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

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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.

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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.

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