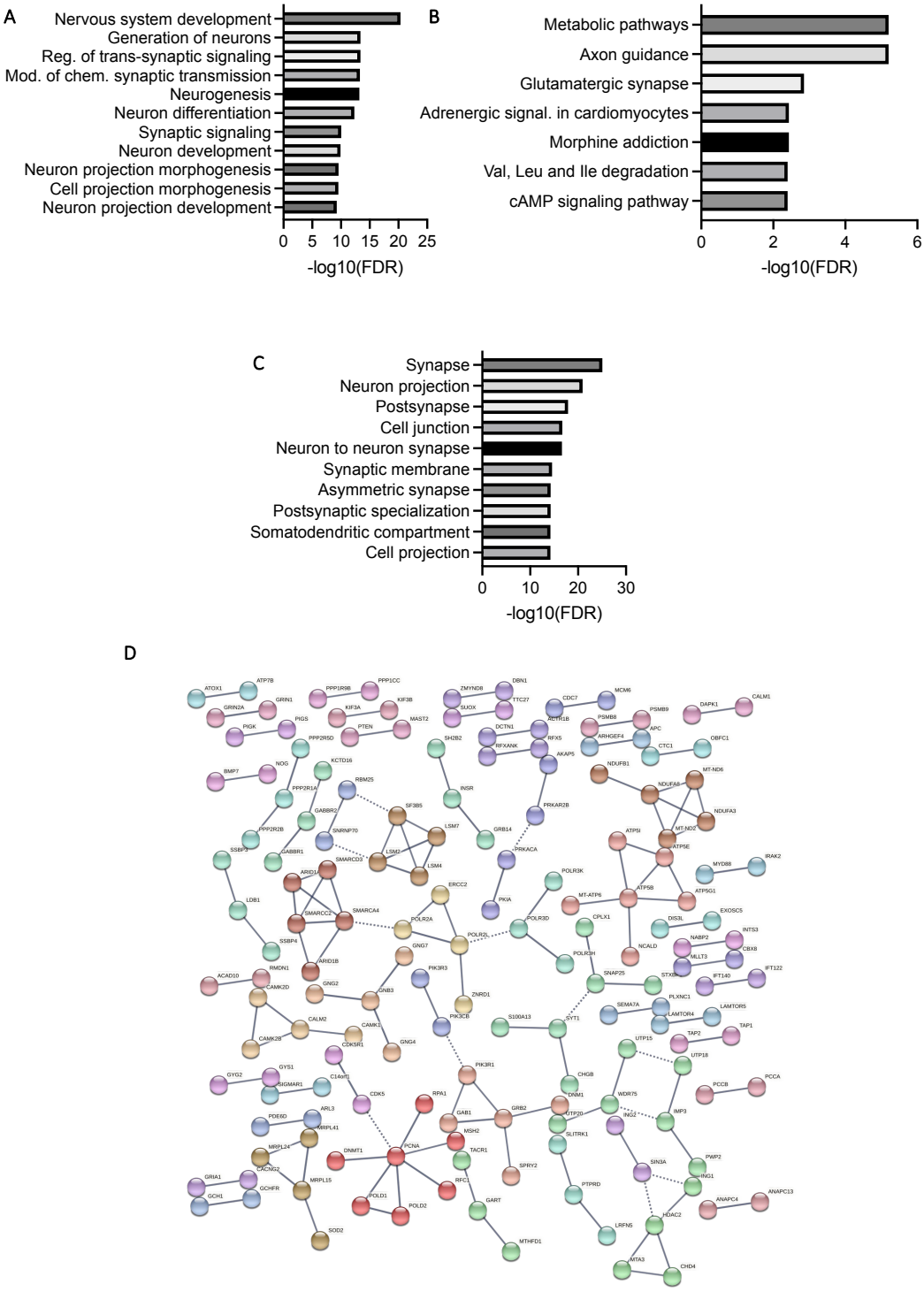


Fig. 3 | Genes downregulated by proteasome inhibition are connected to synaptic biology. (A-C) Gene Ontology Analysis of upregulated genes for Biological Porcesses (A), KEGG pathways (B) and Cellular Components (C). (D) Protein-protein interaction network of genes downregulated after proteasome inhibition.

Proteostatic stress responses *in vitro* replicate alterations in patients' neurons

To investigate the correlation between transcriptomic signatures identified *in vitro* with neurons from sporadic ALS patients, we compared genes misregulated after treatment with genes differentially misregulated in in excitatory neurons from a previously published dataset from sALS patients' motorcortices. This comparison showed a discrete overlap between genes upregulated upon treatment in hiPSC-dervied neurons and genes upregulated in excitatory neurons of ALS patients (Fig. 4A). Shared genes are connected to proteasome subunits and heat-shock response-associated chaperonins and GO and protein-protein interaction analyses confirmed upregulation of pathways involved in protein folding and RNA biology and the connection to neurodegenerative diseases (Fig. 4B-E and S4). As shown by others, some of these changes also overlapped with the transcriptomic signature altered in human neurons after downregulation of TDP43 *in vitro*[15]. This suggests that changes identified in sporadic ALS neurons are connected to neuronally intrinsic proteostatic alternations that are at least in part connected to TDP-43-dependent dysfunction confirming that

Similar comparison for downregulated genes showed connection to synaptic biology, neuronal projections and modulation of electrical impulses through neuronal cultures (Fig. 5A-D and S5). In order to understand if these changes are specific to neurons, we selected genes misregulated in both conditions and we computed a module score for these get sets, we generated a standardised z-score for the expression of gene each and sum it up as a total score for the gene set. For genes upregulated under proteostatic stress, excitatory neurons and to a lower extent inhibitory neurons from ALS patients showed the highest expression (Fig. 5E) suggesting that neurons are the only cell types upregulating unfolded protein responses in ALS patients. Conversely, for genes downregulated under protesostatic stress the biggest differences are seen in neurons, where as expected neurons in controls express high levels of synaptic genes whereas cells from patients downregulate the expression of transcripts connected to synaptic biology (Fig. 5F). This suggests that changes identified in sporadic ALS neurons are connected to neuronally intrinsic proteostatic alternations that are at least in part connected to TDP-43-dependent dysfunction and that trigger changes in electrophysiological properties of neurons.

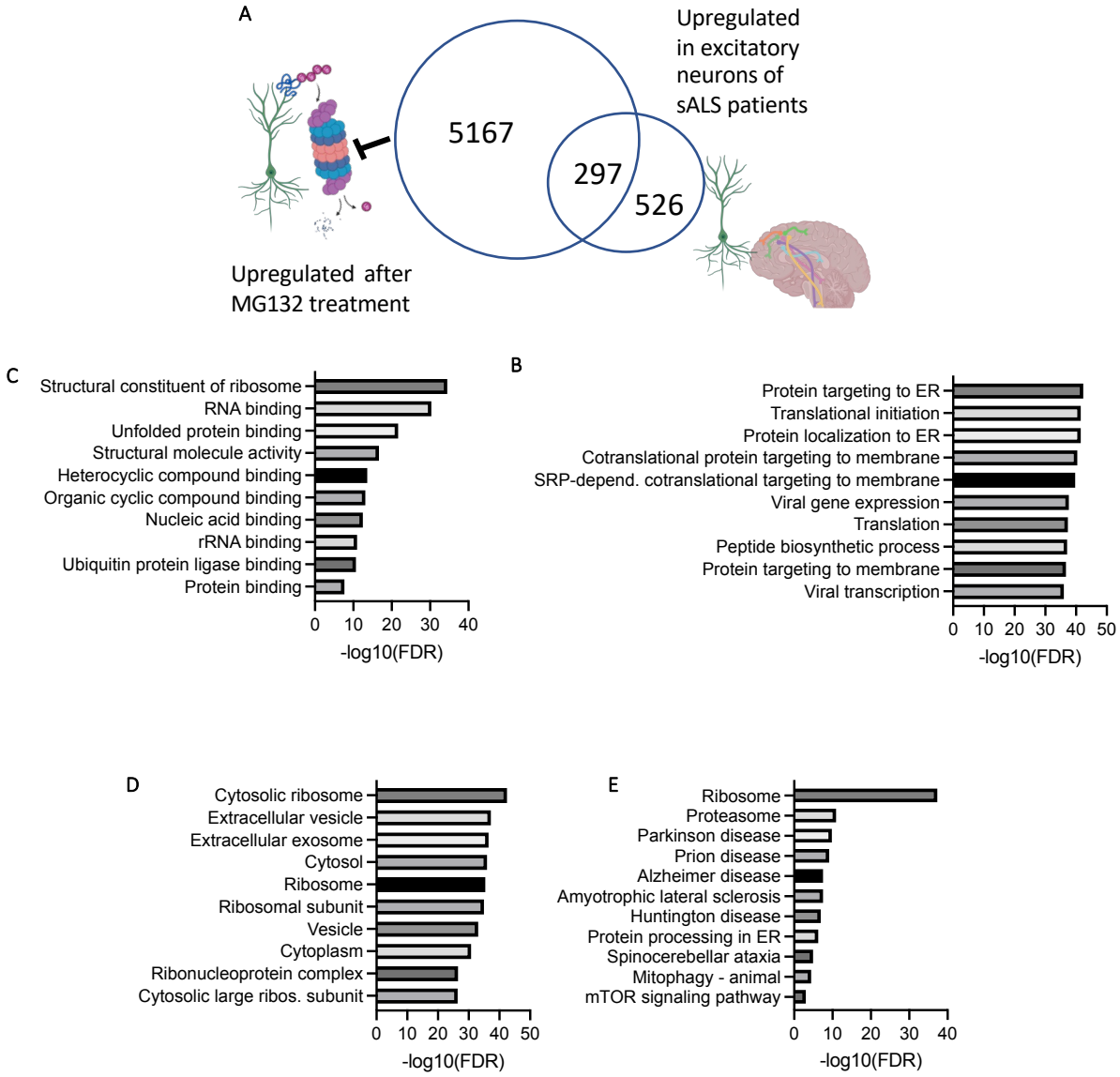


Fig. 4 | Shared transcriptomic signature of neuronal proteostatic stress and from sporadic ALS patients. (A) Shared upregulated genes between neurons under proteasome inhibition and excitatory neurons from sporadic ALS patients. **(B-E) Gene Ontology Analysis of shared upregulated genes for Biological Porcesses (B),Molecular Function (C), Cellular Components (D), KEGG pathways (E).**

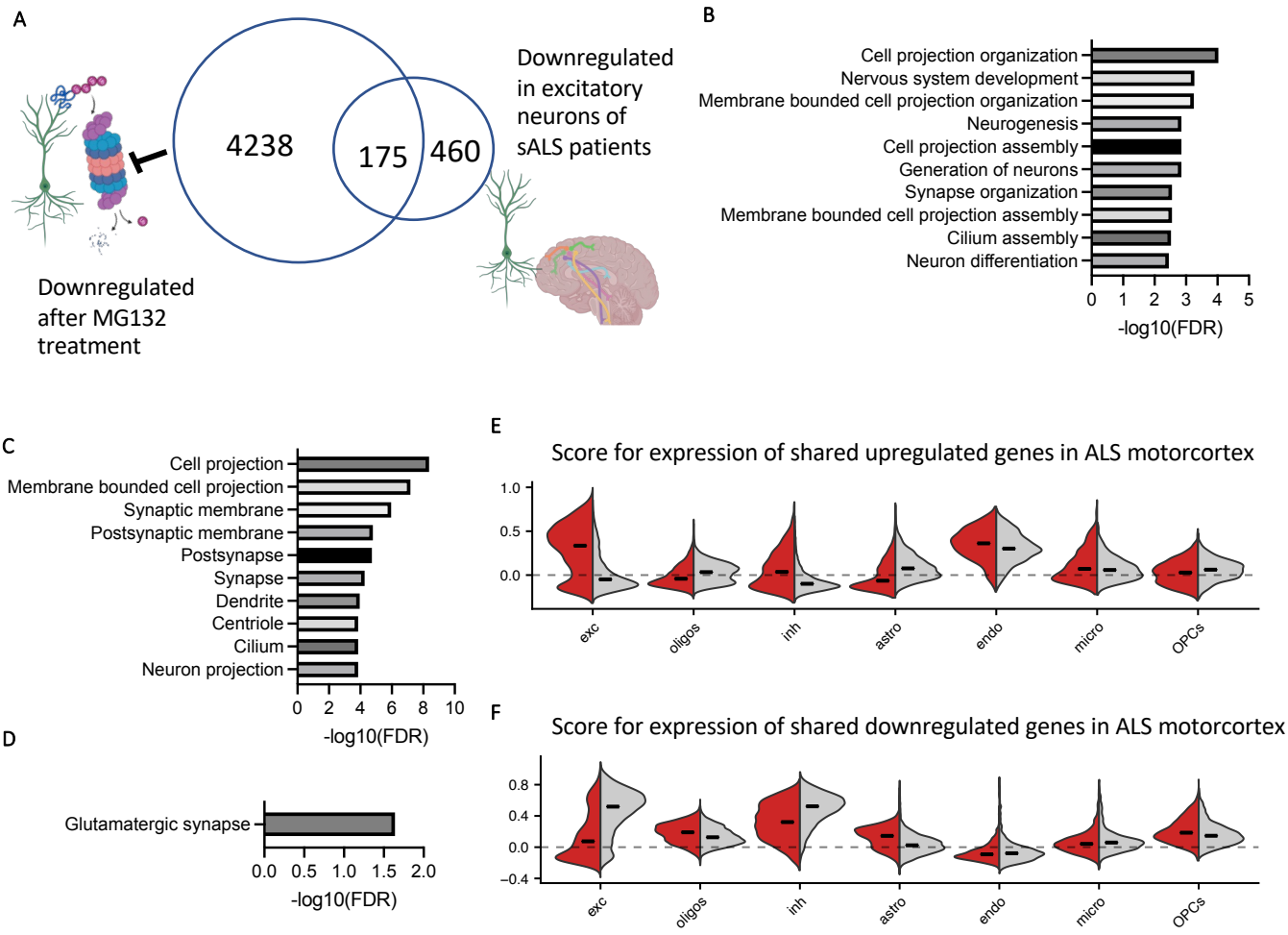


Fig. 5 | Neurons under proteostatic stress downregulate genes related to synaptic function. (A) Shared downregulated genes between neurons under proteasome inhibition and excitatory neurons from sporadic ALS patients. (B-D) Gene Ontology Analysis of shared downregulated genes for Biological Processes (B), Cellular Components (C), KEGG pathways (D). (E) Protein-protein interaction network of shared genes downregulated after proteasome inhibition.

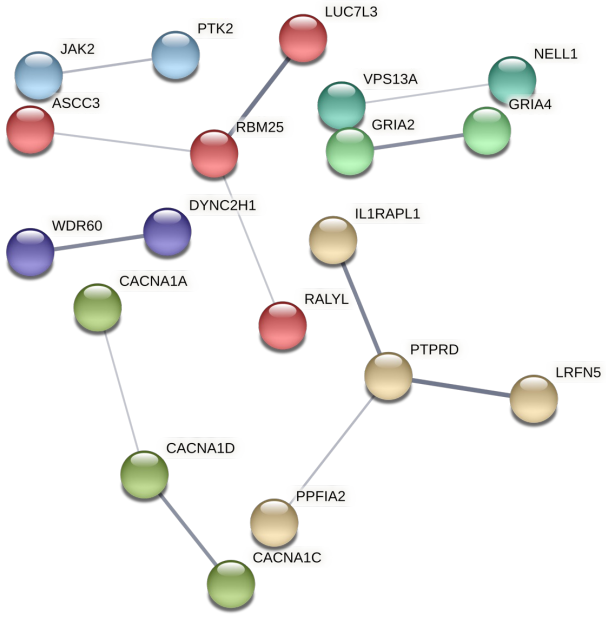


Fig. S5 | Neurons under proteostatic stress downregulate genes related to synaptic function. Protein-protein interaction network of shared genes downregulated after proteasome inhibition.

Neuronal proteosomal activity is tightly regulated in response to stress

We then wondered whether manipulation of these genes could alter neuronal stress responses. We first decided to exclude heat-shock related chaperonins and translational machinery because of their pivotal role in basic biological functions so we focused on the proteasome related proteins (Fig. S4, left). We excluded core proteasomal subunits (PSMAs, PSMBs) because of the necessity of maintaining its intact enzymatic activity and focused on regulatory subunits (PSMCs and PSMDs) which might change proteasomic influx without impeding core catalytic function. We centred our interest ion PSMD12 for the recent identification of genomic deletions in the PSMD12 gene leading to a rare form of neurodegenerative disease[21] but also for intriguing evidence arising from studies in cancer cells. As mentioned above, MG132 was first discovered as a chemotherapeutic, one side effect of proteasome inhibitors as chemotherapy is the induction of peripheral neuropathies in patients[22] prompting its use in research to mimic neuronal degeneration *in vitro*. Even though quite susceptible to proteasome inhibition, myeloma often develops resistance to these drugs. One study ran an almost-genome-wide genetic screen to identify genes whose downregulation would increase survival to proteasome inhibitors and showed that downregulation of subunits of the 19S regulatory proteasome could confer increased survival[23]. In the same year, another group used a similar strategy and found that PSMDs subunits could confer increased survival of cells under proteotoxic stress and suggested that resistance might be mediated by upregulation of other protein degradation pathways, such as autophagy[24]. We therefore decide to manipulate PSMD12 levels in neurons under proteostatic stress and investigate its effect in neuronal survival.

We proceeded by implementing an experimental set up that allows efficient downregulation of transcript in postmitotic neurons devised in our lab and previously described[10]. Briefly, neurons were first transiently transfected with siRNA against *PSMD12* or a non-targeting scramble control, after that both WT and KD cells were treated with proteasome inhibition to look at proteasomal activity, cell survival and protein degradation dynamics (Fig. 6A). First, we ensured that knock-out strategy worked and confirmed that siRNA has a >95% efficiency (Fig. 6B), but also that treatment with proteasome inhibition upregulated *PSMD12* RNA (Fig 6B), as detected by RNA-sequencing (Fig. S1), and that KD was still efficient upon treatment (Fig 6B). We then proceeded to test whether knock-down could manipulate proteasome activity. To our surprise, KD-cells presented reduced proteasomal activity even without treatment with MG132 (Fig. 6C), highlighting the importance of integral proteasomal stoichiometry for functional catalytic activity. These changes do not seem to be connected to cell loss since KD and MG132 do not result in gross cell death (Fig. S4A) but cells treated with siRNA against *PSMD12* do show typical enlarged cytosolic morphology seen in WT cells treated with MG132.

Neuronal proteosomal activity is tightly regulated in response to stress

We then wondered whether these changes might be connected to different ratios between core, catalytic 20S proteasome and regulatory subunits. Western blot analyses showed that, even though extremely efficient, siRNA treatment only modestly decrease PSMD12 protein levels in untreated cells and that proteasome inhibition itself strongly decreased expression of PSMD12 protein independently of siRNA treatment (Fig. 6D), suggesting that PSMD12 levels are tightly controlled by post-translational mechanisms. On the other hand, levels of the core catalytic 20S subunit do not different between WT and KD and are upregulated to similar levels in both conditions after MG132 treatment (Fig. 6E). Therefore, changes seen in proteasome activity upon KD seem to be connected to regulatory activity of PSMD12 itself, rather than loss of proteasomal core integrity. We then wondered these alterations in proteasome activity might proteins towards other degradation pathways and indeed we identify altered expression of autophagy-related proteins p62 and LC3 under proteostatic stress (Fig. 6F). These results are consistent with previous reports showing that initial loss of regulatory subunits of the proteasome might result in impaired protein flux in the proteasome without impacting its core catalytic subunit[23] and that this decrease might be supplemented by increased inputs through the autophagic system[24]. These changes do not seem to be connected to drastic alterations in cell numbers (Fig. S4B).

Taken together, these results suggest that loss of regulatory 19S proteasome subunits can change the direction of degradative protein flux in human neurons and that under proteostatic stress loss of PSMD12 might elicit autophagy as an alternative pathway. Unfortunately, others have reported how these balance between loss of proteasomal regulation and autophagic influx can have both a beneficial or detrimental effect depending on other factors involved[23]. Moreover, given the association of autophagy related genes with ALS/FTD (e.g p62, TBK1, OPTN), the switch to autophagy as a degradation pathway might be an initially beneficial response to proteostatic stress but might still result in ALS-related neurodegeneration. Nonetheless, this small report adds a little piece in the puzzle of the role that proteostasis plays in neurodegeneration in ALS.

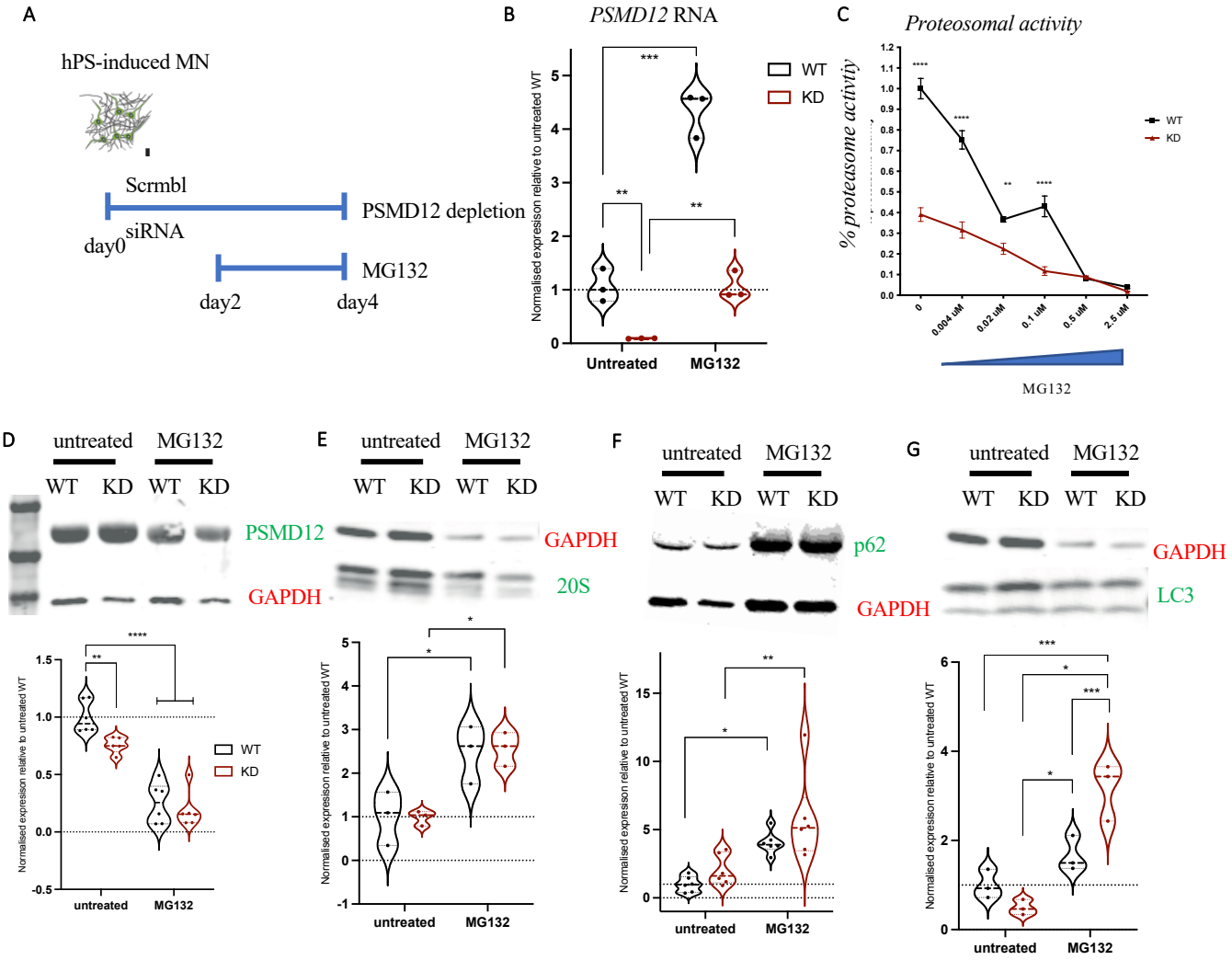


Fig. 6 | Manipulation of proteasome flux results in upregulated autophagy. (A) Workflow for downregulation of proteasome subunit PSDM12 under proteostatic stress. (B) Quantification of *PSMD12* RNA by RT-qPCR. (C) Proteosomal activity under inhibition in WT and KD. (D-G) Western blot quantifications for proteins involved in protein degradation.

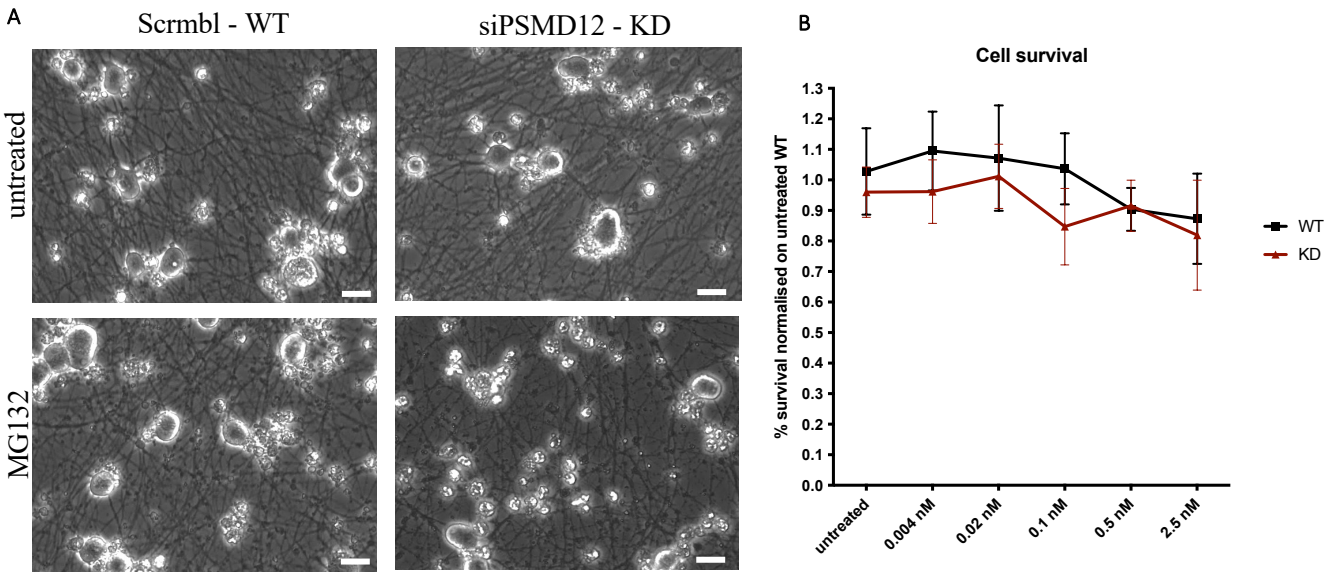


Fig. S6 | Neuronal survival under proteasome manipulations. (A) Brightfield images of neurons subjected to proteasomal manipulations. (B) survival of neurons under proteasomal manipulations.

DISCUSSION

In this report, we carry out a meta-analysis of published RNA-sequencing studies and validations in human iPSC-derived models to understand responses to proteostatic stress in human neurons. Firstly, we characterised a robust system to model proteostatic stress in human neurons *in vitro*. We confirmed that proteasome inhibition activates canonical stress pathways such as proteasome subunits upregulation, activation of Unfolded Protein Responses (UPR) and heat-shock chaperonins. These changes are remarkably accompanied by alterations in splicing factors and translational machinery but also with the upregulation of genes associated with ALS and other neurodegenerative diseases. Surprisingly, these stress responses are associated with downregulation in transcripts encoding synaptic molecules, drawing a connection between proteostatic stress and neuronal excitability, which is the only diagnostic tools for ALS. By leveraging comparison with single-nucleus RNA-sequencing dataset from ALS patients and unaffected individuals, we were able to show that not only these changes in our *in vitro* models mimic biology disrupted in patients, but also that these alterations are specific to excitatory neurons. Finally, we intersect these analyses to nominate a proteasomal subunit, *PSMD12*, whose manipulation might be neuroprotective. Unfortunately, changing levels of PSMD12 results in alterations of proteostatic influx that result themselves in proteasome inhibition and activation of autophagic pathways that are unfortunately also disrupted in ALS.

The connection between ALS and proteostatic stress is a long-lasting theme in the field since the very first connection of ALS-related gene SOD1 to protein degradation pathways[25] and many others have then confirmed this disruptions in ALS patients[3,13], especially in neurons[26]. What is surprising is that in our system these alterations are connected to 1. RNA metabolism and 2. ALS-associated genes. The connection to RNA biology in a human neurons is extremely relevant because of the changes connected to alterations of splicing functions identified by us and others specifically in neurons[9,10] and the emerging need for humanised models in the field[27], with this reproducible and robustly characterised system we know provide a platform to investigate these changes in a human context. Moreover, the upregulation of ALS-associated genes in neurons undergoing proteostatic stress is extremely relevant given that disease-relevant classes of neurons intrinsically express higher levels of ALS genes in both the cortex[15] and the spinal cord[28]. The upregulation of similar sets of genes in response to proteostatic stress might explain the higher susceptibility of these classes of neurons to ALS.

Another striking mechanism disrupted by proteostatic stress is neuronal excitability. Alterations in electrical circuits is a well-characterise symptom and as of today, the most reliable diagnostic tool for ALS[18]. Moreover, one of the three drugs approved to use in patients, Riluzole, acts on modulating neuronal firing[11] and many familial mutations have been shown

to result in neuronal hyperexcitability[29]. Our system could provide a platform to further dissect mechanisms connection between ALS-driven proteostatic stress and synaptic function in human neurons. The specifically neuronal expression of these genes and the fact that these genes are differentially expressed only excitatory neurons in ALS patients, underlies the usefulness of our system to not only broaden our understanding of neurodegeneration in ALS, but also provides a platform for the discovery of new therapeutic approaches to the disease.

Because of this, we attempted to nominate one target that could be neuroprotective under proteostatic stress, PSMD12. Given the chemotherapeutic-resistance acquired by cancer cells following loss of PSMD12[22-24], we hoped to replicate this pro-survival phenotype in neurons by boosting proteasome activity. Indeed, modulating PSMD12 levels resulted in changes in proteasome activity but in the opposite direction. The connection between modulating levels of proteasomal subunits in favours of other protein degradation pathways was demonstrated before[24] and others have recently suggested that, at least in cancer, these changes might also modulate endosomal trafficking[30], a pivotal mechanism for health synaptic function, which draws a connection in between proteostasis and synaptic biology in neurons revealed by our study. Unfortunately, this mechanism, that might be initial beneficial for neuronal survival under stress, might not be feasible for the development of therapeutics for ALS given that autophagy is also a degradative pathway disrupted in both familial and sporadic cases of the disease[4]. Nonetheless, our platform remains a useful tool to nominate other targets that could be investigated for therapeutical strategy in ALS.

References

1 Brown, R. H., Jr. & Al-Chalabi, A. Amyotrophic Lateral Sclerosis. *N Engl J Med* **377**, 1602, doi:10.1056/NEJMc1710379 (2017).

2 Taylor, J. P., Brown, R. H., Jr. & Cleveland, D. W. Decoding ALS: from genes to mechanism. *Nature* **539**, 197-206, doi:10.1038/nature20413 (2016).

3 D'Erchia, A. M. *et al.* Massive transcriptome sequencing of human spinal cord tissues provides new insights into motor neuron degeneration in ALS. *Sci Rep* **7**, 10046, doi:10.1038/s41598-017-10488-7 (2017).

4 Mordes, D. A. *et al.* Dipeptide repeat proteins activate a heat shock response found in C9ORF72-ALS/FTLD patients. *Acta Neuropathol Commun* **6**, 55, doi:10.1186/s40478-018-0555-8 (2018).

5 Prudencio, M. *et al.* Distinct brain transcriptome profiles in C9orf72-associated and sporadic ALS. *Nat Neurosci* **18**, 1175-1182, doi:10.1038/nn.4065 (2015).

6 Tam, O. H. *et al.* Postmortem Cortex Samples Identify Distinct Molecular Subtypes of ALS: Retrotransposon Activation, Oxidative Stress, and Activated Glia. *Cell Rep* **29**, 1164-1177 e1165, doi:10.1016/j.celrep.2019.09.066 (2019).

7 Leigh, P. N. *et al.* Ubiquitin-immunoreactive intraneuronal inclusions in amyotrophic lateral sclerosis. Morphology, distribution, and specificity. *Brain* **114 (Pt 2)**, 775-788, doi:10.1093/brain/114.2.775 (1991).

8 Neumann, M. *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**, 130-133, doi:10.1126/science.1134108 (2006).

9 Melamed, Z. *et al.* Premature polyadenylation-mediated loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration. *Nat Neurosci* **22**, 180-190, doi:10.1038/s41593-018-0293-z (2019).

10 Klim, J. R. *et al.* ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair. *Nat Neurosci* **22**, 167-179, doi:10.1038/s41593-018-0300-4 (2019).

11 Wainger, B. J. *et al.* Effect of Ezogabine on Cortical and Spinal Motor Neuron Excitability in Amyotrophic Lateral Sclerosis: A Randomized Clinical Trial. *JAMA Neurol* **78**, 186-196, doi:10.1001/jamaneurol.2020.4300 (2021).

12 Giacomelli, E. *et al.* Human stem cell models of neurodegeneration: From basic science of amyotrophic lateral sclerosis to clinical translation. *Cell Stem Cell* **29**, 11-35, doi:10.1016/j.stem.2021.12.008 (2022).

13 Brown, R. H. & Al-Chalabi, A. Amyotrophic Lateral Sclerosis. *N Engl J Med* **377**, 162-172, doi:10.1056/NEJMra1603471 (2017).

14 Guo, Q. *et al.* In Situ Structure of Neuronal C9orf72 Poly-GA Aggregates Reveals Proteasome Recruitment. *Cell* **172**, 696-705 e612, doi:10.1016/j.cell.2017.12.030 (2018).

15 Limone, F. *et al.* Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neurons to degeneration in ALS. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2021.07.12.452054 (2021).

16 Baxi, E. G. *et al.* Answer ALS, a large-scale resource for sporadic and familial ALS combining clinical and multi-omics data from induced pluripotent cell lines. *Nat Neurosci* **25**, 226-237, doi:10.1038/s41593-021-01006-0 (2022).

17 Limone, F. *et al.* Efficient generation of lower induced Motor Neurons by coupling Ngn2 expression with developmental cues. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2022.01.12.476020 (2022).

18 Nakata, M. *et al.* Distal excitability changes in motor axons in amyotrophic lateral sclerosis. *Clin Neurophysiol* **117**, 1444-1448, doi:10.1016/j.clinph.2006.04.005 (2006).

19 Kanai, K. *et al.* Altered axonal excitability properties in amyotrophic lateral sclerosis: impaired potassium channel function related to disease stage. *Brain* **129**, 953-962, doi:10.1093/brain/awl024 (2006).

20 Bensimon, G., Lacomblez, L. & Meininger, V. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med* **330**, 585-591, doi:10.1056/NEJM199403033300901 (1994).

21 Kury, S. *et al.* De Novo Disruption of the Proteasome Regulatory Subunit PSMD12 Causes a Syndromic Neurodevelopmental Disorder. *Am J Hum Genet* **100**, 352-363, doi:10.1016/j.ajhg.2017.01.003 (2017).

22 Kaplan, G. S., Torcun, C. C., Grune, T., Ozer, N. K. & Karademir, B. Proteasome inhibitors in cancer therapy: Treatment regimen and peripheral neuropathy as a side effect. *Free Radic Biol Med* **103**, 1-13, doi:10.1016/j.freeradbiomed.2016.12.007 (2017).

23 Tsvetkov, P. *et al.* Compromising the 19S proteasome complex protects cells from reduced flux through the proteasome. *Elife* **4**, doi:10.7554/eLife.08467 (2015).

24 Acosta-Alvear, D. *et al.* Paradoxical resistance of multiple myeloma to proteasome inhibitors by decreased levels of 19S proteasomal subunits. *Elife* **4**, e08153, doi:10.7554/eLife.08153 (2015).

25 Saxena, S., Cabuy, E. & Caroni, P. A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice. *Nat Neurosci* **12**, 627-636, doi:10.1038/nn.2297 (2009).

26 Kiskinis, E. *et al.* Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. *Cell Stem Cell* **14**, 781-795, doi:10.1016/j.stem.2014.03.004 (2014).

27 Guerra San Juan, I. *et al.* Loss of mouse Stmn2 function causes motor neuropathy. *Neuron*, doi:10.1016/j.neuron.2022.02.011 (2022).

28 Yadav, A. *et al.* A Cellular Taxonomy of the Adult Human Spinal Cord. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2022.03.25.485808 (2022).

29 Wainger, B. J. *et al.* Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. *Cell Rep* **7**, 1-11, doi:10.1016/j.celrep.2014.03.019 (2014).

30 Oron, M. *et al.* The molecular network of the proteasome machinery inhibition response is orchestrated by HSP70, revealing vulnerabilities in cancer cells. *Cell Rep* **40**, 111428, doi:10.1016/j.celrep.2022.111428 (2022).

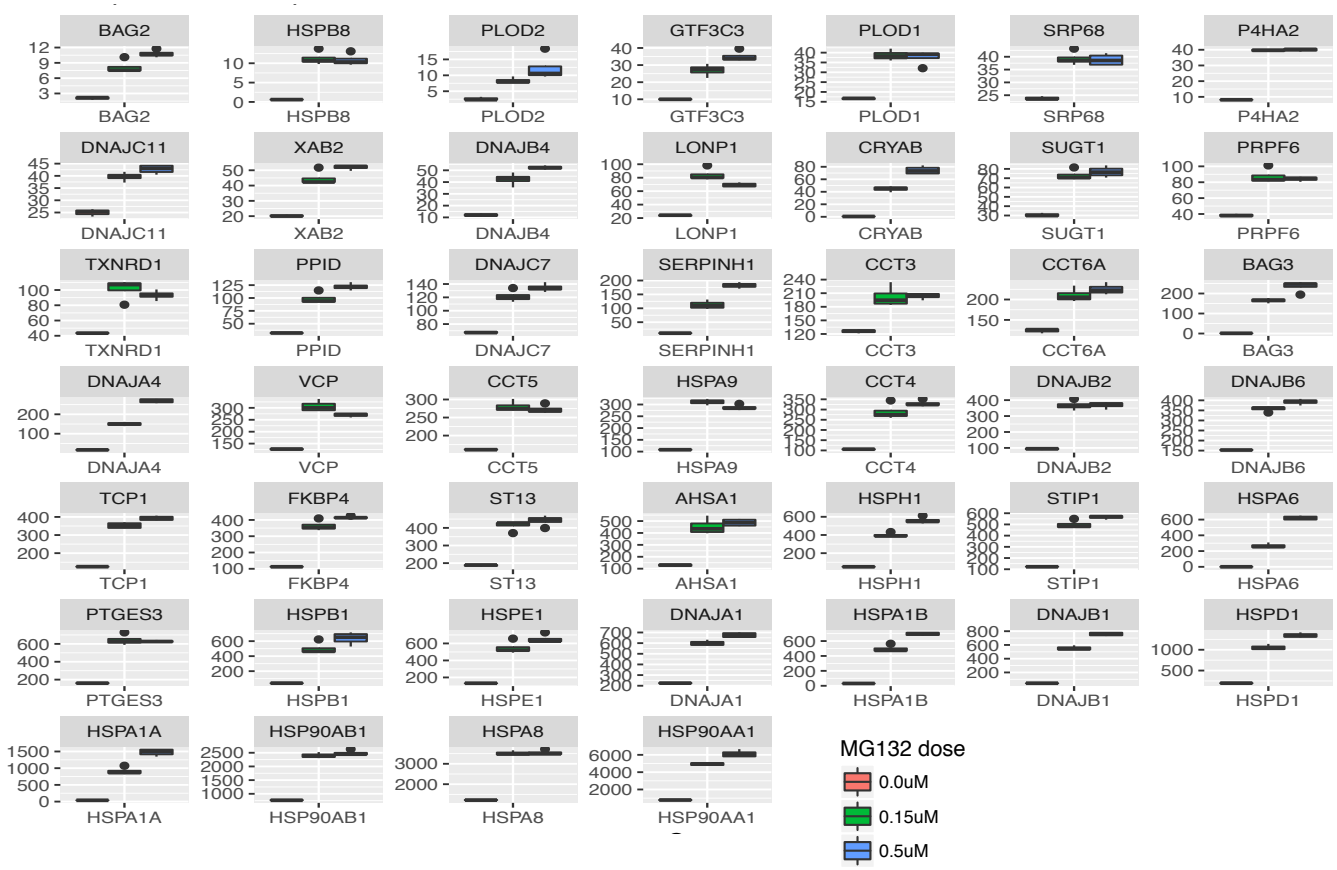


Fig. S2 | Genes upregulated by proteasome inhibition. Genes upregulated after treatment are connected to protein homeostasis.

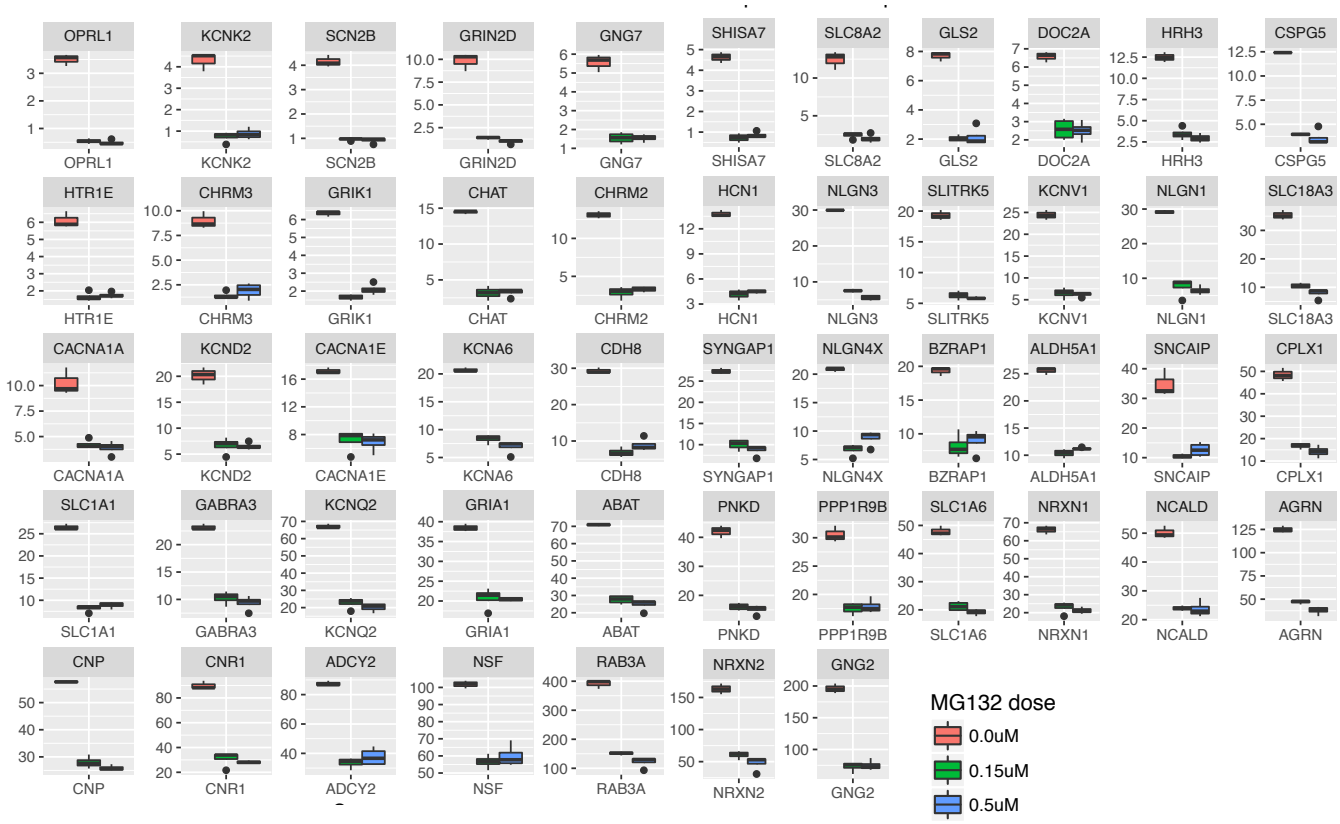


Fig. S3 | Genes downregulated by proteasome inhibition. Genes downregulated after treatment are connected to synaptic biology.

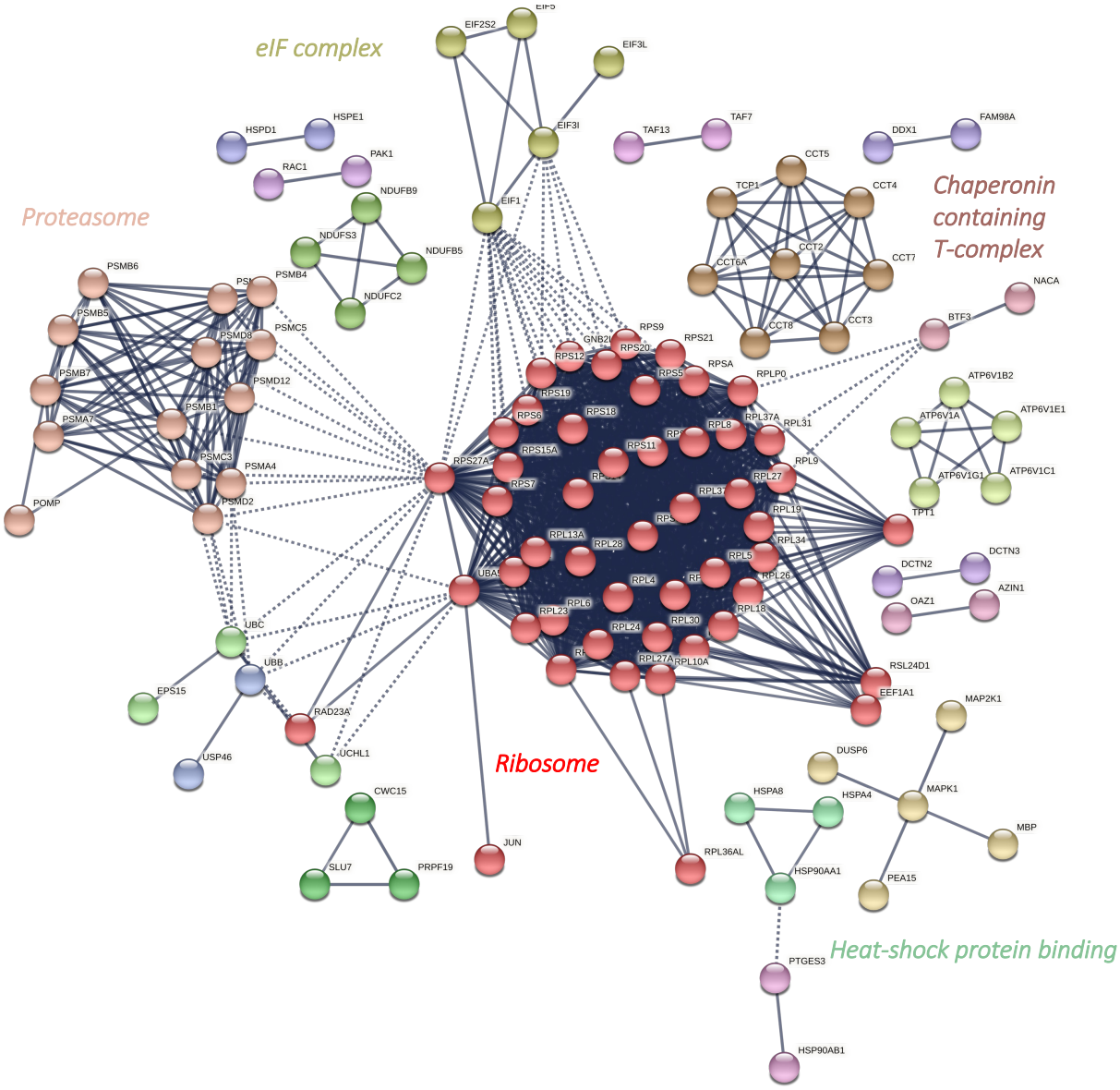


Fig. S4 | Shared transcriptomic signature of neuronal proteostatic stress and from sporadic ALS patients. Protein-protein interaction network of shared genes upregulated after proteasome inhibition.

Establishment of human glial co-culture systems for the study of cell-to-cell interactions

As we highlight in chapter 1 and 3, besides being a purely neurocentric disease many other cell types take part into the initiation and/or exacerbation of neurodegeneration in ALS. Because of the impossibility to readily access brain tissue and the human specificity of certain aspects of ALS biology, we decided to establish differentiation protocols for other cells of the brain, i.e. microglia and oligodendrocytes, and start characterizing their interactions in a dish. A small summary of this work will be presented below.

Microglia

Microglia are the resident macrophages of the brain and play a pivotal role in several mechanisms during development, adult homeostasis and disease by shaping synaptic function, removing debris, performing immune surveillance and defend us against pathogens. Here we present a protocol to generate human Microglia-like cells (iMGLs), adapted from previous schemes[1] and already applied for modelling dysfunctions in neurodegenerative disease contexts[2,3]. To mimic primitive haematopoiesis[4], hPSCs are cultured in three-dimensional embryoid bodies in reduced oxygen levels and subjected to small molecules to push them towards a haemogenic fate. These primitive structures are then stimulated to produce haematopoietic progenitors that rapidly bud out as single cells thanks to a cocktail of stem cell factors, these cells can be harvested and replated to generate myeloid progenitors through M-CSF and specifically brain-like cells thanks to brain-specific IL34 and CNS-enriched TGBβ. After a few weeks, CNS-resident-like myeloid progenitors are further matured by adding microglial ligands CD200 and fractalkine (CX3CL1) (Fig. 7A). After 14 days >95% of cells express myeloid specific markers PU.1 and Iba1 (Fig. 7B). Their expression is maintained till the end of differentiation at day 40 with the addition of microglia-enriched markers Trem2 and CX3CR1 (Fig. 7C). Moreover, cells express surface markers typical of immune myeloid cells and specifically microglia (Fig. 7D).

By day 40 of differentiation, cells assume typical ramified morphology and are highly motile throughout the culture, they contact each other and extend plenty of protrusions and filipodia typical of myeloid cells and reminiscent of brain-resident microglia (Fig. 7F). To further test their functionality, we fed iMGLs with beads covered with *E. coli* extracts and a pH sensitive dye that shows no fluorescence at neutral pH and fluoresces brightly in acidic formations like phagosomes and lysosomes and allows detection of phagocytosis (Fig. 7G). After only 10 minutes from feeding, iMGLs show high degree of internalization demonstrating their highly phagocytic activity (Fig. 7G).

With this preliminary characterization (and other data not shown), we present a highly scalable system to generate microglial cells *in vitro*. We hope to utilise this platform to widen our knowledge in human microglial functions and further investigate its misregulation in ALS.

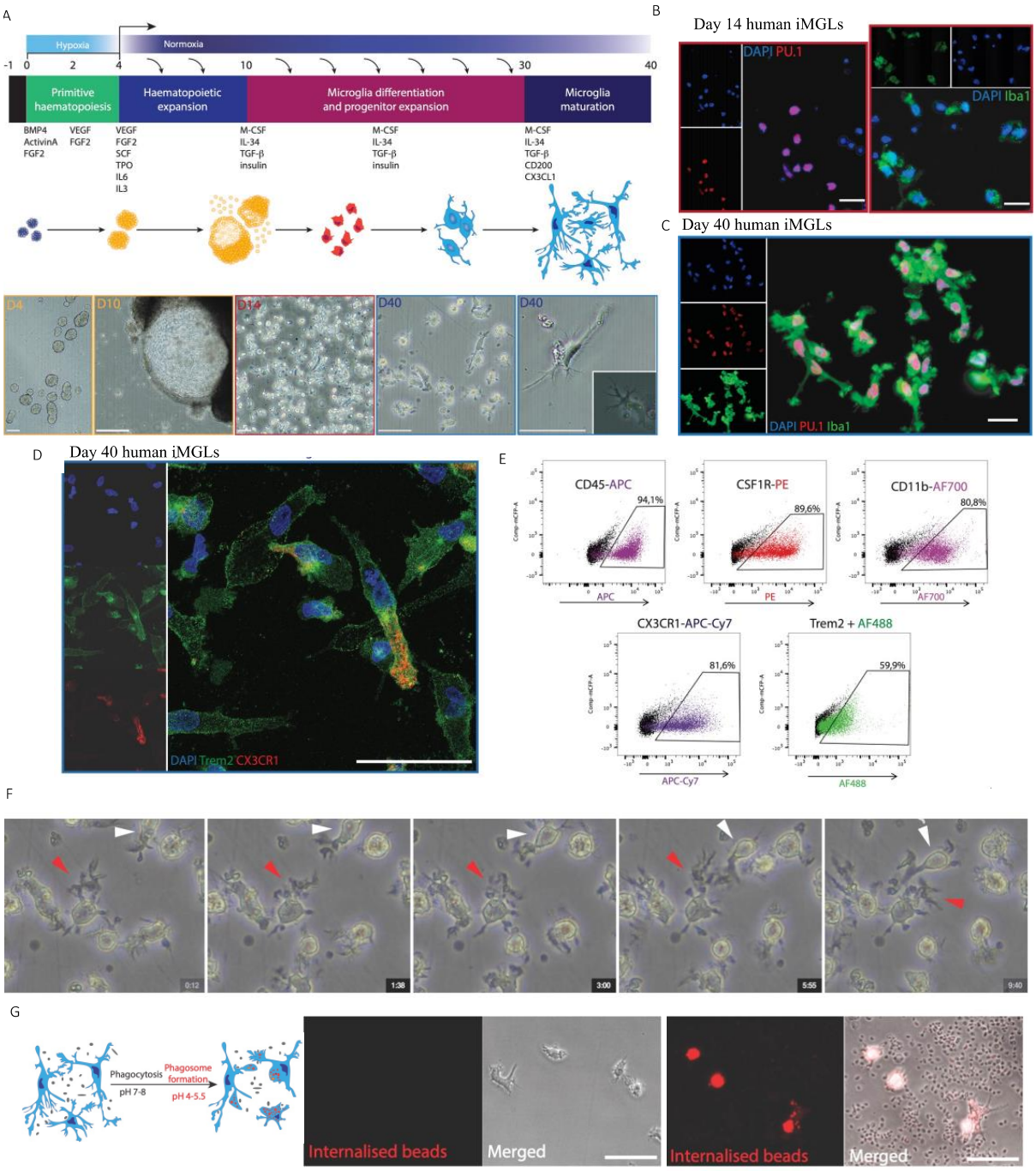


Figure 7 Characterization of human Microglia-like Cells (iMGLs). *(A) Diagram of differentiation protocol from primitive haematopoiesis to early myeloid progenitors into microglia-like cells. (B) immunofluorescence staining of myeloid markers PU.1 and Iba1 at day 14. (C) Immunofluorescence staining of myeloid markers PU.1 and Iba1 at day 40. (D) Immunofluorescence staining of microglia-enriched markers Trem2 and CX3CR1 at day 40. (E) Flow cytometry analysis of immune (CD45), myeloid (CSF1R and Cd11b) and microglial markers (CX3CR1 and Trem2) at day 40. (F) hiMGLs motility and branching at day 40. (G) hiMGLs rapidly phagocytose fluorescent beads.*

As described in chapter 3, intrinsically higher expression of ALS-related genes in neurons and their connection to proteostasis is at the bottom of the susceptibility of these cells to ALS and triggers responses in other cell types, i.e. microglia[3]. We wondered if MG132 treatment could be sufficient to induce these changes in microglia. We established a co-culture system of hiPSC-neurons and human iMGLs, where separately generated iMGLs[1-3] were add on top of neuronal networks[5,6]. In steady state, microglia assume a ramified morphology and interact mostly with neurites removing debris (Fig. 8A). When neurons are pre-treated with MG132 and microglia are added on top, iMGLs acquire an amoeboid morphology, typically associated with reactive states, appear to interact with neuronal cell bodies (Fig. 8B), potentially phagocytosing these cells. As mentioned, iMGLs can be stimulated to express reactive genes by feeding them apoptotic neurons. Pre-activation of iMGLs before co-culture was sufficient to induce amoeboid morphology, as seen when co-cultured with stressed neurons, suggesting that reactive microglia might be deleterious to healthy neurons just as much as proteostatic stress in neurons can trigger reactive microglial phenotypes (Fig. 8C). As expected, co-cultures of both MG132-treated neurons and stimulated microglia resulted in drastic changes in cell morphology with loss of neurite networks (Fig. 8D).

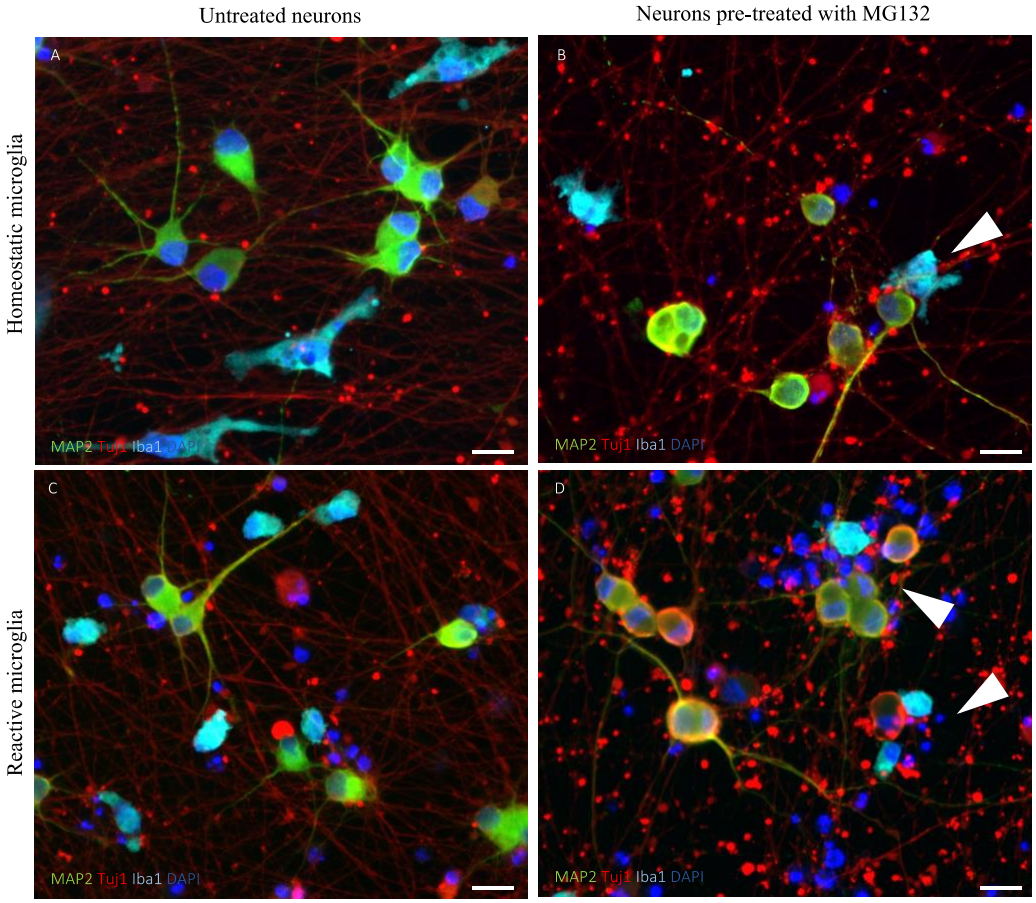


Figure 8 Pre-treatment of neurons with MG132 induces reactivation in microglia. *(A) Untreated neurons and homeostatic iMGLs co-culture. (B) Neurons pretreated with MG132 and homeostatic iMGLs. (C) iMGLs pre-stimulated with apoptotic cells co-cultured with untreated neurons. (D) iMGLs pre-stimulated with apoptotic cells co-cultured with MG132-treated neurons.*

Oligodendrocytes

Oligodendrocytes are responsible for myelinating axons in the CNS, thereby maintaining strong electrical connectivity of brain circuitry. Oligodendrogenesis happens quite late during development and most myelination occurs postnatally, therefore most differentiation protocols published as of now are extremely long and quite complex[7,8]. In an effort to make these processes more amenable to scalability, we decided to adapt a protocol that differentiated oligodendrocytes from hPSCs[7,9] to floating three-dimensional cultures in bioreactors, a well-established method to produce neuronal cells in big scale settings[10]. Briefly, hiPSCs/hESCs cultured in 3D bioreactors are first converted to neural stem cells with small molecules inhibition of double Smad (as described extensively in chapter 5) and are then ventralised through SHH activation and posteriorized through retinoids (Fig. 9A). These progenitors are then pushed towards an oligodendrocyte progenitors (OPCs) identity by the addition of PDGF-AA and IGF and subsequently induced to further differentiate into myelinating oligodendrocytes with T3 (Fig. 9A). Initial analysis of gene expression by RT-qPCR showed upregulation of neuronal progenitor gene PAX6 in early stages of differentiation and of OPC-specific genes in mid-stage of the protocol in spinner bioreactors at comparable levels than the original monolayer protocol (Fig. 9B).

Throughout the differentiation some of the spheroids were periodically harvested and as separate cultures to form monolayers and monitor differentiation of OPC/oligodendrocytes through live immunofluorescent staining for O4, an antibody that recognizing a sulfoglycolipid specific to OPC/early oligodendrocytes. As early as day 50 (week 7) of the protocol, cultures generated $O4^+$ cells that presented typical oligodendroglial morphology (Fig. 9C). By the end of the differentiation protocol (week 10/day 80), we could readily detect in these cultures a mixed of cell types of the CNS: $O4^+MBP^+$ early myelinating oligodendrocytes cells (Fig. 9C), which were interacting with neurons (Fig. 9E) and with astrocytes (Fig. 9F), which are both typically generated in this differentiation protocols[7].

We then decided to integrate microglia in these complex oligodendroglial cultures. To do so we plated oligodendrocytes spheres around week 7 and let them maturate in 2D while in parallel differentiating iMGLs, by day 20 when cells reach a microglial-progenitor like state, we harvested them and added them on the oligodendroglial co-cultures for the last 20 days of maturation (Fig. 10A). Microglial cells fully integrate into the milieu of cells (Fig. 10B), acquire a complex morphology reminiscent of their *in vivo* counterparts (Fig. 10C) and are highly motile throughout the culture interacting very closely with the neuronal network (Fig. 10D). Immunofluorescence analysis revealed that neurons, oligodendrocytes and microglia interact with each other and assume typical morphology (Fig. 8F-G). We believe that this system could be suitable to further model and dissect some of the interactions seen in the brains of ALS patients.

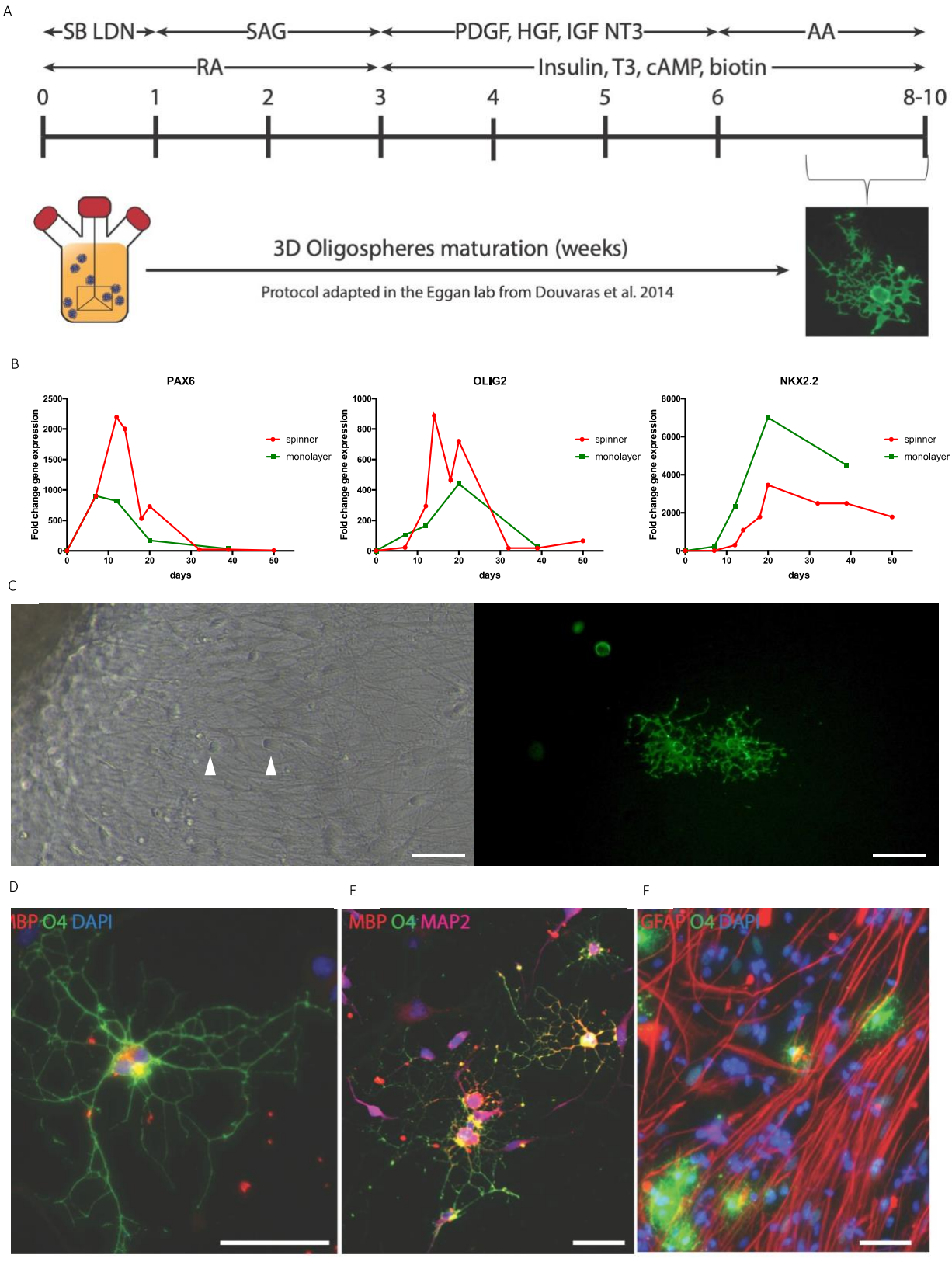


Figure 1 Characterization of 3D human Oligodendrocytes differentiation protocol. *(A) diagram of differentiation protocol. (B) RT-qPCR analysis of Oligodendrocyte Progenitors Markers at early stages of differentiation. (C) Live O4 immunofluorescence staining of committed oligodendrocytes day 50 (week 7). (D-F) immunofluorescence staining of brain cells markers from 2D cultured spheres (day 80, week 10): O4 – OPCs and oligodendrocytes, MBP – myelinating oligodendrocytes, MAP2 – neurons, GFAP – astrocytes.*

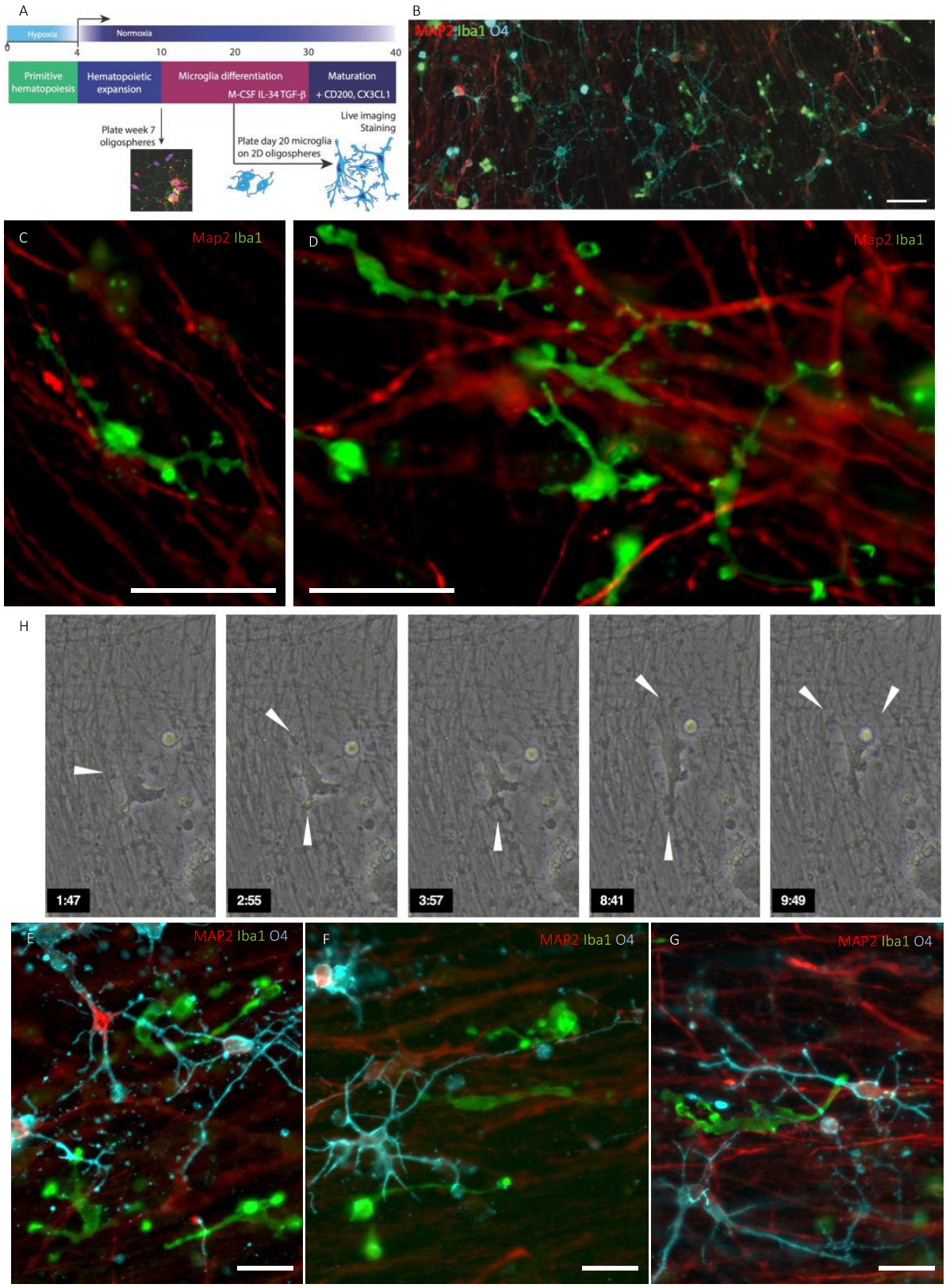


Figure 2 Characterization of Oligodendrocytes and microglial co-cultures. *(A) diagram of co-culture protocol. (B) immunofluorescence staining of brain cells markers from spheres cultures in 2D: O4 – OPCs and oligodendrocytes, Iba1 – iMGLs, MAP2 – neurons. (C-D) immunofluorescence staining of microglia and 2D neurosphere cultures shows full integration of myeloid cells in neuronal network. (E) microglia show mature morphology and motility in co-cultures. (F-H) immunofluorescence staining of microglia and 2D neurosphere cultures shows diverse cell interactions in the mixed cultures.*

References

1 Abud, E. M. *et al.* iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron* **94**, 278-293 e279, doi:10.1016/j.neuron.2017.03.042 (2017).

2 Dolan, M. J. *et al.* A resource for generating and manipulating human microglial states in vitro. *bioRxiv [PREPRINT]*, doi:10.1101/2022.05.02.490100 (2022).

3 Limone, F. *et al.* Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neurons to degeneration in ALS. *bioRxiv [PREPRINT]*, doi:10.1101/2021.07.12.452054 (2021).

4 Ginhoux, F. *et al.* Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science* **330**, 841--845, doi:10.1126/science.1194637 (2010).

5 Limone, F. *et al.* Efficient generation of lower induced Motor Neurons by coupling Ngn2 expression with developmental cues. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2022.01.12.476020 (2022).

6 Nehme, R. *et al.* Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission. *Cell Rep* **23**, 2509-2523, doi:10.1016/j.celrep.2018.04.066 (2018).

7 Douvaras, P. *et al.* Efficient Generation of Myelinating Oligodendrocytes from Primary Progressive Multiple Sclerosis Patients by Induced Pluripotent Stem Cells. *Stem Cell Reports* **3**, 250--259, doi:10.1016/j.stemcr.2014.06.012 (2014).

8 Wang, S. *et al.* Human iPSC-Derived Oligodendrocyte Progenitor Cells Can Myelinate and Rescue a Mouse Model of Congenital Hypomyelination. *Cell Stem Cell* **12**, 252--264, doi:10.1016/j.stem.2012.12.002 (2013).

9 Douvaras, P. & Fossati, V. Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. *Nature Protocols* **10**, 1143--1154, doi:10.1038/nprot.2015.075 (2015).

10 Rigamonti, A. *et al.* Large-Scale Production of Mature Neurons from Human Pluripotent Stem Cells in a Three-Dimensional Suspension Culture System. *Stem Cell Reports* **6**, 993-1008, doi:10.1016/j.stemcr.2016.05.010 (2016).

Overview

With this thesis, we offer solutions to two barriers faced by scientist approaching neurodegenerative diseases: first, a wider understanding of cellular and molecular pathways disrupted in primary samples from patients affected by Amyotrophic Lateral Sclerosis at a single cell resolution; and second, we put forward novel systems to model these disfunctions in laboratory settings and the ability to study cells normally hard to reach.

In **chapter 2**, we review our work on single nucleus RNA sequencing of ALS cortices. By examining cell-type specific expression of genetic risk factors for ALS/FTD, we found that specific subclasses of excitatory neurons intrinsically express higher levels of these disease-associated genes. We propose that these genes may be most essential in extratelencephalic (L5-ET) motor neurons and that mutations might have large ramifications within them. To further dissect the vulnerability of these cells we unbiasedly investigated transcriptomic changes triggered by the disease and found that shared sets of genes are altered in groups of L5-ETNs. The pathways that these genes play a role in includ unfolded protein responses, proteosomal subunits and RNA metabolism. These disruptions revealed another contributor to susceptibility: the genes identified, like genetic risk factors, are constitutively expressed at higher levels in L5-ETNs. We hypothesize that these forms of sensitivity collaborate to make L5-ETNs the “first over the line” to degenerate in ALS. These alterations are accompanied by concurrent effects in other cells: neurons of upper layers upregulate synaptic genes, probably to compensate for lost inputs to the cord; oligodendrocytes switch to a neuronally-engaged state to the expense of myelinating abilities; microglia acquire a pro-inflammatory signature associated with vesicles biology, likely triggered by neuronal apoptosis. We propose that the intrinsic vulnerability of classes of neurons to ALS/FTD initiates responses in other cells but at the same time show that genetic risk factors are involved in processes altered in different cell types. This makes the promotion of neuronal survival undoubtedly crucial but suggests that targeting other cell types might be as important in restoring a neuroprotective environment.

These novel insights are essential in widening our knowledge on disease mechanisms. However, they are only a snapshot of disease, they are based on a small cohort not allowing the full investigation of a highly heterogeneous disease like ALS/FTD, and they leave no room for manipulations. To overcome these obstacles, we provide *in vitro* tools that would allow the manipulations of human brain cells in a dish from a variety of individuals.

In **chapter 3,** we put together a compendium of protocols to differentiate hPSCs into different brain cell types that could be used to further dissect multicellular contributions to neurodegeneration. In this instance, we focus on studies that tested efficacy of transplantation of these products in an effort to repopulate cells lost to degeneration but we also provide a

detailed and valuable resource for researchers to draw from to model biology of the central nervous system *in vitro* and dissect mechanisms disrupted by ALS in different cell types.

In **chapter 4**, we show that the combination of Ngn2 overexpression and ventralising and caudalising factors can generate populations of cervical and brachial motor neuron (MNs) from at least 47 human pluripotent stem cell lines, with extremely high reproducibility and the amenability to expand to hundreds of lines. These MN-like cells, liMNs or liMoNes, express canonical MN markers, resemble other hiPSC-derived MN and exhibit formation of synapsis both in-network with muscle cells *in vitro*. Our pooled and multiplexed sequencing approaches, *Census-seq* and *Dropulation*, revealed the exceptional reproducibility of this system in forming anatomically distinct MN classes that in part resemble their cervical and brachial *in vivo* counterpart. Moreover, our preliminary data provides new tools and technologies that can be used as platforms to manipulate postmitotic neurons *in vitro* and to establish co-culture system with glial cells to investigate biology disrupted in disease. We conclude that combining small molecule patterning with Ngn2 overexpression can facilitate the high-yield, robust and reproducible production of multiple disease-relevant MN subtypes, which is fundamental in propelling our knowledge of motor neuron biology and its disruption in disease.

In **chapter 5**, we describe how we are using the models established in chapter 3 and 4 in conjunction with changes identified in chapter 2 for the nomination of neuroprotective targets under proteostatic stress conditions. Moreover, this section presents preliminary data on the characterization of co-culture systems of different brain cell types derived from human iPSCs described in chapter 3 that could be used to study changes identified in sporadic ALS brain samples described in chapter 2. With this section we hope to provide new, more complex *in vitro* systems to model degeneration and multicellular interactions disrupted in ALS.

Future Perspectives and concluding remarks

1. Expanding knowledge of neurodegeneration at a single cell resolution

In the introduction to this work we have highlighted how different cell types might play pivotal roles in initiation, progression, exacerbation and/or resistance to ALS and even though not described in this work this concerted dissonance has emerged for many other neurodegenerative diseases. In order to understand the disruptions underlying these complex interactions, several groups have undertaken the endeavour to dissect neurodegeneration using single cell/nucleus RNA sequencing technologies.

In the last five years various groups have reported studies using primary samples from patients of Multiple Sclerosis (MS)[1,2], Alzheimer's Disease (AD)[3-8], Parkinson's Disease (PD)[9]

and Frontotemporal Dementia (FTD)[10]. Many others have also looked at specific cell types identifying disease relevant biology, however, only by looking at the complex composition of the multicellular environment of the brain we might get further insights into disease pathology.

These studies have contributed answering a long-lasting question in the field: why are certain classes of neurons selectively sensitive to specific diseases? Some of these reports unravelled susceptibility of specific neuronal subtypes: mid-layer RORB$^+$ neurons accumulate tau aggregates and are depleted in AD[6]; upper layer CUX2-neurons are more affected by meningeal inflammation in MS[1]; ventral dopaminergic neurons in Parkinson's Disease[9]; our study that points at heightened intrinsic susceptibility and select vulnerability of classes of cortical L5-ET neurons in ALS/FTD[11]; recent reports that highlighted a similar scenario for spinal MN in ALS[12]. These findings are milestone in the quest to defining a disease-associated signature that might be at the base of selective neuronal death and at the same time provide a repository that should be compared and further investigated, marking the beginning of a new era in the understanding of selective neuronal vulnerability to degeneration.

At the same time, these studies have provided insights into the role of other cell types in degeneration. In some cases, the culprits might have been hypothesised suspects like astrocytes[3,5,7,8] and microglia[4,7] in AD or oligodendrocytes[2] in MS, pointing at cell-type-specific molecular dysfunctions. In other instances, however, these reports highlighted new rolesfor certain cell types in disruptions generated by the disease such as microglia[1] in MS, oligodendrocytes in AD[5] and brain vasculature in AD[8] and FTD[8]. We contribute by identifying patient-specific changes in oligodendrocytes and microglia in ALS[11].

Parts of this work specifically aim to highlight the multicellular complexity of neurodegenerative diseases and the diverse role that various characters of the cerebral milieu might play in the great masterpiece that is the human brain. The main point that we would like to deliver is that, besides the central role that one cell type might play in a disease, no cell reaches the role of soloist in the commonwealth that shapes the nervous system lyrical ensemble and focusing on only one of them clashes with the final goal of understanding how the harmony of the CNS is disrupted by degeneration.

2. hiPSC modelling: building complex, reproducible systems to mimic *in vivo* function and encompass the diversity of the human brain

Despite the immense potential for *in vitro* modelling brought about by human induced Pluripotent Stem Cells, many issues are still to be resolved: technical variability between differentiations and immaturity of cultures, non-cell autonomous effects and cell-to-cell

interactions but also variability between cell lines that highlight the need for increased scalability to correctly represent the genomic, genetic and phenotypic diversity of humankind.

Several groups have developed methods to improve consistency between differentiations and to increase line-to-line reproducibility like the inclusion of small molecules to boost the representation of a cell type[13], the use of enrichment strategy through the identification of cell surface markers for cell types of interest[14,15] or transgenic reporters[16]. Cellular maturity is a delicate subject in the field given the lack of consensus on the definition of “maturity” and what criteria identify a cellular transition from a foetal to an adult state. However, many have used co-cultures of cell type of interest with other cells of the brain, isolated from primary rodent extracts or human foetal samples, to increase maturation through exogenous factors[17,18]. Parts of our work aim at increasing reproducibility and standardization of differentiation protocols to overcome many of these issues.

In recent years many have developed systems that allow differentiation of hiPSCs into complex 3D structures composed of different cell types called organoids. These methods allow a more physiological development of several cell types together, implementing maturation stages driven by cell-to-cell contacts and allowing the possibility to widen our knowledge on human cells interactions. Even though most research has been focusing on anterior regions of the nervous system[19], a few groups have developed methods to differentiate organoids of posterior identity for the isolation of specific cell types like spinal motor neurons[20], astrocytes[21] and oligodendrocytes[22,23]. The first report of a human iPSC-derived organoid generated complex 3D structures containing motor neurons, inhibitory neurons and astrocytes of spinal cord identity[24] has now been followed by more advanced models of spinal organoids with skeletal muscles that can form neuromuscular junctions[25]. However, for the modelling of ALS, more complex systems are needed since the motor circuit is shaped by cells in the cortex, spinal cord and muscles. The ability of cortical organoids to extend axons towards muscles was first proven by their co-culture with murine spinal cord extracts[26] and later cortical, spinal and muscular organoids were fused together proving a full human motor circuits can be built *in vitro*[27]. These models are extremely complex and not fully standardized yet efforts towards the generation of more reproducible organoids[25,28,29] give hope that one day these structures could be used for the modelling of motor circuit. The work presented in this thesis tries to add a block into the building of complex, multicellular, human systems.

Another caveat of using human stem cells for disease modelling is the current inability to scale up and analyse cell lines from a high number of individuals. Currently, *in vitro* modelling of ALS, especially sporadic, requires big number of lines and high-throughput methods and needs further standardization[30]. This is why we believe the generation of more efficient differentiation paradigms amenable to hundreds of stem cell lines could be highly beneficial to the field. Moreover, the generation of different MN subtypes in our system could

allow the identification of factors that renders certain motor neuron pools more susceptible or more resistant to ALS[31]. Finally, the intermediate penetrance of ALS-associated genes and the complex genetics behind sporadic cases, generated by small effects from common variants and stronger effects from rare variants, suggest that we do not fully understand the full pictures of the genetics underlying ALS. Multiplexed, pooled approaches described in this work[32-34] might allow the simultaneous analysis of neurons from hundreds of patient-derived hiPSCs and might allow firstly the hypothesis-driven assessing of disease relevant perturbations in disease relevant cells but also shade a light on the complex interplay underlying sporadic cases.

Confronting ALS: understanding multicellular contribution to neurodegeneration

Computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

Overall, with this work we hope to open the field to a more holistic approach to the study of ALS and neurodegeneration as a whole, where multi-disciplinary techniques and the use of different models might expand our knowledge on disease. This effort is just the beginning and a lot more work is necessary to translate this knowledge into effective changes for ALS but I do hope to have offered a different view on the need to merge new technologies in innovative ways, stressed the importance of considering primary samples as the orchestra maestro and main driver of our work and use models while being aware of their advantages but also their shortcomings. Not all questions can be answered using one system just like different instruments are needed to create a melody.

1 Schirmer, L. *et al.* Neuronal vulnerability and multilineage diversity in multiple sclerosis. *Nature* **573**, 75-82, doi:10.1038/s41586-019-1404-z (2019).

2 Jakel, S. *et al.* Altered human oligodendrocyte heterogeneity in multiple sclerosis. *Nature* **566**, 543-547, doi:10.1038/s41586-019-0903-2 (2019).

3 Mathys, H. *et al.* Single-cell transcriptomic analysis of Alzheimer's disease. *Nature* **570**, 332-337, doi:10.1038/s41586-019-1195-2 (2019).

4 Zhou, Y. *et al.* Human and mouse single-nucleus transcriptomics reveal TREM2-dependent and TREM2-independent cellular responses in Alzheimer's disease. *Nat Med* **26**, 131-142, doi:10.1038/s41591-019-0695-9 (2020).

5 Sadick, J. S. *et al.* Astrocytes and oligodendrocytes undergo subtype-specific transcriptional changes in Alzheimer's disease. *Neuron* **110**, 1788-1805 e1710, doi:10.1016/j.neuron.2022.03.008 (2022).

6 Leng, K. *et al.* Molecular characterization of selectively vulnerable neurons in Alzheimer's disease. *Nat Neurosci*, doi:10.1038/s41593-020-00764-7 (2021).

7 Grubman, A. *et al.* A single-cell atlas of entorhinal cortex from individuals with Alzheimer's disease reveals cell-type-specific gene expression regulation. *Nat Neurosci* **22**, 2087-2097, doi:10.1038/s41593-019-0539-4 (2019).

8 Lau, S. F., Cao, H., Fu, A. K. Y. & Ip, N. Y. Single-nucleus transcriptome analysis reveals dysregulation of angiogenic endothelial cells and neuroprotective glia in Alzheimer's disease. *Proc Natl Acad Sci U S A* **117**, 25800-25809, doi:10.1073/pnas.2008762117 (2020).

9 Kamath, T. *et al.* Single-cell genomic profiling of human dopamine neurons identifies a population that selectively degenerates in Parkinson's disease. *Nat Neurosci* **25**, 588-595, doi:10.1038/s41593-022-01061-1 (2022).

10 Gerrits, E. *et al.* Neurovascular dysfunction in GRN-associated frontotemporal dementia identified by single-nucleus RNA sequencing of human cerebral cortex. *Nat Neurosci* **25**, 1034-1048, doi:10.1038/s41593-022-01124-3 (2022).

11 Limone, F. *et al.* Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neurons to degeneration in ALS. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2021.07.12.452054 (2021).

12 Yadav, A. *et al.* The Human Motoneuron Expression Signature is Defined by ALS-Related Genes. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2022.03.25.485808 (2022).

13 Boulting, G. L. *et al.* A functionally characterized test set of human induced pluripotent stem cells. *Nat Biotechnol* **29**, 279-286, doi:10.1038/nbt.1783 (2011).

14 Klim, J. R. *et al.* ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair. *Nat Neurosci* **22**, 167-179, doi:10.1038/s41593-018-0300-4 (2019).

15 Barbar, L. *et al.* CD49f Is a Novel Marker of Functional and Reactive Human iPSC-Derived Astrocytes. *Neuron* **107**, 436-453 e412, doi:10.1016/j.neuron.2020.05.014 (2020).

16 Kiskinis, E. *et al.* Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. *Cell Stem Cell* **14**, 781-795, doi:10.1016/j.stem.2014.03.004 (2014).

17 Di Giorgio, F. P., Boulting, G. L., Bobrowicz, S. & Eggan, K. C. Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. *Cell Stem Cell* **3**, 637-648, doi:10.1016/j.stem.2008.09.017 (2008).

18 Nehme, R. *et al.* Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission. *Cell Rep* **23**, 2509-2523, doi:10.1016/j.celrep.2018.04.066 (2018).

19 Del Dosso, A., Urenda, J. P., Nguyen, T. & Quadrato, G. Upgrading the Physiological Relevance of Human Brain Organoids. *Neuron* **107**, 1014-1028, doi:10.1016/j.neuron.2020.08.029 (2020).

20 Rigamonti, A. *et al.* Large-Scale Production of Mature Neurons from Human Pluripotent Stem Cells in a Three-Dimensional Suspension Culture System. *Stem Cell Reports* **6**, 993-1008, doi:10.1016/j.stemcr.2016.05.010 (2016).

21 Douvaras, P. & Fossati, V. Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. *Nat Protoc* **10**, 1143-1154, doi:10.1038/nprot.2015.075 (2015).

22 Douvaras, P. *et al.* Efficient generation of myelinating oligodendrocytes from primary progressive multiple sclerosis patients by induced pluripotent stem cells. *Stem Cell Reports* **3**, 250-259, doi:10.1016/j.stemcr.2014.06.012 (2014).

23 Marton, R. M. *et al.* Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. *Nat Neurosci* **22**, 484-491, doi:10.1038/s41593-018-0316-9 (2019).
24 Ogura, T., Sakaguchi, H., Miyamoto, S. & Takahashi, J. Three-dimensional induction of dorsal, intermediate and ventral spinal cord tissues from human pluripotent stem cells. *Development* **145**, doi:10.1242/dev.162214 (2018).
25 Faustino Martins, J. M. *et al.* Self-Organizing 3D Human Trunk Neuromuscular Organoids. *Cell Stem Cell* **26**, 172-186 e176, doi:10.1016/j.stem.2019.12.007 (2020).
26 Giandomenico, S. L. *et al.* Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. *Nat Neurosci* **22**, 669-679, doi:10.1038/s41593-019-0350-2 (2019).
27 Andersen, J. *et al.* Generation of Functional Human 3D Cortico-Motor Assembloids. *Cell* **183**, 1913-1929 e1926, doi:10.1016/j.cell.2020.11.017 (2020).
28 Velasco, S. *et al.* Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. *Nature* **570**, 523-527, doi:10.1038/s41586-019-1289-x (2019).
29 Paulsen, B. *et al.* Autism genes converge on asynchronous development of shared neuron classes. *Nature* **602**, 268-273, doi:10.1038/s41586-021-04358-6 (2022).
30 Baxi, E. G. *et al.* Answer ALS, a large-scale resource for sporadic and familial ALS combining clinical and multi-omics data from induced pluripotent cell lines. *Nat Neurosci* **25**, 226-237, doi:10.1038/s41593-021-01006-0 (2022).
31 Kanning, K. C., Kaplan, A. & Henderson, C. E. Motor neuron diversity in development and disease. *Annu Rev Neurosci* **33**, 409-440, doi:10.1146/annurev.neuro.051508.135722 (2010).
32 Limone, F. *et al.* Efficient generation of lower induced Motor Neurons by coupling Ngn2 expression with developmental cues. *bioRxiv [PREPRINT]*, doi:doi.org/10.1101/2022.01.12.476020 (2022).
33 Mitchell, J. M. *et al.* Mapping genetic effects on cellular phenotypes with “cell villages”. *bioRxiv [PREPRINT]*, doi:https://doi.org/10.1101/2020.06.29.174383 (2020).
34 Limone, F. *et al.* Efficient generation of lower induced motor neurons by coupling Ngn2 expression with developmental cues. *Cell Rep*, 111896, doi:10.1016/j.celrep.2022.111896 (2022).

Nederlandse Samenvatting

De confrontatie aangaan met ALS: meercellige bijdrage aan neurodegeneratie begrijpen

Computationele analyse en hiPSC's in vitro modellering als een multidisciplinaire aanpak

Amyotrofische laterale sclerose (ALS) is een dodelijke neurodegeneratieve aandoening die wordt gekenmerkt door een progressief verlies van motorische functies. Hoewel het bekend staat om de gelijknamige sclerose van het ruggenmerg die te zien is bij een autopsie, is ALS het resultaat van de degeneratie van extratelencefale cortico-spinale motorneuron (CSMN), en het daaraan gelinkte geleidelijk verlies van cortico-spinale banen en de degeneratie van motorneuronen van het ruggenmerg, wat resulteert in controle verlies over spieren en ademhalingsfalen. Het blijft onduidelijk waarom specifieke soorten motorneuronen selectief door de ziekte worden aangetast. Hoewel genetische studies van familiale ALS ons begrip van deze aandoening enorm hebben vergroot, is de overgrote meerderheid van de ALS-gevallen sporadisch (90%), zonder familiegeschiedenis en meestal zonder bekende genetische oorzaak. Het is nog onbekend of familiale mutaties en sporadische mutaties leiden tot vergelijkbare moleculaire veranderingen en hoe verschillende cellulaire subtypes kunnen bijdragen aan deze veranderingen, wordt nog nader onderzocht. De beperkte beschikbaarheid van hersenweefsel en de onduidelijkheid over de veranderingen in specifieke celtypen vormen twee belangrijke hindernissen: ten eerste dienen de moleculaire processen die bij patiënten worden verstoord te worden begrepen op cellulair niveau per celtype; ten tweede, is er een noodzaak om verschillende systemen te bouwen om deze mechanismen in vitro te modelleren.

Dit proefschrift probeert aspecten van deze vragen te beantwoorden door te beginnen met een poging om de unieke moleculaire eigenschappen te begrijpen die motorneuronen gevoelig maken voor ALS. Met dit doel voerden we RNA-sequencing uit van 79.169 losse kernen van cortices van patiënten en controles van vergelijkbare leeftijd. Bij gezonde personen ontdekten we dat de expressie van ALS-risicogenen significant verrijkt was in THY1+ laag-5, extratelencefale (L5-ET) neuronen en niet in andere celtypes. Bij patiënten werden deze genetische risicofactoren, evenals genen die betrokken zijn bij eiwithomeostase en stressreacties, significant geïnduceerd in THY1+ L5-ET en een bredere verzameling extratelencefale neuronen. Onderzoek van oligodendrogliale en microgliale kernen legde patiëntspecifieke genexpressieveranderingen bloot die op zijn minst gedeeltelijk een reactie waren op ziektegerelateerde veranderingen in neuronen. Onze bevindingen suggereren dat de selectieve kwetsbaarheid van extratelencefale neuronen gedeeltelijk verband houdt met

de intrinsieke moleculaire eigenschappen die hen gevoeliger maken voor genetische en veranderingen die worden veroorzaakt door ALS-pathologie.

In een poging om nieuwe modellen te creëren om deze mechanismen te bestuderen, bespreken we vervolgens de generatie van een nieuw protocol om motorneuronen te genereren uit menselijke pluripotente stamcellen (hPSC's). In deze studie hebben we de overexpressie van de neuraliserende transcriptiefactor Neurogenin2 (*Ngn2*) gecombineerd met kleine moleculen om hPSC's te veranderen in geïnduceerde onderste motorneuronen (liMoNes/liMN's). Met behulp van een *Hb9*::GFP-cellijn toonden we aan dat deze aanpak activatie van de spinale motorneuron (MN)-specifieke transcriptiefactor *Hb9/MNX1* induceerde, waarbij tot 95% van de cellen *Hb9*::GFP positief werd. Deze cellen verkregen en behielden expressie van bekende vroege en late MN-markers. liMN's waren vergelijkbaar met hPSC-afgeleide MN die met een conventionele methode gegenereerd waren, vertoonden spontane elektrische activiteit, brachten synaptische markers tot expressie en vormden in vitro contacten met spiercellen. Samengevoegde, multiplex RNA-sequencing van losse cellen uit 50 cellijnen toonde aan dat populaties van verschillende MN-subtypen reproduceerbaar gegenereerd werden. Namelijk populaties van cervicale, brachiale en spierweefsel-innerverende MN’s die lijken op hun *in vivo* tegenhangers in het menselijke embryonale ruggenmerg. We concluderen dat de combinatie van kleine moleculen met overexpressie van *Ngn2* zorgt voor een hoge opbrengst, en een robuuste en reproduceerbare productie van meerdere ziekterelevante MN-subtypen. Dit is van fundamenteel belang voor het vergroten van onze kennis van motorneuronbiologie en de verstoring ervan bij ziekte.

Ten slotte hebben we een verzameling van protocollen samengesteld om hPSC's te differentiëren in verschillende hersenceltypen die kunnen worden gebruikt om de bijdrage van meerdere celtypen aan neurodegeneratie verder te onderzoeken. In dit geval richten we ons vooral op studies die de werkzaamheid van transplantatie van deze cellulaire producten hebben getest in een poging om cellen die verloren zijn gegaan door degeneratie te vervangen, maar we bieden ook een gedetailleerde en waardevolle bron van informatie om de biologie van het centrale zenuwstelsel *in vitro* te modelleren en om onderliggende mechanismen van ALS in verschillende celtypes te onderzoeken.

Het doel van dit proefschrift is om op celniveau inzicht te krijgen in de moleculaire mechanismen die verstoord zijn in de ziekte, om opties aan te bieden om deze verstoringen in menselijke modellen te bestuderen, en om te bediscussiëren hoe toekomstige experimenten met een multidisciplinaire aanpak onze kennis over de ziekte kunnen vergroten om uiteindelijk nieuwe therapieën voor behandeling of herstel te vinden.

Summary in English

Confronting ALS: understanding multicellular contribution to neurodegeneration

Computational analysis and hiPSCs in vitro modelling as a multidisciplinary approach

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder characterised by a progressive loss of motor function. While it is known for the eponymous sclerosis of the spinal cord observed upon autopsy, ALS is the result of extratelencephalic Cortico-Spinal Motor Neuron (CSMN) degeneration, connected to gradual loss of cortico-spinal tracts and degeneration of spinal cord motor neurons which results in loss of control over muscles and respiratory failure. It remains unclear why classes of motor neurons are selectively affected by the disease. Although genetics studies of familial ALS have tremendously increased our understanding of this condition, the vast majority of ALS cases are sporadic (90%), occurring without a family history and most often without a known genetic cause. Indeed, whether familial mutations and sporadic insurgence might converge on similar molecular pathways is still unknown and how different cellular subtypes might contribute to these changes remain a subject of investigation. The fundamental inaccessibility of brain tissue and the uncertainty surrounding the disturbances triggered by the disease in specific cell types poses two main hurdles: first, the need for a better understanding of what molecular processes are disrupted in patients at a cell type resolution; second, the need to build different systems to model these mechanisms *in vitro*.

This body of work seeks to answer aspects of these questions by starting with an effort to understand the unique molecular properties that sensitise motor neurons to ALS. To do so, we performed RNA sequencing of 79,169 single nuclei from cortices of patients and age-matched, controls. In unaffected individuals, we found that expression of ALS risk genes was significantly enriched in THY1$^+$ layer-5, extratelencephalic (L5-ET) neurons 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 THY1$^+$ L5-ET and a wider collection of extratelencephalic neurons. Examination of oligodendroglial and microglial nuclei revealed patient-specific gene expression changes that were at least in part a response to disease-associated alterations in neurons. Our findings suggest that the selective vulnerability of extratelencephalic neurons is partly connected to their intrinsic molecular properties sensitising them to genetic alterations produced by ALS pathology.

In an effort to create new paradigms to study these mechanisms we will then discuss the generation of a new protocol to differentiate motor neurons from human Pluripotent Stem

Cells (hPSCs). In this study, we coupled the overexpression of the neuralising transcription factor Neurogenin2 (*Ngn2*) with small molecule patterning to differentiate hPSCs into lower induced Motor Neurons (liMoNes/liMNs). Using an *Hb9*::GFP-reporter line, we showed that this approach induced activation of the spinal motor neuron (MN) specific transcription factor *Hb9/MNX1* with up to 95% of cells becoming *Hb9*::GFP^{+}. These cells acquired and maintained expression of canonical early and mature MN markers. liMNs resembled *bona fide* hPSC-derived MN differentiated by conventional small molecule patterning, exhibited spontaneous electrical activity, expressed synaptic markers and formed contacts with muscle cells *in vitro*. Pooled, multiplex single-cell RNA sequencing on 50 cell lines revealed reproducible populations of multiple anatomically distinct MN subtypes of cervical and brachial, limb-innervating MNs that in part resemble their *in vivo* counterparts in the human embryonic spinal cord. We conclude that combining small molecule patterning with Ngn2 overexpression can facilitate the high-yield, robust and reproducible production of multiple disease-relevant MN subtypes, which is fundamental in propelling our knowledge of motor neuron biology and its disruption in disease.

Finally, we put together a compendium of protocols to differentiate hPSCs into different brain cell types that could be used to further dissect multicellular contributions to neurodegeneration. In this instance, we mostly focus on studies that have tested the efficacy of transplantation of these cellular products in an effort to repopulate cells lost to degeneration but we also provide a detailed and valuable resource for researchers to draw from to model biology of the central nervous system *in vitro* and dissect mechanisms disrupted by ALS in different cell types.

This work aims to shed a light on what molecular mechanisms are disrupted in the disease at single-cell resolution, offer options on tools to study these disruptions in human models and further discuss how future experiments using multi-disciplinary approaches could expand our knowledge on the disease in an effort to find new therapies and a possible cure.

Riassunto in italiano

Affrontare la SLA: comprendere il contributo multicellulare alla neurodegenerazione

Analisi computazionali e modelli di hiPSC in vitro come approccio multidisciplinare

La sclerosi laterale amiotrofica (SLA) è una malattia neurodegenerativa fatale caratterizzata da una progressiva perdita della funzione motoria. Sebbene sia nota per l'omonima sclerosi del midollo spinale osservata in autopsia, la SLA è il risultato della degenerazione dei motoneuroni cortico-spinali extratelencefalici (CSMNs), collegata alla graduale perdita dei sistemi cortico-spinali e alla conseguente degenerazione dei motoneuroni del midollo spinale che sfocia nella perdita del controllo muscolare e nell'insufficienza respiratoria. Non è chiaro perché alcune classi di motoneuroni siano più selettivamente colpite da questa malattia. Sebbene gli studi genetici sulla SLA familiare abbiano notevolmente aumentato la nostra comprensione di questa malattia, la stragrande maggioranza dei casi di SLA è sporadica (90%), si verifica cioè senza una storia familiare e molto spesso senza una causa genetica nota. In effetti, non è ancora noto se le mutazioni familiari e l'insorgenza sporadica possano convergere su meccanismi molecolari simili e la maniera in cui i diversi sottotipi cellulari contribuiscano a questi cambiamenti rimane oggetto di indagine. L'inaccessibilità fondamentale del tessuto cerebrale e l'incertezza che circonda i disordini innescati dalla malattia in specifici tipi di cellule pone due ostacoli principali: in primo luogo, la necessità di una migliore comprensione di quali processi molecolari vengono interrotti nei pazienti a una risoluzione sul singolo tipo cellulare; in secondo luogo, la necessità di costruire diversi modelli per studiare questi meccanismi *in vitro*.

Questo corpus di lavoro cerca di rispondere ad aspetti di queste domande iniziando con uno sforzo per comprendere le proprietà uniche molecolari che sensibilizzano i motoneuroni alla SLA. Per fare ciò, abbiamo eseguito il sequenziamento dell'RNA di 79.169 singoli nuclei estratti da cortecce di pazienti e controlli di stessa età. In individui non affetti, l'espressione di geni connessi al rischio di SLA era significativamente arricchita nei neuroni extratelencefalici $THY1^+$ dello strato 5 (L5-ET) e non in altri tipi di cellule. Nei pazienti, questi fattori di rischio genetici, così come geni coinvolti nell'omeostasi proteica e nelle risposte allo stress, sono indotti in modo significativo in $THY1^+$ L5-ET e in un più ampio gruppo di neuroni extratelencefalici. L'esame dei nuclei derivanti da oligodendroglia e microglia ha rivelato cambiamenti di espressione genica specifici di pazienti che sono, almeno in parte, una risposta alle alterazioni associate a cambiamenti connessi alla malattia nei neuroni. I nostri risultati suggeriscono che la vulnerabilità selettiva dei neuroni extratelencefalici è in parte

collegata alle loro proprietà molecolari intrinseche che li sensibilizzano alle alterazioni genetiche prodotte dalla patologia della SLA.

Questa tesi prosegue con un compendio di protocolli per differenziare le hPSC in diversi tipi di cellule cerebrali che potrebbero essere utilizzate per spiegare ulteriori contributi multicellulari alla neurodegenerazione. In questo caso, ci concentriamo principalmente su studi che hanno testato l'efficacia del trapianto di questi prodotti cellulari nel tentativo di ripopolare le cellule perse a causa della degenerazione, ma forniamo anche una risorsa dettagliata e preziosa per i ricercatori da cui attingere per modellare la biologia del sistema nervoso centrale sistema in vitro e sezionare i meccanismi interrotti dalla SLA in diversi tipi di cellule.

Nel tentativo di creare nuovi paradigmi per studiare questi meccanismi, discuteremo quindi un nuovo protocollo per differenziare motoneuroni spinali dalle cellule staminali pluripotenti umane (hPSC). In questo studio, abbiamo affiancato la sovraespressione del fattore di trascrizione neuralizzante Neurogenina-2 (Neurogenin2, *Ngn2*) con il patterning di morfogeni per differenziare le hPSC in motoneuroni somatici indotti (liMoNes/liMNs). Utilizzando una linea reporter *Hb9*::GFP, abbiamo dimostrato che questo approccio induce l'attivazione del fattore di trascrizione specifico del motoneurone spinale (MN) *Hb9/MNX1*, con fino al 95% delle cellule che diventano Hb9::GFP+. Queste cellule acquisiscono e mantengono l'espressione di marcatori canonici di motoneuroni precoci e maturi. I liMN somigliano a MN derivati da hPSC differenziati dal protocolli convenzionale che usano solo morfogeni, mostrano attività elettrica spontanea, esprimono marcatori sinaptici e formano contatti con cellule muscolari *in vitro*. Il sequenziamento multiplo di RNA a singola cellula in pool su 50 linee cellulari ha rivelato popolazioni riproducibili di più sottotipi MN anatomicamente distinti a livelli cervicali e brachiali, che innervano gli arti e che in parte assomigliano alla loro controparte nel midollo spinale embrionale umano *in vivo*. Concludiamo che la combinazione di morfogeni e la sovraespressione di *Ngn2* facilita la produzione robusta, riproducibile ed ad alto rendimento di più sottotipi di MN rilevanti per lo studio di malattie, che è fondamentale per promuovere la nostra conoscenza della biologia dei motoneuroni e la sua alterazioni in neurodegenerazione.

Questo lavoro mira a far luce su quali meccanismi molecolari sono interrotti nella malattia alla risoluzione di una singola cellula, offrire opzioni sugli strumenti per studiare queste interruzioni in modelli umani e discutere ulteriormente come futuri esperimenti che utilizzano approcci multidisciplinari potrebbero espandere le nostre conoscenze sul malattia in uno sforzo verso la scoperta di nuove terapie e possibilmente una cura.

Acknowledgements

I want to thank my advisors, Prof. *Kevin Eggan* and Prof*. Niels Geijsen*. Kevin taught me that science is not just work, it is exciting and fun, and showed me that I can endure a lot more than I thought. I want to thank Niels for his kindness, support, refreshing views and for dealing with time changes and improbable situations in the calmest way.

Thanks to many collaborators that made this possible. Prof.s *Ralda Nehme, Olli Pietiläinen, Daniel Mordes* and *Aaron Burberry*, for supporting the young scientist and person in me through an extremely challenging journey and for teaching me that surrendering is not an option if the right people have your back. Immense gratitude to Prof. *Beth Stevens*, my adoptive mentor that helped me transform present into future. Thanks Dr. *Martine Therrien*, great companion in a journey filled with tiny monsters. Thank you to Prof. *Steven McCarroll,* Dr. *Sulagna Dia Ghosh* and the dropulation team for their help with a computerised world I was absolutely unprepared for.

Thanks to Prof**.** *Lee Rubin* for welcoming me in his lab and colleagues and friends Dr. *Caterina Gasperini,* Dr. *Kaylee Wells*, Dr. *Richard Giadone*, for making me feel at home.

An exceptional thank you to members of the *Eggan lab* that I had the pleasure to meet. Being the last Egganite standing I want to say that it's been an honour, I will cherish our memories. To the first I have met on a Friday in January 2016, Dr. *Jana(imal) Marie Mitchell*, a big MEOW! no words can describe how important our friendship is to me. To the best companion through this torturous journey called PhD and loca perdida, (almost Dr) *Irune Guerra San Juan*, there aren't enough eskarrik asko that could show my gratitude. To my found twin, fellow nerd (your words) and fellow New Yorker, *Janell (Janellee) L.M. Smith*, so lucky I am to have found you. To my other fellow New Yorker with whom I spent hours bickering but also sciencing, Dr. *Brian J. Joseph*, thanks for showing me that I can embrace my neuroticism. To the two younglings, *Kavya Raghunathan* and *Alexander Couto*, thanks for keeping me young, reminding me I've always been young and that I always will be, Charlie's (VT) will never be forgotten. Thanks to Dr. *Maura Charlton* for making sure I wouldn't "slip through the cracks". Unfortunately, space is running out but I want to thank: *Dr. Kevin Sean (K) Smith, Dr. Martin Berryer, Dr. Marcel(ito) Leyton, Prof. Atsushi (STAP) Fukuda, Dr. Kanchana (Kanchi) Gamage, Dr. Joseph (Joe) Klim, Dr. Pratibha (Prati) Tripathi, Dr. Jin Hao, Dr. Sophie DeBoer, Dr. Yenal Lakes, Dr. Greta Pintacuda, Dr. Mina Tsuji, Dr. Luis Williams, B. Mackenzie Smith.*

Bedankt best paranimfen en muggenzifters *Jessie W. Brummer en Irune Guerra San Juan.*

A massive thank you to Dr. *Francesca (Franca) Rapino,* Dr. *Bruno (Capo) DiStefano* and Dr. *Patrizia (Patri) Pessina* for being a constant across an ocean, especially *Capo* for making me the scientist I am today.

Grazie to my fans in the old country: *Paola (Polli) Sabatino, Sara (boLzi) Bolzon, Francesco (Bubu) Verdone, Elisa (Nani) De Siena.* Merci bonica to Dr. *Tamara (Tamarindo) Cruz Santa Cruz.* To my companion in misfortune, Dr. *Elena Jacinta Sobrino,* thanks for pointing out error in word #1 of page #1. A giant hug to my red-headed soulmate *Jessica (Jess) Tapp*.
Grazie to *my family* for making me who I am today and for supporting me through a life of roaming around the globe even though they don't fully understand why I chose it. Me neither, honestly. To my mother *Bruna,* for teaching me that love can fill an ocean. To my brother *Giorgio*, for reminding me that our bond can exist on astral planes unknown to many. To the strong women that raised me, *Rosina, Elvira, Monica, Barbara,* thank you for teaching me how to get through anything with love and laughter.

I want to end with a quote:

> "Il peggio non è il dolore ma la consapevolezza di tutto. 'Sta malattia è 'na bestiaccia."
> "The worst isn't the pain but the awareness of it all. This disease is an ugly beast."
>
> *-Mattia Limone*
>
> Traveller, sambuca drinker, ALS patient, but mostly my uncle
>
> (Massafra, 17 giugno 1956 – Latina, 30 Maggio 2022)

A reminder that when talking about ALS "the best possible scenario" is shorter than a five-year PhD, which felt impossibly long to me but definitely felt impossibly long and excruciating to you, ciao zio.
Endless thanks to the people that stand besides ALS patients, especially my father, *Angelo*. Thank you for showing me that love can be stronger than any pain if you are taking care of somebody close to your heart. I would have preferred not to learn it this way but an invaluable life lesson, nonetheless.

"Insurmountable" has definitely acquired a new meaning to me…

Curriculum Vitae

Francesco Limone was born in Latina, Italy, on the 12th of May 1992. He pursued his four year bachelor's degree in Molecular and Cell Biology at the University of York (United Kingdom) where he got interested in stem cell and developmental biology. During his undergraduate studies, he spent a year working in Prof. Thomas Graf's lab, at Center for Genomic Regulation (Barcelona, Spain), where he started working with induced Pluripotent Stem Cells (iPSCs) and got interested in their use for modelling biological processes in vitro. After graduating with honours on the 7th of July 2015, he moved to Paris to start a one-year Master in Developmental and Stem Cell Biology at Université Pierre et Marie Curie (Paris 6, Sorbonne Université, France). For his master internship he moved to Cambridge (Massachusetts, United States) and worked on his thesis in Dr. Kevin Eggan's lab at the Harvard Stem Cell and Regenerative Biology Department (HSCRB). Here he started using human pluripotent stem cells to generate human neurons in vitro to model brain-specific biology and neurodegeneration, with a focus on Amyotrophic Lateral Sclerosis (ALS). After graduating second in his class on 30th of June 2016, he kept on working at HSCRB as a technician to further dissect the role of multiple cell types in disruptions in ALS.

In January 2018, Francesco embarked in his doctoral studies in a shared PhD mentorship between Dr. Kevin Eggan and Prof. Niels Geijsen at Leiden University Medical Center (LUMC, formerly at Hubrecht Institute, University of Utrecht, Netherlands). In his doctoral work, in collaboration with Prof. Beth Stevens's group (Children's Hospital, Boston, United States), he focused on projects using single-cell RNA-sequencing and human pluripotent stem cell models for the understanding of disease mechanisms in ALS with a focus on the contribution of glial cells. Francesco also worked on the development of new in vitro differentiation schemes for motor neurons from human pluripotent cells with Prof. Steven McCarroll's and Dr. Ralda Nehme's group at the Stanley Center for Psychiatric Research (Broad Institute, Cambridge, USA) as well as the characterization and optimization of differentiation protocols for microglia and Oligodendrocytes Progenitor Cells from hiPSCs. He also contributed to studying immune system's involvement in ALS and the role of cytoskeletal proteins and ion channels in motor neuron's function. Since 2023 he is started as a postdoctoral fellow in Dr. Shane Liddelow's lab at the Neuroscience Institute, NYU Grossman School of Medicine (New York University Langone Health, New York, USA).

List of publications

In press, pre-prints and manuscripts in preparation

Limone F*, Couto A*, Wang JY, Zhang Y, McCourt B, Huang C, Jani M, Paladino M, Li QZ, Keaney J, Gillet G, Goodman WA, Kadiu I, Eggan K, Burberry A. "*C9orf72* promotes tolerance via restriction of co-stimulation". Under revision, *Science Translational Medicine*, *2023*.

Joseph BJ, Marshall KA, Mann JR, Prudencio M, ***Limone F***, Vanoye CG, Simkin D, Leyton M, Nash L, Guerra San Juan I, Gold J, Zhang C, Wichterle H, George AL Jr, Petrucelli L, Eggan K, Kiskinis E. "*TDP-43 Dysfunction Triggers Exon Skipping and Aggregation of the Epilepsy Gene KCNQ2 in ALS/FTD*". Under revision, *Nature Neuroscience, 2023*.

Limone F*, Mordes D*, Couto A, Burberry A, Pietiläinen O, Joseph B, Ghosh S, Meyer D, Goldman M, Bortolin L, Kadiu I, McCarroll S, Eggan K. "Single-nucleus sequencing reveals enriched expression of genetic risk factors sensitises Motor Neuron to degeneration in ALS". Under revision*, Nature Aging, 2022*. *bioRxiv,* https://doi.org/10.1101/2021.07.12.452054

Hao J, Wells M, Niu G, Guerra San Juan, ***Limone F***, Fukuda A, Leyton-Jaimes M, Joseph B, Qian M, Mordes D, Budnik B, Dou Z, Eggan K. "Loss of TBK1 activity leads to TDP-43 proteinopathy through lysosomal dysfunction in human motor neurons". Under revision*, Nature Communications, 2021*. *bioRxiv* https://doi.org/10.1101/2021.10.11.464011

Publications

Donal MJ, Therrien M, Jereb S, Kamath T, Atkeson T, Marsh SE, Goeva A, Lojek N, Murphy S, Joung J, Liu B, ***Limone F,*** Eggan K, Hacohen N, Bernstein BE, Glass KC, Leinonen V, Blurton-Jones M, Zhang F, Epstein CB, Macosko EZ, Stevens B. "A resource for generating and manipulating human microglial states *in vitro*". Accepted*, Nature Immunology, 2023*. *bioRxiv* https://doi.org/10.1101/2022.05.02.490100

Limone F $^{corresp.}$, Mitchell JM, Guerra San Juan I, Ghosh S, Smith JLM, Raghunathan K, Couto A, Meyer D, Mello C, Nemesh J, Smith BM, McCarroll S, Pietiläinen O, Nehme R, Eggan K$^{corresp.}$. "Efficient generation of lower induced Motor Neurons by coupling Ngn2 expression with developmental cues". *Cell Reports*, *2022*, https://doi.org/10.1016/j.celrep.2022.111896

Rapino F, Natoli T, ***Limone F,*** O'Connor E, Black J, Tegtmeyer M, Chen W, Norabuena E, Narula J, Hazelbaker D, Angelini G, Barrett L, O'Neil A, Beattle UK, Thanos JM, de Rivera H, Sheridan SD, Perlis RH, McCarroll SA, Stevens B, Subramanian A, Nehme R, Rubin LL. "Small molecules screen reveals pathways that regulate C4 secretion in stem-cell derived astrocytes". *Stem Cell Reports, 2022, https://doi.org/10.1016/j.stemcr.2022.11.018*

Limone F, Klim JR, Mordes D. "Pluripotent Stem Cell Strategies to Rebuild the Human Brain". *Frontiers in Aging Neuroscience*, *2022*. 10.3389/fnagi.2022.1017299

Guerra San Juan I, Nash L, Smith K, Qian M, Burberry A, Dorr A, Couto A, Klim J, Pintacuda G, ***Limone F***, Whisenant E, Noble C, Melnik V, Potter D, Holmes A, Verhage M, Eggan K. "Loss of STMN2 Function Causes Motor Neuropathy". *Neuron*, *2022,* 10.1016/j.neuron.2022.02.011.

Ashoti A*, ***Limone F****, van Kranenburg M, Alemany A, Baak M, Vivié J, Piccioni F, Creyghton M, Eggan K, Geijsen N. "Considerations and practical implications of performing a phenotypic CRISPR/Cas survival screen". *PLoS ONE, 2022*, 10.1371/journal.pone.0263262

Fukuda A, Hazelbaker D, Motosugi N, Hao J, ***Limone F***, Beccard A, Mazzucato P, Messana A, Okada C, Guerra San Juan I, Qian M, Umezawa A, Akutsu H, Barrett L, Eggan K. “The de novo DNA methyltransferases DNMT3A and DNMT3B initiate erosion of dosage compensation in human pluripotent stem cells”. *Stem Cell Reports, 2021,* 10.1016/j.stemcr.2021.07.015

Burberry A, Wells M, ***Limone F***, Couto A, Smith K, Keaney J, Gillet G, van Gastel N, Wang J, Pietiläinen O, Qian M, Eggan P, Cantrell C, Mok J, Kadiu I, Scadden D, Eggan K. “C9orf72 suppresses systemic and neural inflammation induced by gut bacteria”. *Nature, 2020* 10.1038/s41586-020-2288-7

Klim J*, Williams L*, ***Limone F***, Guerra San Juan I, Davis-Dusenbery B, Mordes D, Burberry A, Steinbaugh MJ, Gamage K, Kirchner R, Moccia R, Cassel S, Chen K, Wainger B, Woolf C, Eggan K. “ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair”. *Nature Neuroscience*, *2019* 10.1038/s41593-018-0300-4

Mordes D, Prudencio M, Goodman L, Klim J, Moccia R, ***Limone F***, Pietilainen O, Chowdhary K, Dickson D, Rademakers R, Bonini N, Petrucelli L, Eggan K. “Dipeptide repeat proteins activate a heat shock response found in C9orf72-ALS/FTLD patients”, *Acta Neuropathological Comm., 2018* 10.1186/s40478-018-0555-8

Nehme R*, Zuccaro E*, Ghosh S, Li C, Sherwood J, Pietiläinen O, Barrett L, ***Limone F***, Worringer K, Kommineni S, Zang Y, Cacchiarelli D, Meissner A, Adolfsson R, Haggarty S, Madison J, Muller M, Arlotta P, Fu Z, Feng G, Eggan K. “Combining NGN2 programming with developmental patterning generates human excitatory neurons with NMDAR-mediated synaptic transmission”. *Cell Reports*, *2018* 10.1016/j.celrep.2018.04.066

Kriiger PHL*, Di Stefano B*, de Wit E*, ***Limone F***, van Oevelen C, de Laat W, Graf T. “Cell-of-origin specific 3D genome structure acquired during somatic cell reprogramming”. *Cell Stem Cell, 2016* 10.1016/j.stem.2016.01.007

Di Stefano B, Collombet S, Jakobsen J, Wierer M, Lackner A, Sardina JL, Segura Morales C, Stadhouders R, ***Limone F***, Mann M, Porse B, Thieffry D, Graf T. “C/EBPα creates elite cells for iPSC reprogramming by upregulating Klf4 and increasing levels of Lsd1 and Brd4”, *Nature Cell Biology*, *2016* 10.1038/ncb3326