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

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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 death¹. First described by the founder of modern neurology Jean-Martin Charcot and his team of neuroscientists at La Salpêtrière hospital in Paris in 1869²⁻⁶, 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 50s⁷. The scarring of descending corticospinal tracts (sclerosis) is the result of Cortico-Spinal Motor Neuron (CSMN/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.

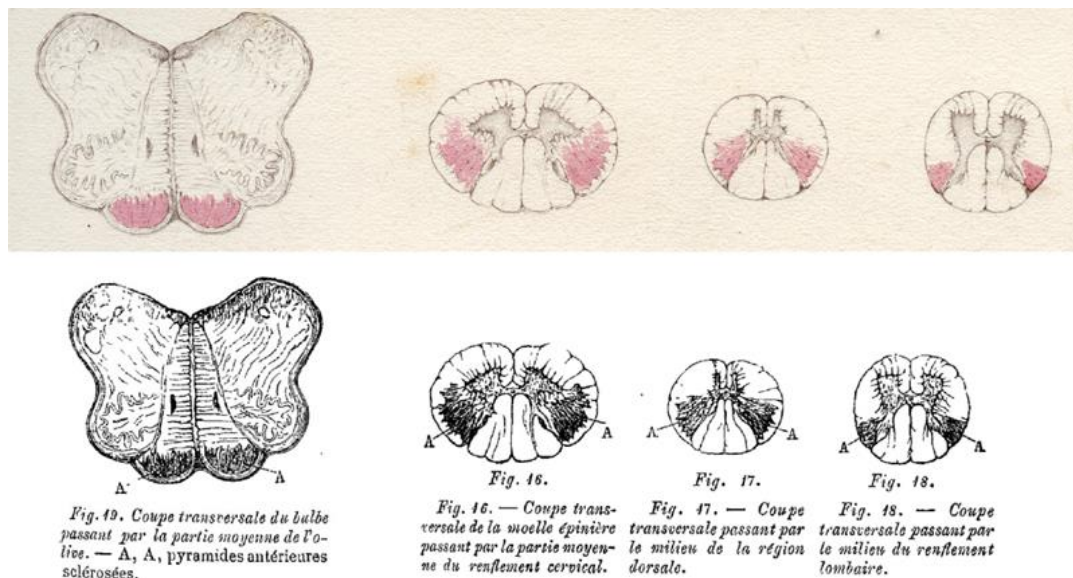


Figure 1 First description of ALS from Joffroy's thesis⁵. Top: positioning of medullar pyramids of the corticospinal tracts. Bottom: cross sections of the medulla supposedly drawn by Charcot himself depicting sclerotic areas (AA) through medulla oblongata (bulbe), cervical, thoracic and lumbar spine (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 ALS⁹. 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 stages¹². 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 ALS¹³.

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 cause⁸. 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 well¹⁶, 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 cases¹⁷. *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 penetrance²⁸ 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 others²⁹⁻³¹. The intermediate penetrance of certain mutations, the cumulative knowledge on disease modifiers and the partial heritability (established at 60% in twins studies³²) result in many cases being more familial clusters rather than classical mendelian inheritance³³ 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 neurons³⁵. These common, skein-like accumulations can be composed of different proteins and are highly ubiquitinated³⁵. 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.

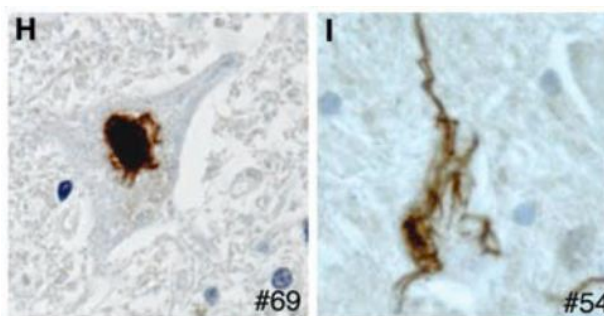


Figure 2. Identification of TDP-43 inclusions in sporadic ALS. Immunostaining with anti-TDP-43 labelling round nuclear aggregates (H) and nuclear loss with skein-like aggregates (I) in spinal cord motor neurons (original images from Neumann et al, 2006, Science³⁵).

Other pathological features are specific to certain genes, e.g. intranuclear RNA foci²⁰ in C9orf72-patients with ubiquitinated aggregates rich in SQSTM1/p62³⁷, 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 gene²² 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 ALS²⁶.

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 function³⁸. Only around 50% of FTD cases present TDP-43 pathology, whereas the rest is characterised by aggregates of either FUS or tau³⁹. Similarities in pathological features include loss of cortical neurons¹ 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 brainstem⁴⁰.

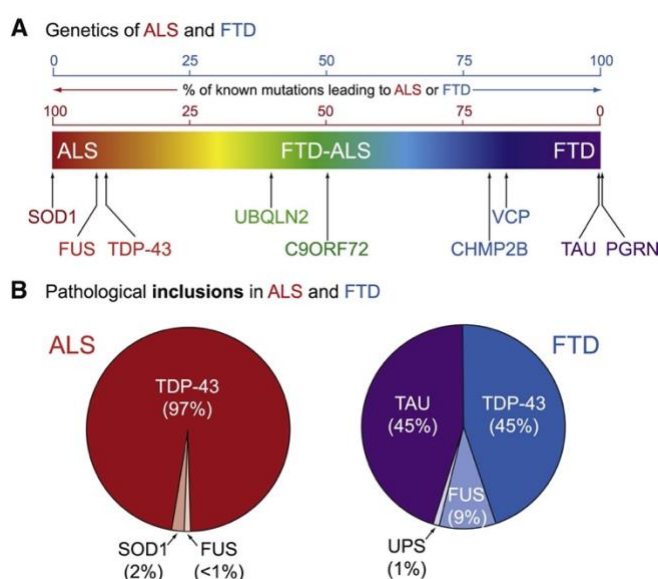


Figure 3. Overlap of pathology and genetics in the ALS and FTD (from Ling et al.¹⁴). (A) Major causative genes of ALS (red) and FTD (blue) positioned along the spectrum of manifestations of the two types of degeneration. (B) Break-down of cases presenting inclusions of different proteins in ALS and FTD.

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 impairment⁴⁰. 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-FTD⁴¹.

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 factors⁴² 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 glia⁴³. 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 proteins⁸. 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 functions¹. 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 recognised⁴⁵ and connected to loss of muscle tone⁵, 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 mRNAs¹⁶ preventing normal translation and act as a self-replicating cytoplasmic sinks¹. 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 proteins⁵⁷. For TDP-43 specifically, this may result in alterations in its splicing activity⁵⁸, 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 axons⁶³ and failed autoregulation⁵⁸, some of these disrupted mechanisms overlap with FUS-dependent aggregation⁵⁷. 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 proteins⁶⁵.

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 neurons⁶⁶. 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 proteostasis⁷⁵. Other organelles associated with dysfunctions of the disease are mitochondria, mostly through the antioxidant role of SOD1 protein¹⁹, the oxidative stress found in spinal cord of patients⁷⁶ and mutations in *CHCHD10*, a mitochondrial gene associated with rare familial cases of ALS⁷⁷.

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 above⁶³ 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 neurofilament⁸⁸ and one of the two drugs approved for treatment of ALS, Riluzole, acts by modulating neuronal firing¹⁰, pointing at axonal biology and synaptic activity as an extremely important aspect of the disease.

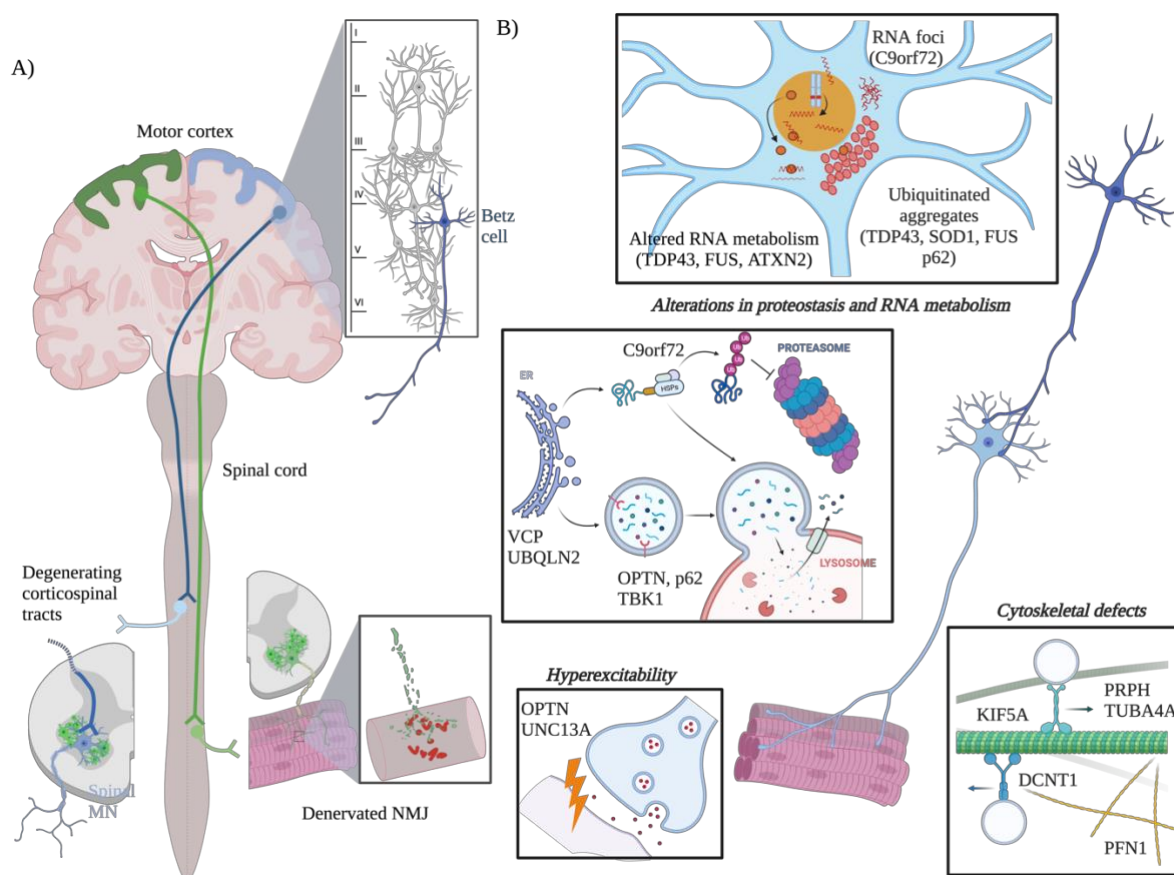


Figure 4 Mechanisms disrupted in neurons by ALS. A) Areas of the nervous system affected by ALS. Upper motor neurons (Betz cells) in the motor cortex start to be affected with consequent degeneration of corticospinal tracts resulting in lower motor neurons being affected and losing muscle control through 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. 2016¹.

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 excitotoxicity⁹².

In vivo and *in vitro* studies have confirmed the view that astrocytes play a role in disease initiation and progression. Astroglia and reactive astrocytes have been identified in several mouse models of SOD1, TDP43 and C9orf72⁴³, and selective deletion of mutant

SOD1^{G85R} from astrocytic lineage delayed disease onset and slowed down progression in animal models⁹³. 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 support⁹⁴. *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 circuitries⁹⁹. Oligodendrocytes pathology has been identified in several studies in ALS patients¹⁰⁰ and the relevance of this cell type in the disease is underscored by findings of TDP-43 inclusions in these cells as well¹⁰¹.

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 tissue¹⁰². 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 survival¹⁰³. 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 patients¹⁰³, 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 ALS²⁹ and FTD¹⁰⁴ 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 well¹⁰⁷.

Mouse models have provided numerous insights into microglial involvement in the disease. Microglial activation is believed to occur even prior to symptoms occurrence in SOD1 mouse models¹⁰⁸. 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

pathogenesis¹⁰⁹. 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 state⁴³. These reports have recently been confirmed by single-cell RNA-sequencing studies (scRNA-seq) that identified reactive microglia in SOD1^{G93A} mouse model¹¹⁰ 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 survival¹¹².

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 microglia¹¹¹. Specifically, *TREM2* has been shown to be one of the main regulators of reactive states in microglia¹¹⁰ and *GRN* deficient mice develop reactive microglia that associate with TDP43 aggregates¹¹³. Not only, recent studies have shown that *TREM2*, *GRN*, *TBK1* and *C9orf72* are highly expressed in microglia¹⁶. Mouse models of *C9orf72* have proven that loss of function in this gene results in several immune phenotypes¹¹⁴, many of which are connected to neuroinflammation¹¹⁵, as shown also in patients¹¹⁶, and these changes are a result of aberrant lysosomal trafficking¹¹⁷. Given the role of *GRN* in lysosomal biology¹¹³ and of *TBK1* in autophagy and vesicle trafficking specifically in microglia¹¹⁸, 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* dysfunction¹²⁰ 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 models¹¹⁵ and suggest that peripheral immunity might play a role in ALS disease progression¹¹⁵. 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 progression¹²¹. 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 modifiers¹¹⁵.

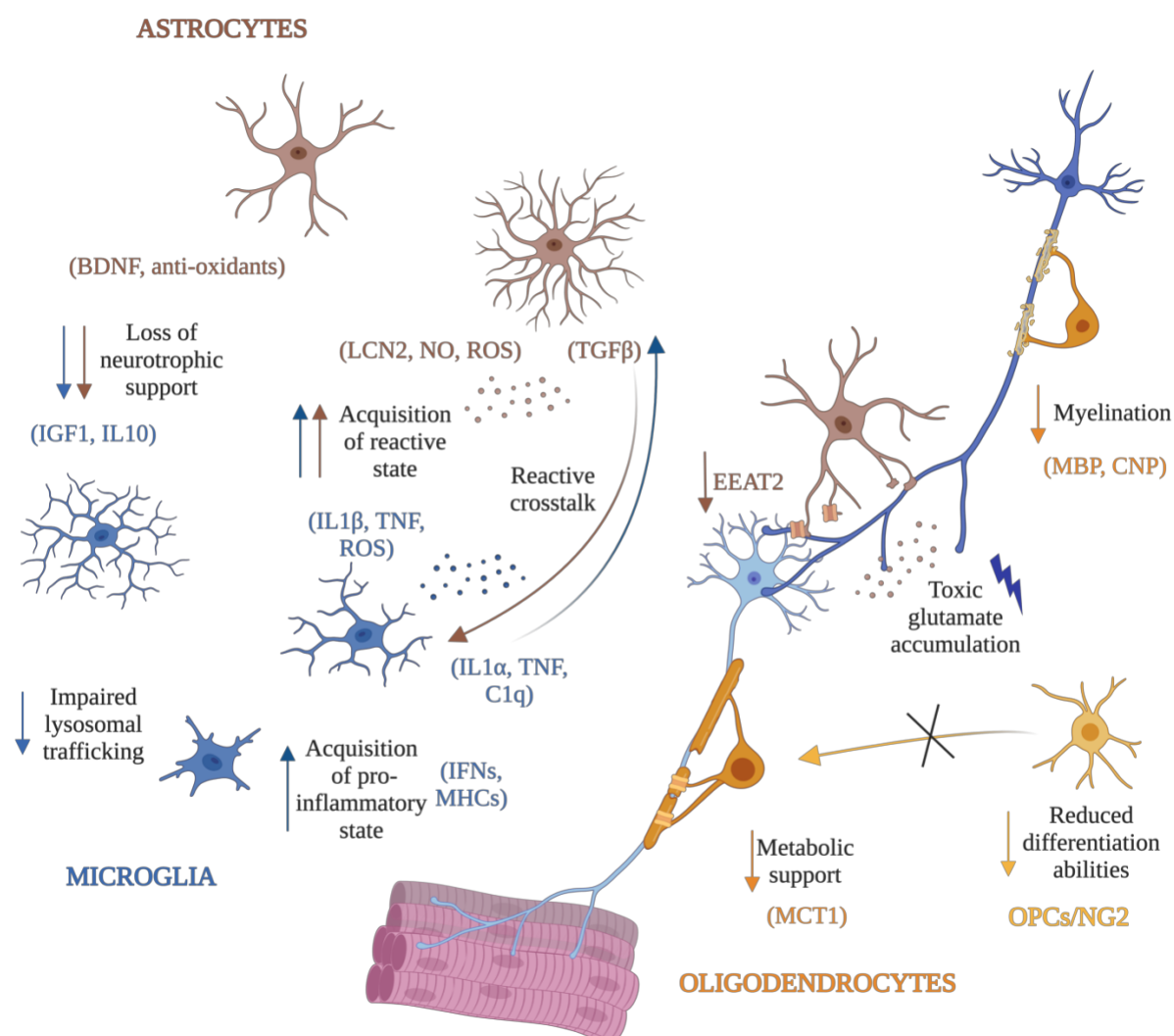


Figure 5 Pathophysiological interactions of glial cells and neurons in Amyotrophic Lateral Sclerosis. Neuronal dysfunctions described have been shown to be associated with activation states of glial cells. Phenotypes associated with ALS in glial cells entail loss of supportive roles for neurons: loss 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. 2016¹ and Vahsen et al. 2021⁴².

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 controls¹²³⁻¹²⁵. 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 control¹²⁵ and alternative splicing¹²³ 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 patients¹²³ and Tam et al. corroborated upregulation of inflammation and reactive glial biology in a subset of patients¹²⁵, 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 patients¹²⁶⁻¹²⁸, 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 microglia¹²⁸, 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 disease¹²⁹. 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 cord¹³⁰. 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 species¹³¹. 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 biology¹³².

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) technologies¹³³. At almost 25 years since their isolation¹³⁴, 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 system¹³⁵. 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 mechanisms¹³³.

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 subtypes¹³⁵⁻¹³⁷. 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 cases¹². Because hiPSCs more easily and spontaneously differentiate into neurons¹³⁵, 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* hiPSCs¹³⁹⁻¹⁴¹ 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-neurons¹⁴³ and similar dysregulations in axonal and neurofilament dynamics in TDP-43 mutated cells⁵⁹ 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* mutants¹⁴⁴ 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* phenotypes¹⁴⁶. More recently, our group and others have started to couple hiPSCs with novel genetic manipulation technologies like CRISPR-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 patients¹⁴⁷. 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 strides 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 identified in 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 multidisciplinary techniques and the use of different models might expand our perspectives on the disease.

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