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

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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:

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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).

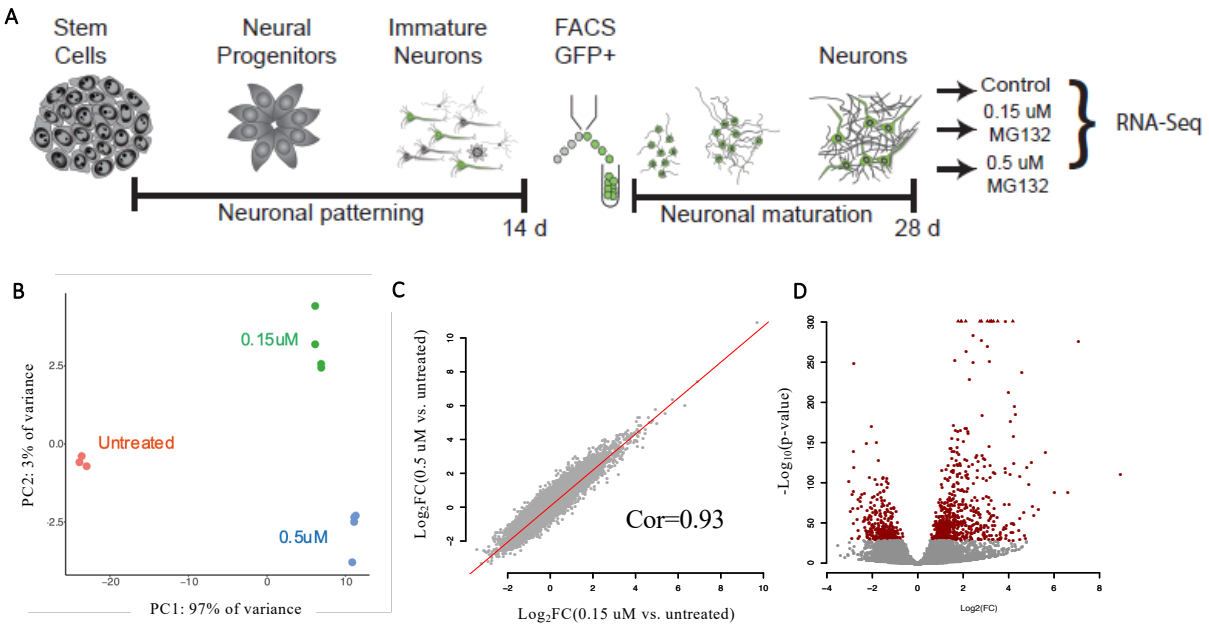

Fig. 1 | Proteasome inhibition in hiPSC-derived neurons drives strong transcriptomic changes. (A) Workflow of sequencing experiment. (B) Principle Component Analysis of sequenced samples. (C) Correlation analysis of different treatments (D) Differential gene expression analysis between treated and untreated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

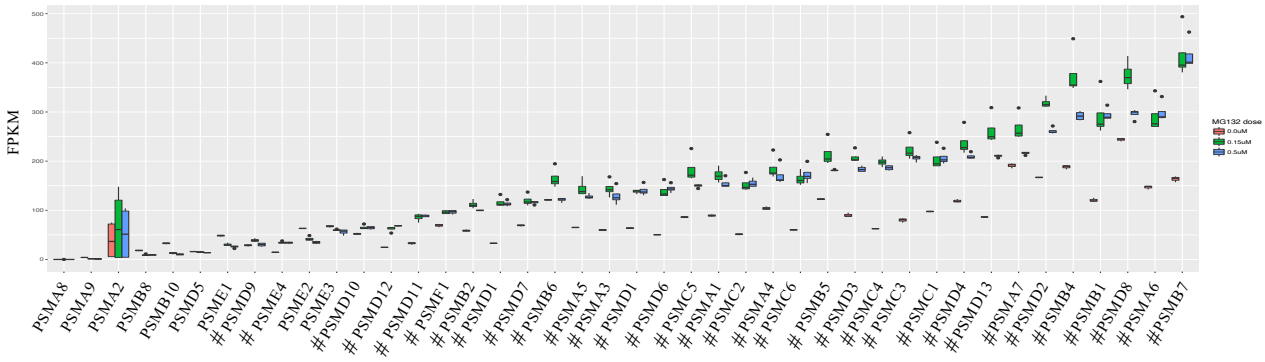

Fig. S1 | Proteasome subunits are significantly upregulated after treatment. # statistically significant

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.

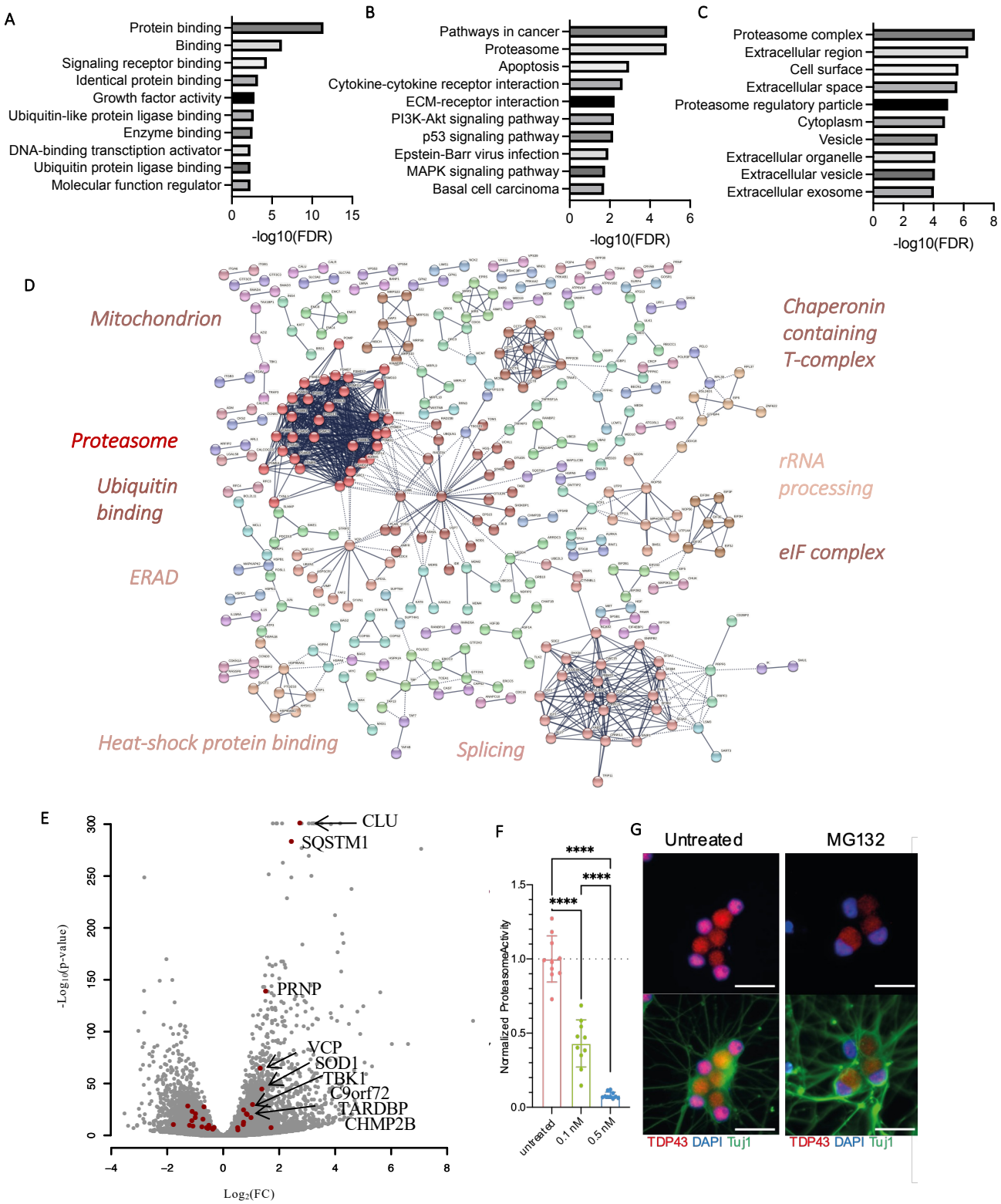

Fig. 2 | Genes upregulated by proteasome inhibition are connected to proteostatic stress and neurodegenerative diseases. **(A-C)** Gene Ontology Analysis of upregulated genes for Molecular Function (A), KEGG pathways (B) and Cellular Components (C). (D) Protein-protein interaction network of genes upregulated 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.

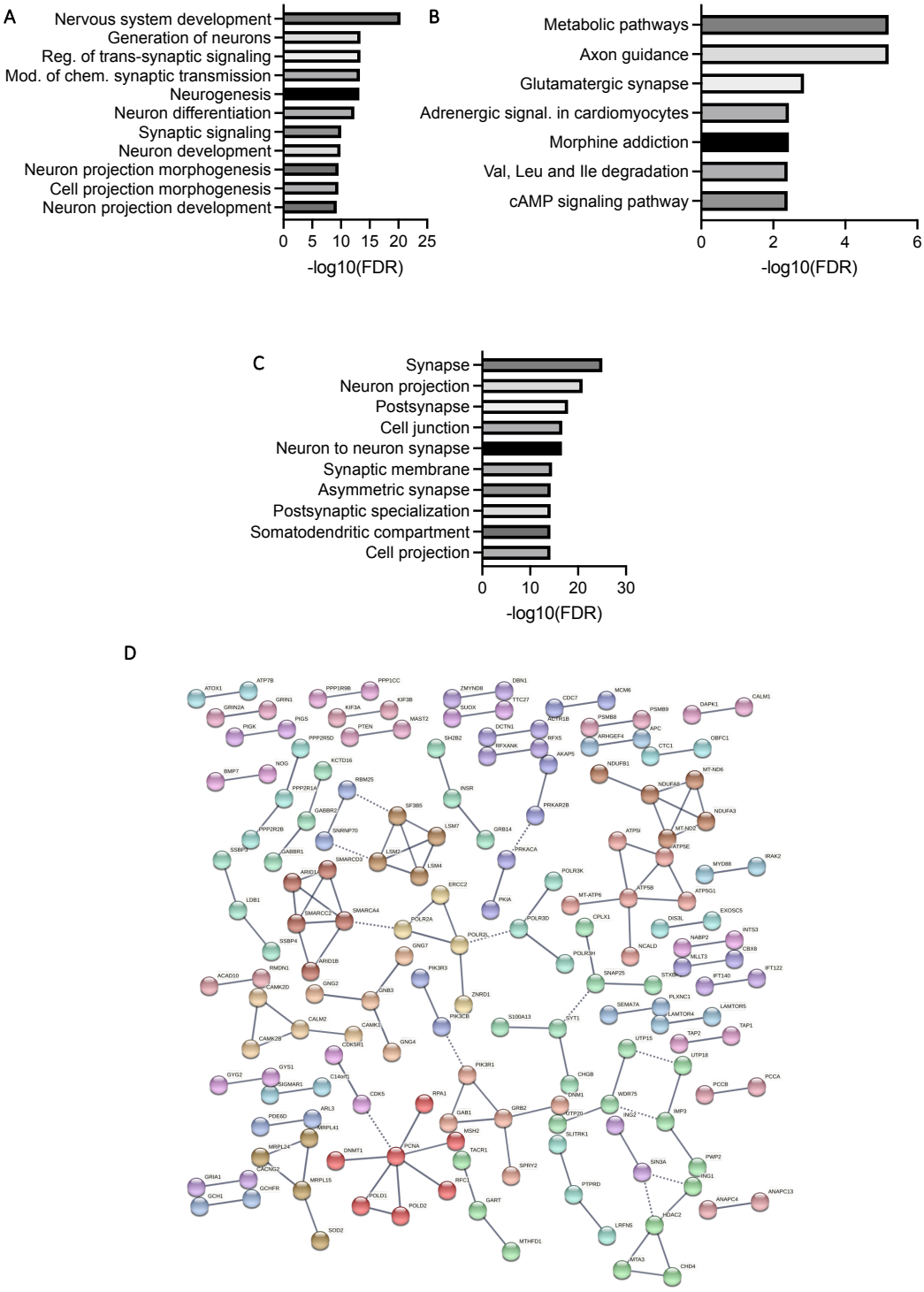

Fig. 3 | Genes downregulated by proteasome inhibition are connected to synaptic biology. (A-C) Gene Ontology Analysis of upregulated genes for Biological Porcesses (A), KEGG pathways (B) and Cellular Components (C). (D) Protein-protein interaction network of genes downregulated after proteasome inhibition.

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.

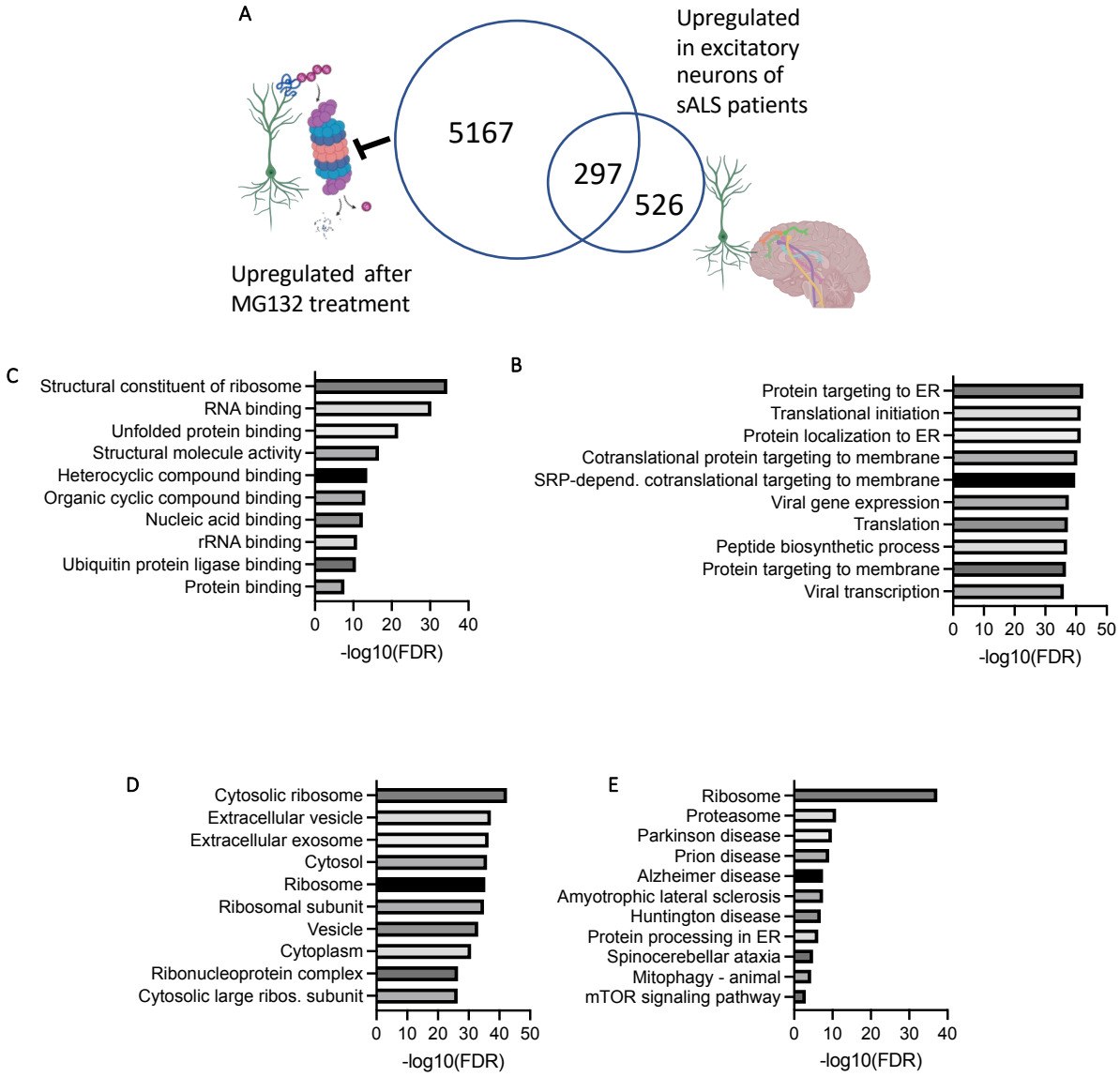

Fig. 4 | Shared transcriptomic signature of neuronal proteostatic stress and from sporadic ALS patients. (A) Shared upregulated genes between neurons under proteasome inhibition and excitatory neurons from sporadic ALS patients. **(B-E) Gene Ontology Analysis of shared upregulated genes for Biological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

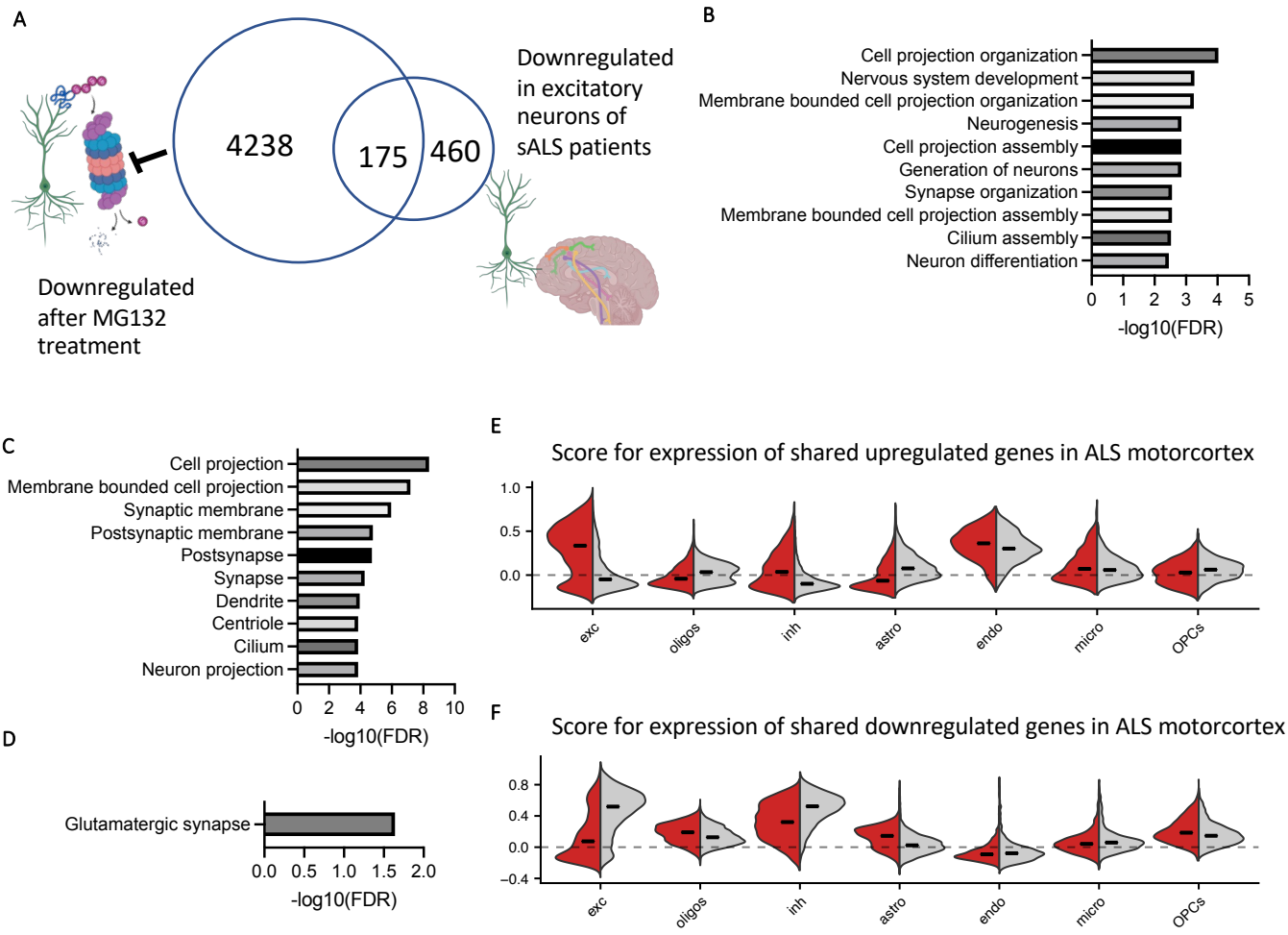

Fig. 5 | Neurons under proteostatic stress downregulate genes related to synaptic function. (A) Shared downregulated genes between neurons under proteasome inhibition and excitatory neurons from sporadic ALS patients. (B-D) Gene Ontology Analysis of shared downregulated genes for Biological Processes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

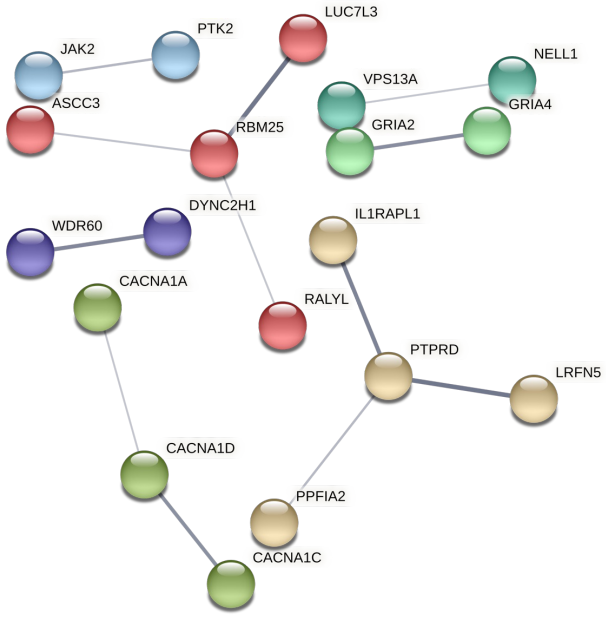

Fig. S5 | Neurons under proteostatic stress downregulate genes related to synaptic function. Protein-protein interaction network of shared genes downregulated after proteasome inhibition.

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.

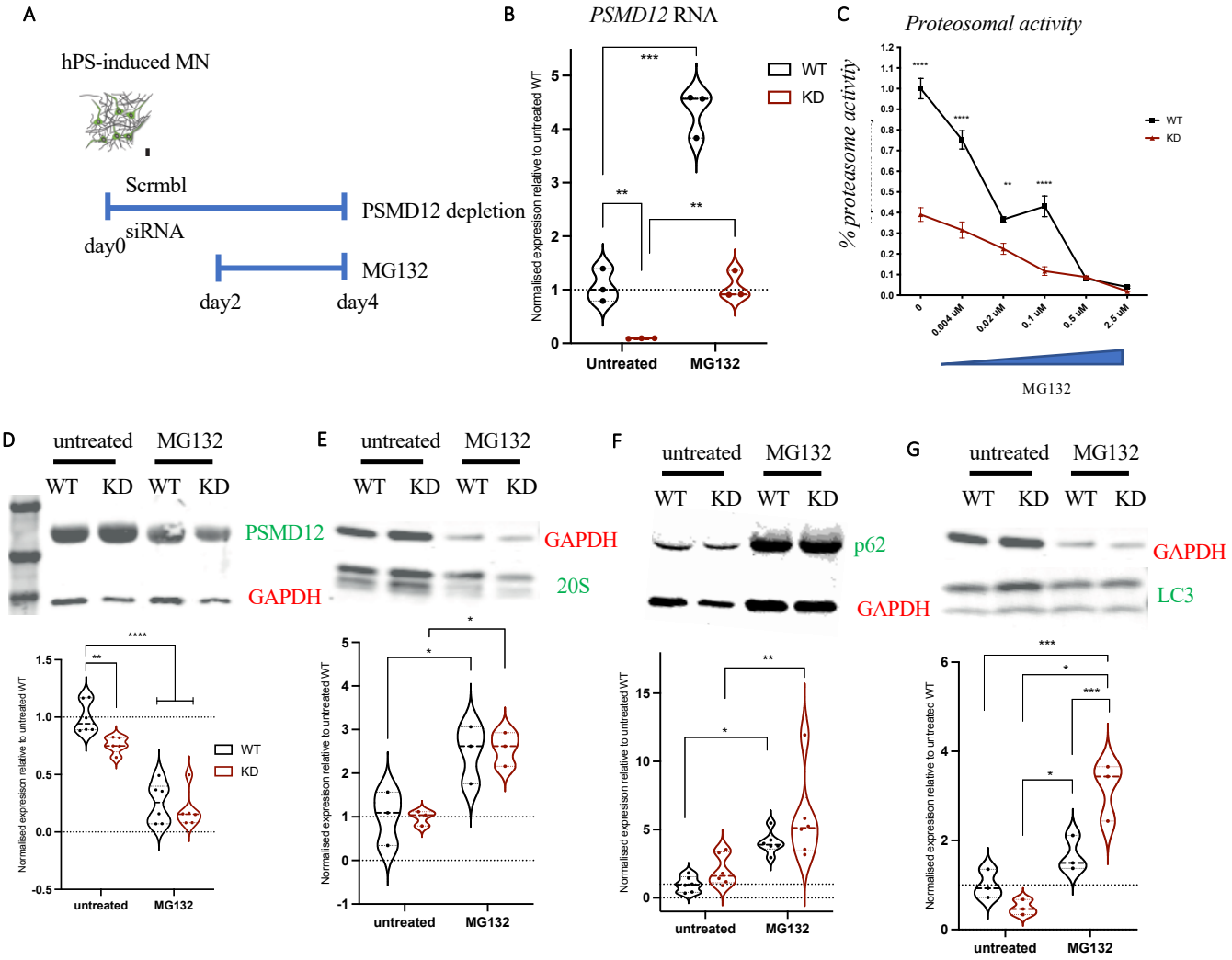

Fig. 6 | Manipulation of proteasome flux results in upregulated autophagy. (A) Workflow for downregulation of proteasome subunit PSDM12 under proteostatic stress. (B) Quantification of *PSMD12* RNA by RT-qPCR. (C) Proteosomal activity under inhibition in WT and KD. (D-G) Western blot quantifications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

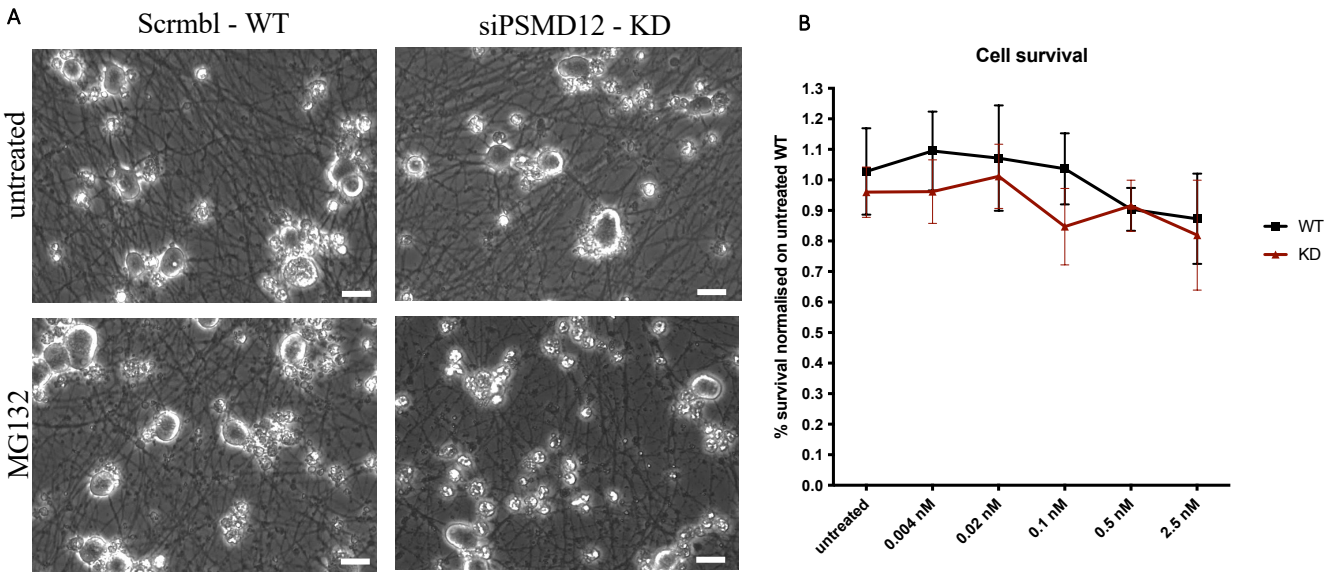

Fig. S6 | Neuronal survival under proteasome manipulations. (A) Brightfield images of neurons subjected to proteasomal manipulations. (B) survival of neurons under proteasomal manipulations.

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.

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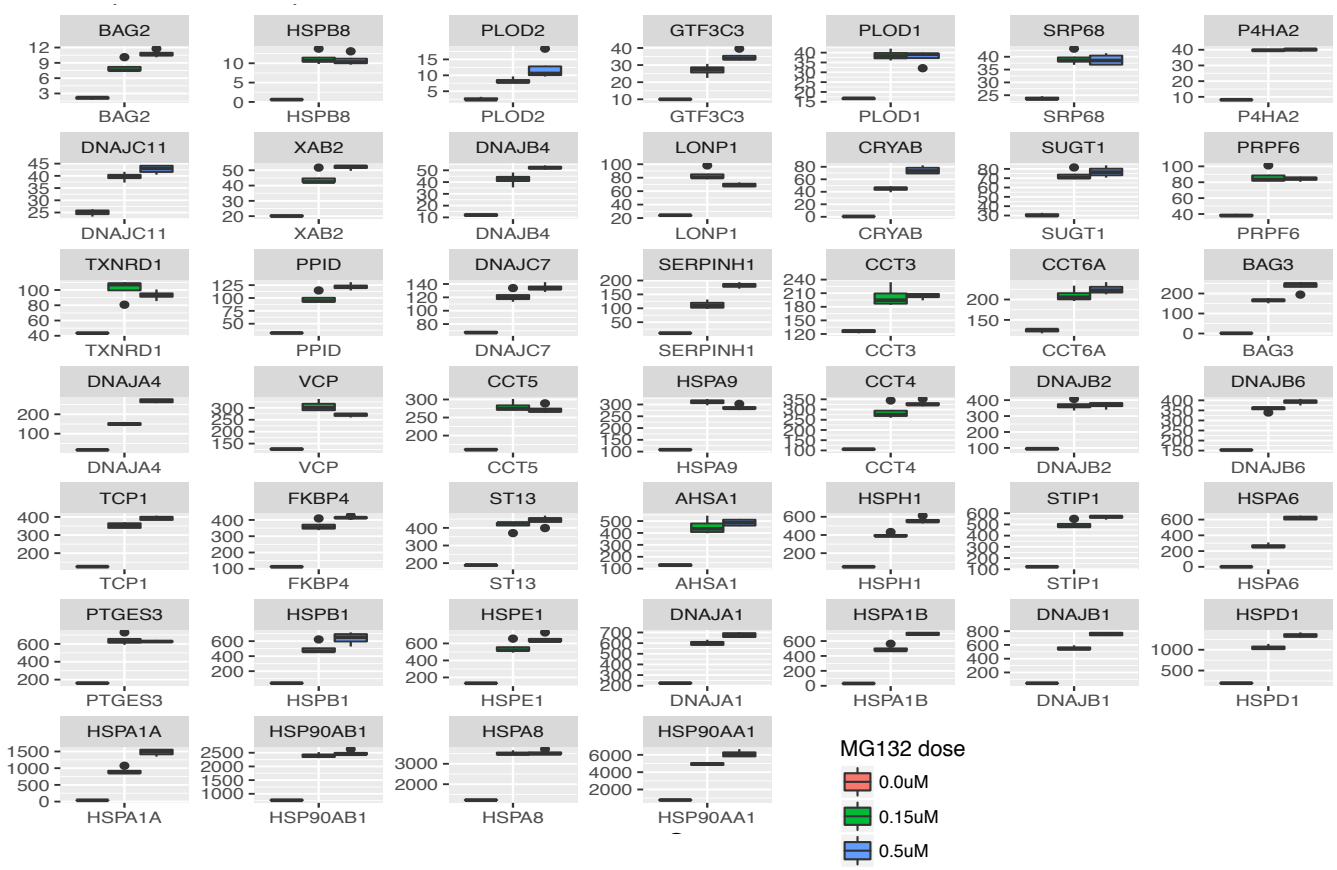

Fig. S2 | Genes upregulated by proteasome inhibition. Genes upregulated after treatment are connected to protein homeostasis.

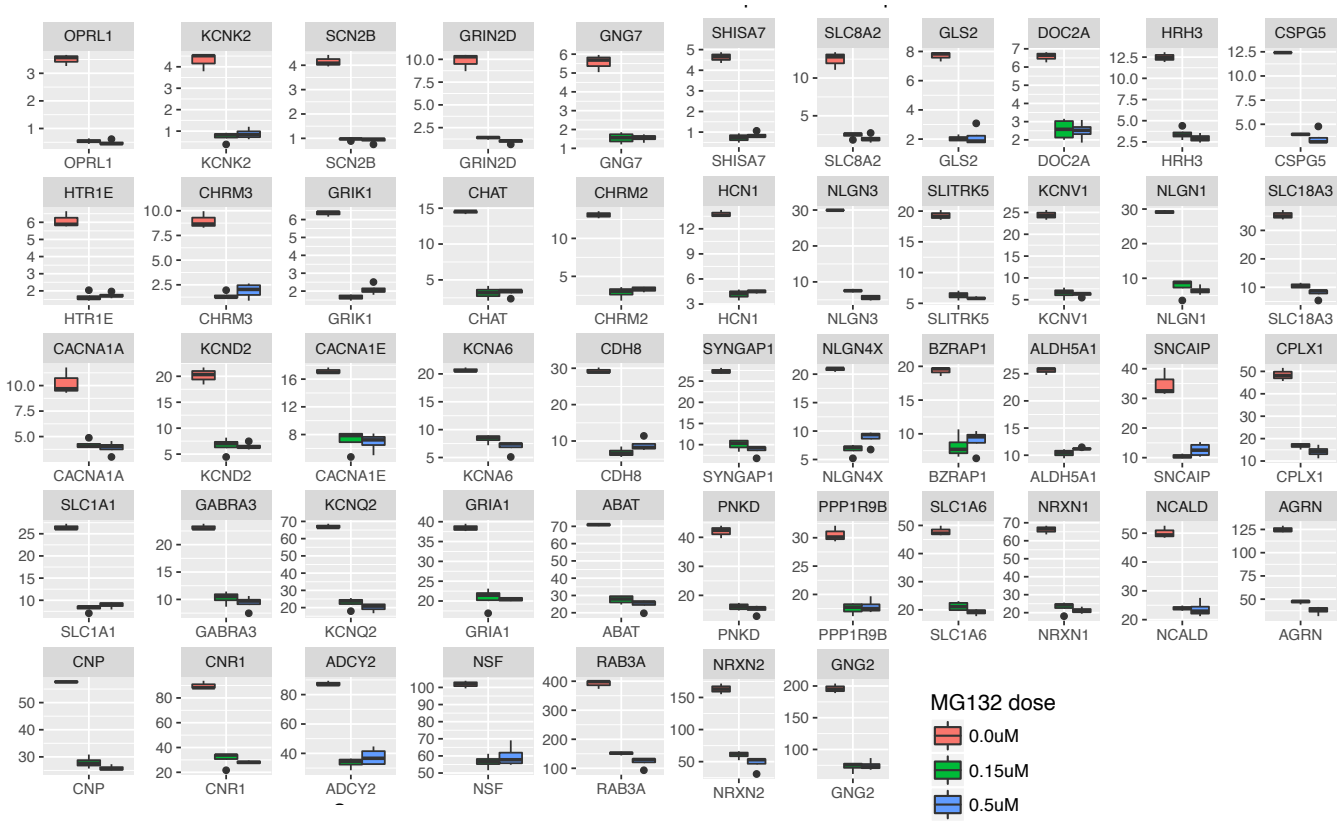

Fig. S3 | Genes downregulated by proteasome inhibition. Genes downregulated after treatment are connected to synaptic biology.

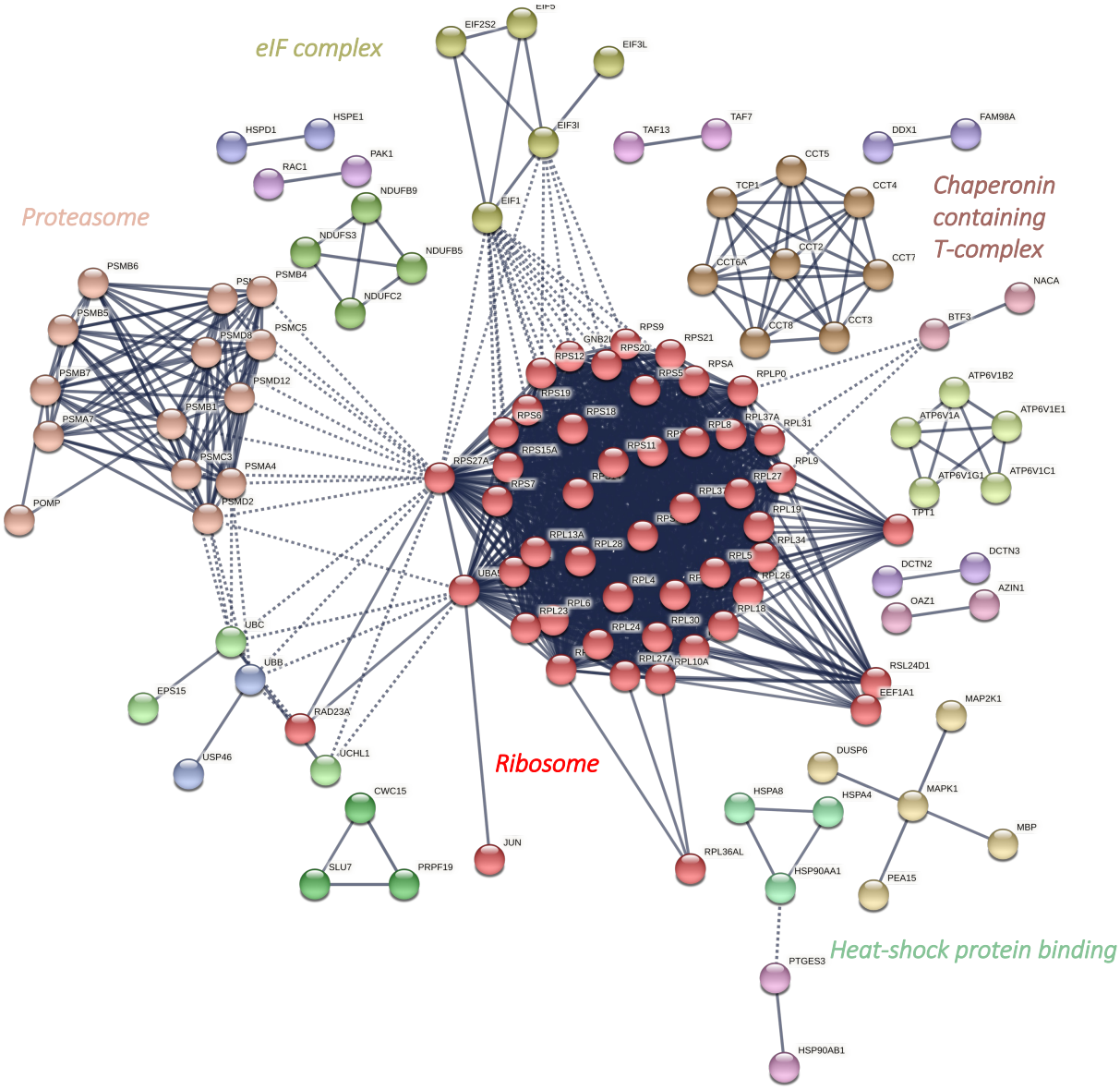

Fig. S4 | Shared transcriptomic signature of neuronal proteostatic stress and from sporadic ALS patients. Protein-protein interaction network of shared genes upregulated after proteasome inhibition.

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.

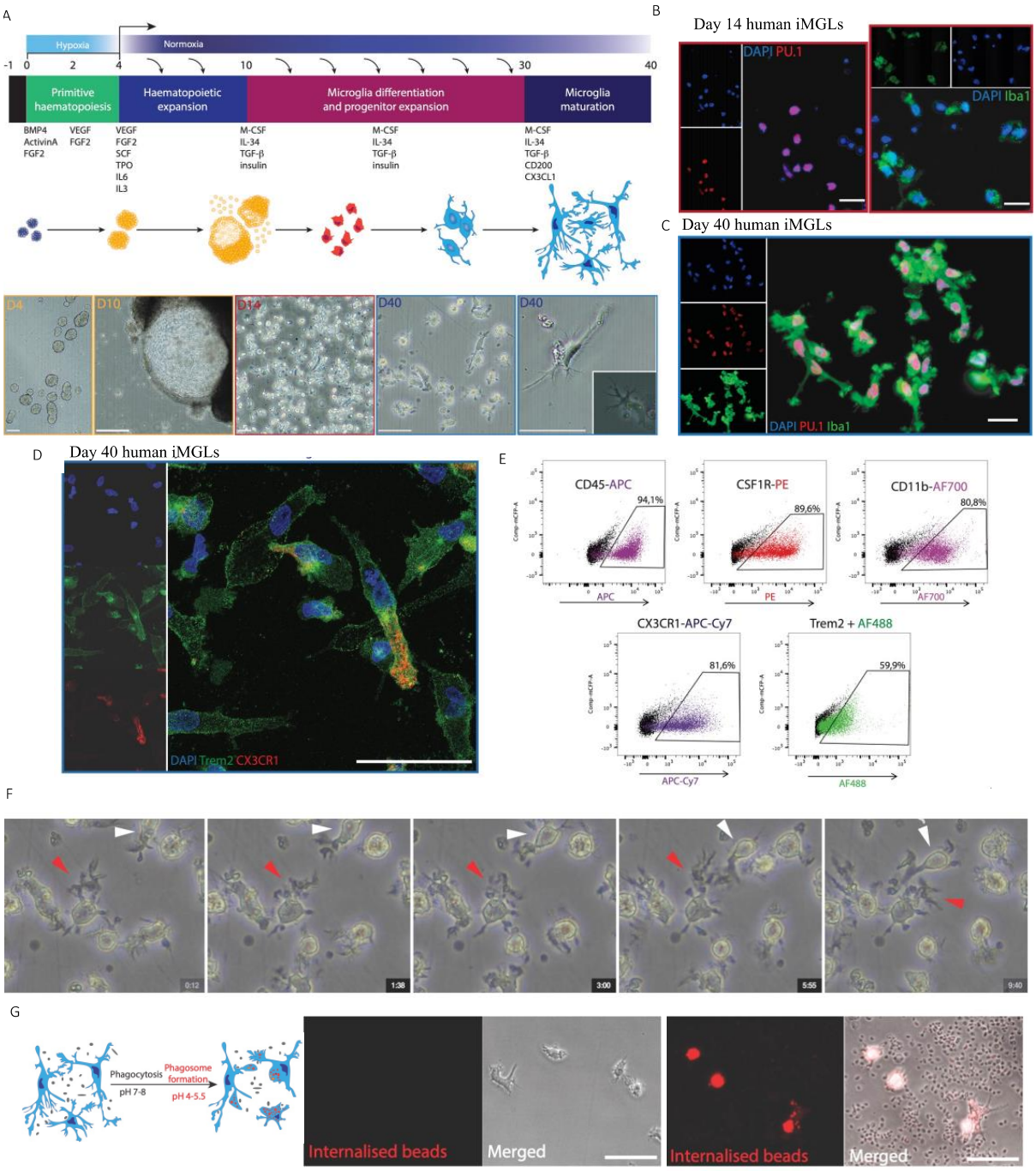

Figure 7 Characterization of human Microglia-like Cells (iMGLs). *(A) Diagram of differentiation protocol from primitive haematopoiesis to early myeloid progenitors into microglia-like cells. (B) immunofluorescence staining of myeloid markers PU.1 and Iba1 at day 14. (C) Immunofluorescence staining of 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).

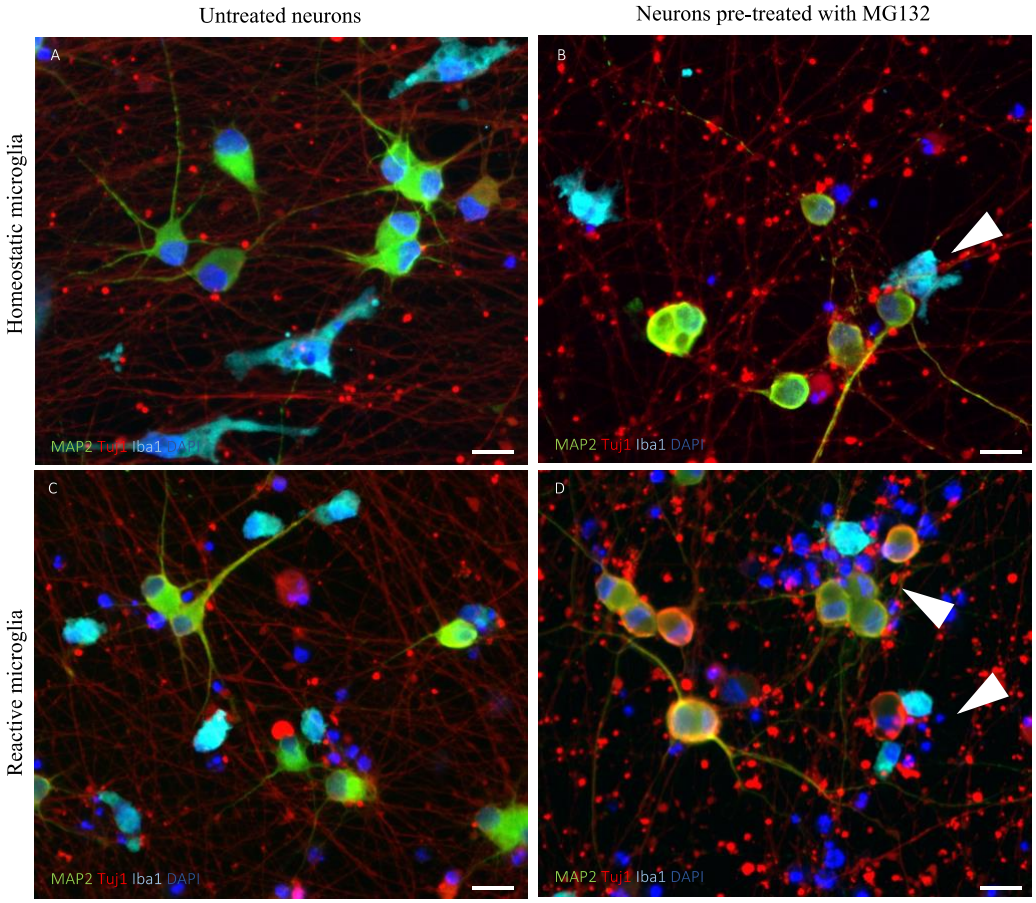

Figure 8 Pre-treatment of neurons with MG132 induces reactivation in microglia. *(A) Untreated neurons and homeostatic iMGLs co-culture. (B) Neurons pretreated with MG132 and homeostatic iMGLs. (C) iMGLs pre-stimulated with apoptotic cells co-cultured with untreated neurons. (D) iMGLs pre-stimulated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

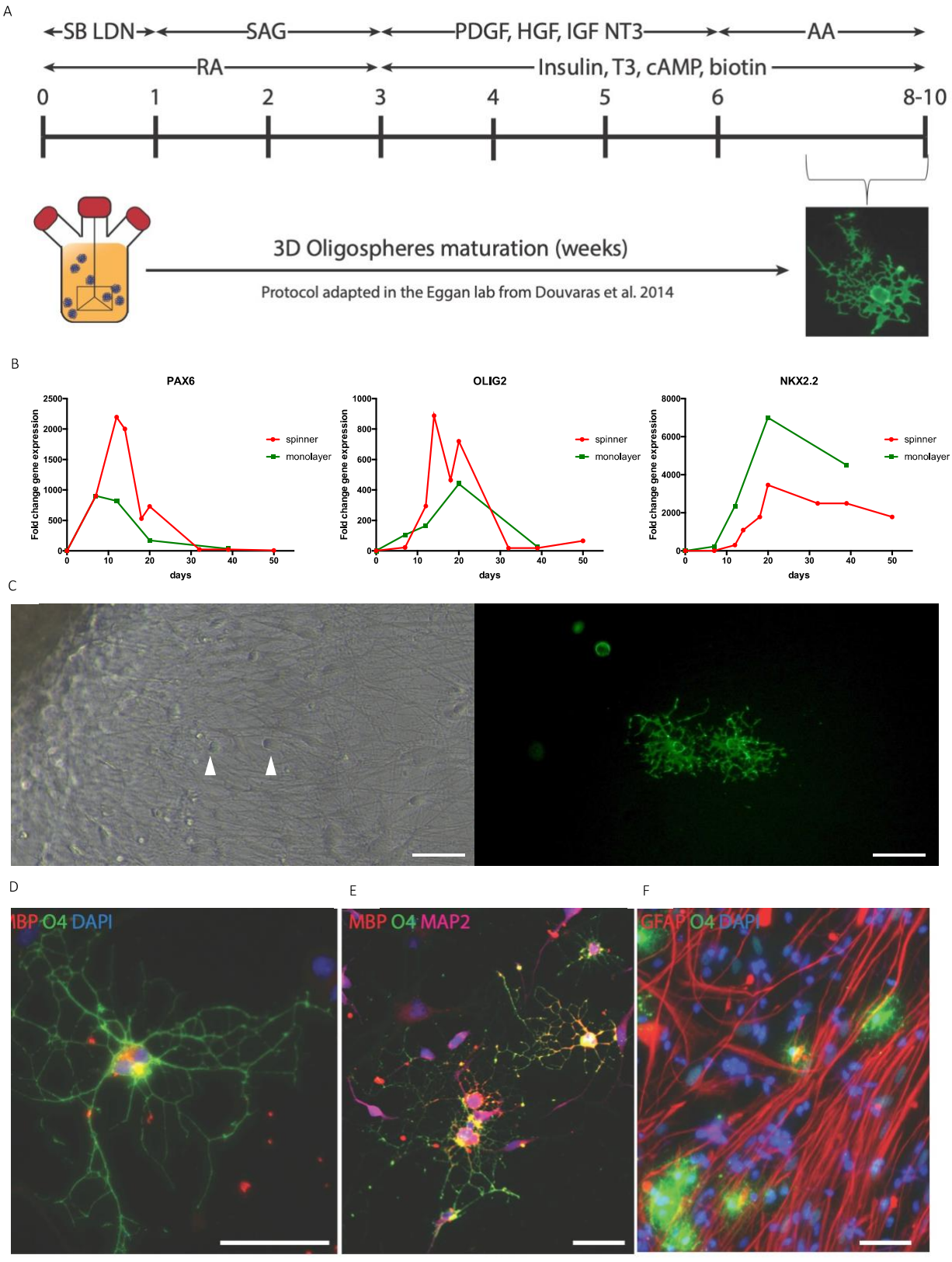

Figure 1 Characterization of 3D human Oligodendrocytes differentiation protocol. *(A) diagram of differentiation protocol. (B) RT-qPCR analysis of Oligodendrocyte Progenitors Markers at early stages of differentiation. (C) Live O4 immunofluorescence staining of committed oligodendrocytes day 50 (week 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.*

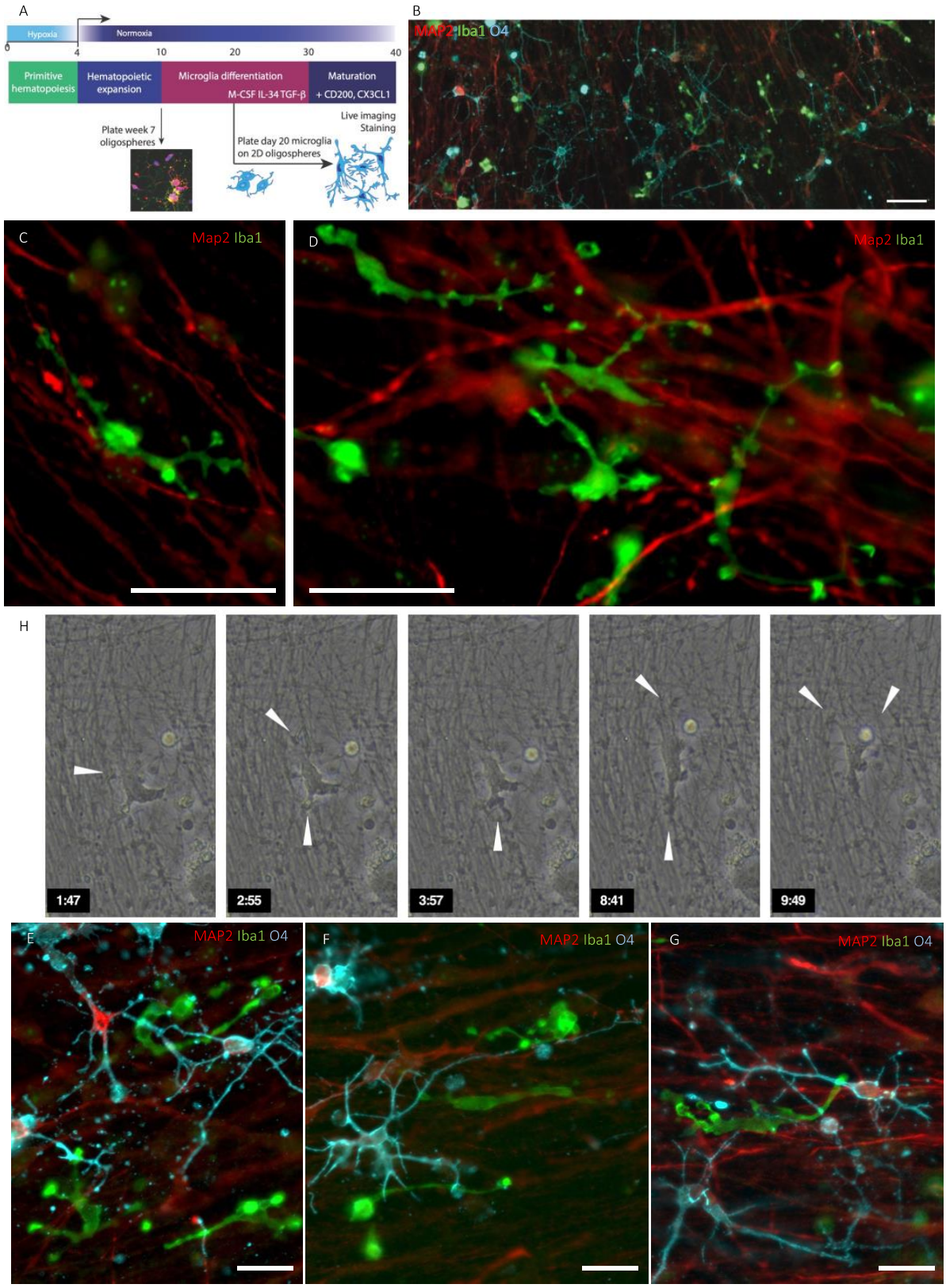

Figure 2 Characterization of Oligodendrocytes and microglial co-cultures. *(A) diagram of co-culture protocol. (B) immunofluorescence staining of brain cells markers from spheres cultures in 2D: O4 – OPCs and oligodendrocytes, Iba1 – iMGLs, MAP2 – neurons. (C-D) immunofluorescence staining of microglia 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.*

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