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Human Stem Cell Models of Neurodegeneration: From Basic Science of Amyotrophic Lateral Sclerosis to Clinical Translation

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Summary

Neurodegenerative diseases are characterized by progressive cell loss leading to disruption of the structure and function of the central nervous system. Amyotrophic lateral sclerosis (ALS) was among the first of these disorders modeled in patient-specific iPSCs, and recent findings have translated into some of the earliest iPSC-inspired clinical trials. Focusing on ALS as an example, we evaluate the status of modeling neurodegenerative diseases using iPSCs, including methods for deriving and using disease-relevant neuronal and glial lineages. We further highlight the remaining challenges in exploiting the full potential of iPSC technology for understanding and potentially treating neurodegenerative diseases such as ALS.

Keywords

Pluripotent stem cells; clinical translation; disease modeling; neurodegenerative diseases; ALS; FTD; motor neurons: cortical neurons; astrocytes; microglia

Introduction

Neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD), are characterised by a high level of etiological heterogeneity, with diverse genetic causes, environmental factors and complex pathophysiologies played out in the multicellular environment of the aging nervous system. This complexity poses significant challenges for *in vitro* modeling. However, the advent of

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Declaration of interests

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human induced pluripotent stem cell (hiPSC) technology (Takahashi and Yamanaka, 2006) has dramatically changed our ability to create physiologically relevant *in vitro* models for such diseases. As the genetic background of the cell donor is maintained during cell reprogramming, hiPSCs provide an excellent means to evaluate the effect of disease-causing mutations on the relevant, otherwise non-accessible cell types in neurodegenerative disorders, such as neurons and glial cells. In addition to monoculture differentiation protocols, more complex *in vitro* models, including multicellular and three-dimensional (3D) culture compositions, are now also becoming available to capture disease-relevant cellular interactions.

ALS has a significant monogenic contribution to causation, and the cell types (principally spinal motor neurons) affected by the disease can be produced to a high level of purity *in vitro* from stem cells. It therefore serves as an exemplar for the use of hiPSCs for discovery and translational neuroscience with implications more widely for the fields of neurodegenerative disease modeling and stem cell differentiation. In this review, we critically evaluate hiPSC differentiation protocols for the most ALS-relevant cell types (spinal motor neurons, cortical neurons, astrocytes and microglia) and summarize the impact of these models on our understanding of ALS pathogenesis. We also provide a perspective on the use of multicellular and 3D models, then review the remaining challenges and possible solutions in hiPSC research, including emerging technologies, and the role of hiPSC-based findings in improving prospects for successful clinical trials in ALS.

Amyotrophic lateral sclerosis: disease mechanisms and pathology

Amyotrophic lateral sclerosis (ALS), is a neurodegenerative disorder with a prevalence of ~5/100,000 people and a mean age of onset of ~64 years (life-time risk ~1:400) in populations of European genetic heritage (Chio et al., 2013; Kiernan et al., 2011). Characterized by the degeneration of MNs in the cortex, brainstem, and spinal cord, ALS results in progressive muscle weakness and paralysis, with death typically due to neuromuscular respiratory failure a median of 2.5 years from symptom onset, although there is considerable heterogeneity, with approximately 5% of cases surviving for more than 10 years (Chio et al., 2011).

Approximately 90% ALS cases are considered sporadic (sALS), in the absence of a family history of ALS or the related condition frontotemporal dementia (FTD). However, in ~10% of all ALS patients, including in a significant minority of apparently sALS cases, a disease-determining genetic variant can be identified, suggesting that the genetic contribution to ALS risk is substantially driven by rare variants (Talbot et al., 2018). The commonest mutation, a dynamic hexanucleotide repeat expansion (HRE) GGGGCC (G4C2) in the first intron of *C9orf72* (DeJesus-Hernandez et al., 2011; Renton et al., 2011), accounts for 20-50% of fALS cases and 3-5% of sALS patients, respectively (Kiernan et al., 2021). In another 10-20% of fALS and 1-2% of sALS cases, mutations are identified in the *SOD1* gene (Rosen et al., 1993). Mutations in *TARDBP* (encoding for the 43 kDa transactive response DNA binding protein TDP-43) and *FUS* are each found in 5% of fALS and less than 1% of sALS cases (Sreedharan et al., 2008; Vance et al., 2009), while a multitude of other, less commonly mutated genes, including *MAT3, OPTN, UBQLN2, TBK1, and*

NEK1, are also known to cause fALS and sALS. Based on founder effects, the contribution of genetic mutations to ALS varies between populations of different geographic origin. For instance, mutations in *C9orf72* are rare in Japan (Ogaki et al., 2012), while mutations in *TARDBP* are common in Sardinia (Borghero et al., 2014).

Neuropathologically, almost all ALS cases (~97%), except for the ~3% caused by mutations in the *FUS* and *SOD1* genes, show nuclear clearing and cytoplasmic aggregation of TDP-43 in neuronal and non-neuronal cells (Neumann et al., 2006). This characteristic TDP-43 pathology is also found in 40-50% of patients with isolated frontotemporal degeneration (FTD), a neurodegenerative disease characterized clinically by deterioration of social cognition and language, with relative preservation of memory, and pathologically by frontotemporal lobar degeneration (FTLD). Clinical overlap exists between ALS and FTD, with 3-5% of ALS patients showing overt behavioral variant FTD, while more subtle forms of loss of executive functions are found in up to 50% of ALS patients (Beeldman et al., 2018; Talbot et al., 2018). This suggests that FTLD-TDP and ALS share substantial biological features and potential treatment strategies (Burrell et al., 2016).

Over the past decades of ALS research, a multitude of *in vitro* and *in vivo* models have been created in an effort to recapitulate and study the processes that lead to MN degeneration in ALS patients. In agreement with data from neuroimaging and postmortem studies, these models suggest that ALS arises through a combination of primary neuronal damage with contributions to pathogenesis from non-neuronal cells such as glia (Vahsen et al., 2021). Despite this increasing understanding of ALS pathophysiology, there are currently only two licensed therapeutic options for ALS patients, riluzole and edaravone, which only very moderately improve survival and rate of progression (Kiernan et al., 2021). A large number of drug candidates showing promising results in preclinical models have so far failed to translate into benefits for patients. With the notable exception of antisense oligonucleotides (ASOs), which are currently undergoing clinical trials in patients carrying mutations in the *SOD1* or *C9orf72* genes, there is no prospect yet of substantial therapeutic options for ALS patients.

The failed development of new drugs that alter natural history can, in part, be explained by a lack of accurate disease models. ALS research has heavily relied on animal models overexpressing mutant forms of *SOD1,* which only occur in a small fraction of ALS cases, and crucially do not show TDP-43 aggregation. Inherent species differences in gene expression and the functional organization of the motor system also contribute to the difficultly in translating findings from animal models to humans (Herculano-Houzel et al., 2015; Lin et al., 2014; Schieber, 2007). hiPSC technology has offered a new and powerful tool to circumvent such issues through the development of *in vitro* differentiation protocols for the most ALS-relevant cell types that are discussed below. Methods which directly re-program fibroblasts into relevant cell types, by bypassing the hiPSC stage, are complementary to hiPSC technology for the modeling of conditions such as ALS, since some age-dependent molecular profiles are preserved while they are lost upon hiPSC reprogramming (Mertens et al., 2015). This concept has been reviewed in detail elsewhere (Mertens et al., 2018).

Differentiation of hiPSCs into motor neurons and cortical neurons

From hiPSCs to spinal motor neurons

The direct differentiation of hiPSCs to spinal MNs, which we will refer here as MNs, is the first essential step toward their use in the study of neurodevelopment, neurodegeneration, and potential future use in the clinic. Mimicking embryonic neurodevelopment, the generation of hiPSC-derived MNs involves several now clearly defined developmental steps:

1. Neural induction starts with inhibition of bone morphogenetic protein BMP and transforming growth factor beta (TGFb) signalling (referred as dual-SMAD inhibition) (Chambers et al., 2009) as well as inhibition of WNT signalling (Wilson et al., 2001), following the neural default model (Munoz-Sanjuan and Brivanlou, 2002). A positive role for fibroblast growth factor (FGF) signaling has been described in the chick embryo (Wilson et al., 2000) but may not be required for anterior neuroecotderm induction in hiPSCs.

2. Rostral neural progenitors can be induced to adopt a more posterior positional identity, governed by a response to caudalizing signals (i.e. retinoic acid, RA) (Durston et al., 1998; Muhr et al., 1999). Alternatively, posterior neural precursors can be induced directly in the presence of FGF and WNT signalling (Peljto et al., 2010) via induction of a transient posterior precursor referred to as neuro-mesodermal precursor (Lippmann et al., 2015). While the RA-based caudalization yields mostly brachial level progenitors, the WNT/FGF based pattern provides access to trunk and lumbar progenitors.

3. The resulting spinal progenitor cells then acquire a MN progenitor identity, induced by the ventralizing action of sonic hedgehog (SHH) signaling (Briscoe and Ericson, 2001). The activation of the SHH pathway inhibits the expression of dorsal progenitors in a concentration-dependent manner that mimics the response of primary spinal progenitor cells (Wichterle et al., 2002).

It has become clear that the systematic variation in the identity, timing and concentration of the patterning factors that hiPSCs are exposed to in the early phases can determine the efficiency, identity, and functional maturity of the final cultures, and may explain the discordant results reported by different groups when modelling ALS in hiPSC-derived MNs. The first reported generation of hiPSCs and successful differentiation to MNs from an ALS patient came from the Eggan laboratory in 2008 (Dimos et al., 2008). The authors used a directed differentiation protocol previously developed for mouse and human embryonic stem cells (hESCs) to produce spinal MNs (Lee et al., 2007; Li et al., 2005; Wichterle et al., 2002). This was soon followed by a comprehensive MN differentiation protocol for hESCs and hiPSCs (Hu and Zhang, 2009), and subsequently by numerous publications describing variations in MN differentiation protocols used in ALS research (Table 1).

Spinal MN differentiation protocols based on dual SMAD inhibition for the neural induction step rely on small molecule compounds, such as SB431542 (SB, an inhibitor of activin-nodal signaling) and LDN193189 (LDN, an inhibitor of BMP signaling). Several alternative BMP inhibitors have been used including dorsomorphin, recombinant noggin and DMH1

(Neely et al., 2012). In some instances, dual SMAD inhibition is combined with CHIR99021 (CHIR, an activator of the Wnt signaling pathway) that promote posterior identities and neuroepithelial proliferation (Li et al., 2011). Although now commonly used in MN differentiation protocols in ALS (Amoroso et al., 2013; Devlin et al., 2015; Kiskinis et al., 2014; Maury et al., 2015), it is noteworthy that the first studies on MN differentiations did not include a SMAD inhibition stage (Dimos et al., 2008; Hu and Zhang, 2009).

After initial neural induction, hiPSCs must be directed towards a caudal and ventral identity using RA and recombinant SHH or its agonist (SAG) respectively (Wichterle et al., 2002). In 2015, Maury and colleagues performed a comprehensive study of the effect of varying the concentration and timing of these two morphogens on differentiation efficiency as well as impact on MN subtype identity (Maury et al., 2015). This protocol has been used in several recent studies for *C9orf72* hiPSC-MNs (Dafinca et al., 2020; Mehta et al., 2021; Selvaraj et al., 2018). Overall, the concentration of RA in different protocols ranges from 100 nM (Hu and Zhang, 2009) to 1 μM (Dafinca et al., 2016; Karumbayaram et al., 2009; Maury et al., 2015), while one study showed the generation of MNs in the absence of activators of retinoid signaling (Patani et al., 2011). Conversely, Calder et al showed in 2015 that efficient MN generation can be obtained by early exposure to RA in the absence of SHH pathway agonists via suppression of GLI3 signaling (Calder et al., 2015).

Before maturation, neurons are generally dissociated and plated at lower densities to stimulate neurite outgrowth. Depending on the protocol, this stage can occur as early as 9 days (Maury et al., 2015; Qu et al., 2014) or as late as 45 days post-induction (Devlin et al., 2015). When final neural maturation is induced, the growth medium is usually supplemented with brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) (Dafinca et al., 2020; Dafinca et al., 2016; Devlin et al., 2015; Dimos et al., 2008; Hu and Zhang, 2009; Kiskinis et al., 2014; Qu et al., 2014; Selvaraj et al., 2018), as well as ciliary neurotrophic factor (CNTF) (Devlin et al., 2015; Dimos et al., 2008; Karumbayaram et al., 2009; Kiskinis et al., 2014) or IGF-1 (Dafinca et al., 2020; Dafinca et al., 2016; Hu and Zhang, 2009; Qu et al., 2014).

Despite the well-defined phases of MN differentiation, the marked variability in differentiation protocols, as well as in the identity of the final cultures, even between different hiPSC lines differentiated in parallel, requires objective assessment of MN identity and purity if studies are to be meaningfully compared. Postmitotic MNs can be identified by specific MN transcription factors, such as ISL1 and HB9, while more mature MNs express choline acetyltransferase (ChAT) and the vesicular acetylcholine neurotransmitter transporter (vAChT) (Amoroso et al., 2013; Karumbayaram et al., 2009). While all MNs will initially follow this pattern of expression, it has become clear that during the differentiation process only a subset of these proteins may be expressed (Amoroso et al., 2013; Maury et al., 2015). Depending on the markers used to define MN identity, the purity of MN cultures reported in the ALS literature ranges from 20-30% (Amoroso et al., 2013; Dimos et al., 2008), 40-50% (Devlin et al., 2015; Hu and Zhang, 2009; Maury et al., 2015) to 95% (Du et al., 2015). One strategy to reduce heterogeneity of MN cultures is to enrich for MNs by fluorescence activated cell sorting (FACS) of neural progenitors, for example by adenoviral transduction with HB9:GFP (Amoroso et al., 2013; Wainger et al., 2014).

However, restriction of HB9 expression to early development favors selection of immature neurons which may not be the most relevant disease models for neurodegenerative disorders.

Assessment of the functional maturity of hiPSC-MNs by electrophysiology involve the presence of action potentials and sustained trains of action potentials which occur only at later stages of MN differentiation (Hu and Zhang, 2009). In heterogeneous cultures MNs are generally identified and patch-clamped on the basis of size, not molecular identity. Studies focusing on electrophysiological properties in ALS hiPSC-MNs therefore typically use extended maturation periods (Devlin et al., 2015). However, it is important to note that different subtypes of MNs have different electrophysiological properties such as rate and amplitude of action potential and the presence of action potentials alone is not sufficient to categorize a MN as mature. Other parameters of functional maturity in MN cultures include evidence of synaptic connectivity such as the presence of miniature excitatory or inhibitory postsynaptic currents. Furthermore, whole cell patch clamping is a technically challenging, low throughout method that may be inefficient at determining the real variation present within a neuronal culture. Therefore, there is growing effort to implement optical readouts of neuronal activity that can be monitored across thousands of neurons in parallel such as Calcium imaging, use of fluorescent voltage indicators combined with optogenetic stimulation (“all optical electrophysiology”) (Kiskinis et al., 2018) or activity measurements using multielectrode arrays (MEA) (Wainger et al., 2014), methods that may provide a better representation of overall network activity and connectivity in heterogeneous cultures (Ronchi et al., 2021).

From hiPSCs to cortical neurons

In many neural conversion protocols, hiPSCs undergo the formation of radially organized neuroepithelia (neural rosettes) (Zhang et al., 2001). The neural rosettes assume by default a primitive anterior identity (Pankratz et al., 2007) and yield glutamatergic forebrain neurons with dorsal telencephalic identity in the absence of morphogens (Elkabetz et al., 2008; Li et al., 2009). A default anterior and dorsal identity is also observed when using dual-SMAD inhibition-based neural induction protocols (Chambers et al., 2009).

Most current cortical neuron differentiation protocols do not selectively yield neurons from a particular cortical layer but lead to a mixture of deep and upper layer neurons. Their relevance to ALS, which is characterized by restricted degeneration of specific subsets of cortical neurons affecting corticofugal neurons in the motor cortex and their connections, is therefore less clear. In addition, there is considerable interest in modelling the related neurodegenerative disease FTD, which can occur as part of the ALS disease spectrum, but even less is understood of the specific cortical neuronal subtypes undergoing degeneration. We will briefly discuss here the specific protocols used in *C9orf72*-ALS/FTD research to generate hiPSC-derived cortical neurons in terms of length and how they differ at various stages (Table 2). However, further efforts are clearly needed in the field to yield cortical neuron differentiation protocols more directly relevant to ALS/FTD research.

Cortical neuron differentiation protocols currently used for ALS/FTD can be broadly defined by three main stages: hiPSCs convert to cortical neurepithelial stem cells, then to progenitor

cells, followed by differentiation and maturation into cortical neurons able to fire action potentials and form synapses.

Several studies in which cortical neurons have been differentiated from ALS hiPSCs were based on a protocol by (Delaloy et al., 2010), and have used cells carrying the G4C2 repeat expansion in *C9orf72* (e.g. (Almeida et al., 2013; Freibaum et al., 2015; Yuva-Aydemir et al., 2019). Similar to the cortical neuron differentiation protocol by Shi et al. (Shi et al., 2012), the first phase involves dissociation of hiPSC colonies and embryoid body (EB) formation. After a week, EBs are typically attached to laminin-coated dishes and allowed to form neural rosettes (e.g. (Almeida et al., 2013; Shi et al., 2012), which are then lifted and maintained as neurospheres in suspension for 1-2 weeks. Final differentiation is initiated by plating dissociated neurospheres on dishes coated with poly-D-lysine and laminin either without further addition of other growth factors (Shi et al., 2012) or with the addition of BDNF and GDNF to support neurite outgrowth (e.g. (Almeida et al., 2013; Dafinca et al., 2016; Selvaraj et al., 2018).

In addition to the extrinsic factor-based MN and cortical differentiation strategies listed in this section, several groups have developed transcription factor-driven differentiation strategies. Those include the forced expression of Neurogenin 2 (NGN2), ISL1 and LHX3 to convert control, sALS, and *C9orf72* ALS/FTD patient-derived iPSCs into induced MNs (iMNs) (Shi et al., 2019; Shi et al., 2018) based on earlier work in mouse ESCs (Mazzoni et al., 2013). Transcription factor-based conversion of iPSCs into iMNs has been used for screening assays and for candidate evaluation studies in the context of *SOD1, TARDBP, C9orf72* and sALS (Imamura et al., 2017). Forced expression of Neurogenin 1 (NGN1) and NGN2 (Busskamp et al., 2014; Lam et al., 2017), or NGN2 alone (Zhang et al., 2013), was also sufficient for the rapid induction of cortical-like neurons (iNs) from hiPSCs and can be combined with extrinsic patterning strategies (Nehme et al., 2018) to enhance cortical marker expression. NGN2-driven differentiation was adapted by Shlevkov et al. to perform a high-content screen which identified 6 small-molecule regulators of the axonal transport of mitochondria, some of which rescued transport deficits in iPSC MNs derived from a SOD1-ALS patient (Shlevkov et al., 2019).

Differentiation of hiPSCs into astrocytes and microglia

In neurodegenerative disorders such as ALS, astrocytes and microglia are thought to play a role in pathogenesis by promoting an inflammatory state in the brain (neuroinflammation). In the brain and spinal cord, astrocytes and microglia exert their primary functions on neurons, but also signal to one other (Matejuk and Ransohoff, 2020). These physiological, tricellular interactions are summarized in Figure 1. While changes in those interactions have been implicated in ALS (Filipi et al., 2020), it remains unclear what triggers neuroinflammation and how those changes contribute to neurodegeneration in ALS. Nevertheless, controlling neuroinflammation by modulating the communication between MNs and glia is under active study as a potential therapeutic strategy for ALS. The recent development of new differentiation protocols for hiPSC-derived astrocytes and microglia has opened up the opportunity to study the effect of ALS-causing mutations on glial cells as well as the interaction between human glial cells and MNs *in vitro.* Because the number of

astrocyte and microglia differentiation protocols used in ALS studies is still rather limited, we will cover differentiation strategies that have been utilized in both ALS and non-ALS studies.

From hiPSCs to astrocytes

Protocols to differentiate astrocytes from hiPSCs (Table 3) typically consist of four steps: (1) differentiation of hiPSCs into neural progenitor cells (NPCs); (2) neural patterning to specify astrocytes to defined regions of the CNS; (3) long-term culture or induction of gliogenic switch; and (4) astrocyte terminal differentiation and maturation (reviewed in (Krencik and Zhang, 2011; Tyzack et al., 2016).

Astrocyte differentiation protocols from hiPSCs are listed in table 3. During development, gliogenesis follows neurogenesis. In the late embryonic stage and early postnatal period, astrocyte progenitors are specified from NPCs via Notch signalling (Chambers et al., 2001). *In vitro,* NPCs can be derived from hiPSCs using 3D differentiation methods (Eiraku et al., 2008; Falk et al., 2012; Watanabe et al., 2005) or 2D monolayer strategies such as dual SMAD inhibition (Chambers et al., 2009. Regional specification of NPCs is obtained through the combination of growth factors and cytokines (i.e. FGF8, RA, SHH, BMP), which allows the generation of spinal cord-, midbrain-, and dorsal or ventral-forebrain specific astrocytes (Hjorth, 1993; Holmqvist et al., 2015; Krencik et al., 2011; Krencik and Zhang, 2011; Li et al., 2018; Roybon et al., 2013). Astrocyte lineage specification (gliogenic switch) via long-term NPC expansion and subsequent terminal astrocytic differentiation are then performed in media supplemented with various factors alone or in combination such as cAMP, CNTF, LIF, EGF, FGF2, BMP, or in presence of serum.

In the initial protocols for the derivation of astrocytes from hiPSCs, NPCs were expanded in the presence of RA, SHH and cAMP, cultured in suspension for two months in media consisting of DMEM/F12, N1 supplement and cAMP, after which they were cultured on plastic for an additional 7 days (Hu et al., 2010). Because gliogenesis follows neurogenesis during embryonic development, generation of astrocytes from hiPSCs can be protracted, requiring differentiation periods ranging from 3 to 6 months, making hiPSC studies laborious, costly and difficult to standardize (Studer et al., 2015). A recent method to efficiently generate astrocytes in 4 to 7 weeks from the hiPSC stage uses CRISPR/Cas9-mediated inducible expression of the transcription factor nuclear factor I A (*NFIA*), or *NFIA* in combination with SRY-box transcription factor 9 (*SOX9*) (Li et al., 2018). The rapid, NFIA and SOX9 mediated transcriptional fate conversion was also reported for directly generating mouse astrocytes from postnatal skin fibroblast (Caiazzo et al., 2015). An alternative strategy is the use of NFIA to rapidly trigger the gliogenic switch in early spinal or cortical patterned NPCs followed by the astrocytic differentiation of those glial competent precursors into spinal cord or cortex- related astrocytes from hiPSCs using LIF or serum-based differentiation protocols (Tchieu et al., 2019).

To selectively enrich for astrocytes, glial-committed NPCs were positively sorted for A2B5 by magnetic selection followed by re-plating in neurobasal medium containing CNTF for an additional 2 weeks (Mormone et al., 2014). Alternatively, a nearly pure astrocyte population can be isolated from 3D human cerebral cortical spheroids (hCS), which, after 590 days

in culture, were dissociated and immunopanned using anti-Thy1 to harvest neurons and anti-HepaCAM to harvest astrocytes (Sloan et al., 2017), methods developed previously for the isolation of astrocytes from the adult brain. *CD49f* is another marker which has been proposed to purify human fetal astrocytes in the spinal cord and spinal astrocytes derived from hiPSCs (Barbar et al., 2020). After FACS purification, *CD49f*$^{+}$ astrocytes showed trophic support of neurons, glutamate uptake and phagocytic capability. In response to inflammatory stimuli, *CD49f*$^{+}$ astrocytes acquired an A1-like reactive state characterized by impaired phagocytosis and glutamate uptake as well as loss of capability to support neuronal maturation. Moreover, conditioned medium from *CD49f*$^{+}$ A1 astrocytes was toxic to human and rodent neurons.

However, for precise modeling of region-specific neurodegenerative disorders, it is important to consider that astrocytes possess heterogeneous region-specific morphologies and functions. In rodents, astrocytes are morphologically classified into two major subtypes: protoplasmic and fibrous, which are widely distributed in the brain and spinal cord (reviewed in (Tabata, 2015)). In the human cortex, two additional astrocyte subtypes exist: interlaminar and varicose projection astrocytes (Oberheim et al., 2012). These four astrocyte subtypes are characterized by different GFAP expression and morphology. In addition, functional differences exist between spinal and cortical, ventral and dorsal regions (as reviewed in (Tyzack et al., 2016). For instance, cortical astrocytes express high levels of the glutamate transporter GLT-1, whereas spinal astrocytes express lower levels of GLT-1 but express glycine receptors. The hiPSC-derived astrocytes described to date still possess immature morphological and functional characteristics compared to adult astrocytes and are most likely mixed populations of different astrocyte subtypes, morphologies, and functions. Although some laboratories have attempted to generate cortical or spinal specific astrocytes, their region-specific functions have not been investigated in depth, and we are still lacking efficient ways to guide the astrocyte differentiation toward a well-defined dorsal vs ventral profile. Proper morphological and functional characterization will be key to understanding the pathogenesis of region-specific neurodegenerative disorders, and critical for precise hiPSC disease modeling.

From hPSCs to microglia

While protocols for the differentiation of hiPSCs into astrocytes have now been available for more than a decade, hiPSC-derived microglia have become available only recently (Muffat et al., 2016). These differentiation protocols principally aim to mimic microglial ontogeny, which is distinct from that of *Myb*-dependent blood macrophages, as microglia arise from *Myb*-independent yolk sac-derived progenitors (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Kierdorf et al., 2013; Schulz et al., 2012). During neurodevelopment, these progenitors migrate into the brain to undergo final maturation to microglia in concert with neurons and then maintain their population by self-renewal (Bruttger et al., 2015). hiPSC-derived microglia are therefore more suitable for disease modelling compared to blood monocyte-derived macrophages, which are employed by some researchers as a proxy for brain macrophages. Several articles have recently reviewed the commonalities and differences between protocols for the differentiation of hiPSC-derived microglia (e.g. (Haenseler and Rajendran, 2019; Hasselmann and Blurton-Jones, 2020; Hedegaard et al.,

2020; Speicher et al., 2019; Wurm et al., 2021)). Here, we briefly focus on key principles and limitations of current methods.

Typically, hiPSC microglial differentiation protocols consist of four steps (Table 4): (1) initial patterning into cells corresponding to the primitive streak ; (2) differentiation into hemangioblasts and primitive hematopoietic stem cells; (3) differentiation into myeloid progenitor cells; (4) terminal microglial differentiation and maturation. These milestones are achieved by protocols of diverging complexity and duration, either by simply exposing hiPSCs to different growth factors and cytokines, or additionally employing sophisticated sorting methods (FACS or MACS), or by inducing hypoxia. The initial patterning step can be performed via 3D EB formation (Haenseler et al., 2017; Muffat et al., 2016), or in 2D monolayers (Abud et al., 2017; Douvaras et al., 2017; Guttikonda et al., 2021; McQuade et al., 2018; Pandya et al., 2017; Takata et al., 2017). The differentiation of hemogenic endothelial cells into primitive haematopoetic stem cells is achieved by the addition of cytokines including IL-3 and M-CSF. This leads to the formation of yolk sac-like structures, progressively releasing myeloid progenitor cells into the culture medium, which can be simply harvested and re-plated for terminal differentiation or further enriched by FACS or MACS. Terminal differentiation into hiPSC-derived microglia is typically achieved by continuous CSF1R engagement, typically using IL-34. The resulting hiPSC-derived microglia generated by different protocols show similar properties: they are motile and functionally active in phagocytosis and secretion, and their transcriptome is similar to human fetal microglia. However, the regional identity of these cells, for instance, whether they represent spinal or cortical microglia, is usually ill-defined, emphasizing that there remains a need for better characterization of cells generated using currently available protocols.

Insights into ALS pathophysiology from hiPSC models

A wide range of cellular phenotypes have been described in models of ALS. A major challenge is how to relate these to the pathophysiology of the human disease in a way that improves the prospect of identifying disease-modifying drugs. It is fundamental to appreciate that ALS is a clinical syndrome in which neurodegeneration occurs in the complex network which produces voluntary movement (Talbot et al., 2018), with multiple genetic and non-genetic contributions. Disease modelling in ALS is likely to uncover common cellular phenotypes across different mutations (eg; alterations in excitability, defects in axonal transport), but these may be driven by distinct differences in upstream biological pathways.

Animal models, particularly rodents, continue to have a place in disease modelling in ALS and have provided important clues into core pathogenic pathways and the relative contribution of neuronal and non-neuronal cells in the context of intact tissue architecture, especially the neuromuscular synapse, which shows common features in its organization across species. However, animal models based on genetic manipulation, such as overexpression of human genes in mice from heterologous promoters designed to force a ‘strong’ phenotype, may not faithfully recapitulate the key pathways in human ALS, and may amplify interspecies differences in biology and response to therapy. Complementing vertebrate models, the fruit fly *Drosophila Melanogaster* and yeast models

have been employed by multiple labs in ALS research to gain additional insights into ALS pathogenesis and for drug discovery due to the recapitulation of specific aspects of ALS pathology and the availability of tools to rapidly manipulate gene and protein expression (reviewed in (Di Gregorio and Duennwald, 2018; Liguori et al., 2021; Zhang et al., 2018). In contrast, post-mortem tissue from ALS patients enables access to the full spectrum of disease heterogeneity but only captures a static snapshot at the end-stage of disease. Not all MNs are equally susceptible to degeneration in ALS, and those cells remaining at autopsy may reflect populations relatively resistant to the disease process, making the differentiation between disease drivers and compensatory mechanisms in post-mortem tissue samples difficult.

hiPSCs present a complementary platform to vertebrate, fly and yeast models and post-mortem tissue, facilitating investigation of relevant disease processes in the context of the specific human cell-types targeted in ALS, across all genotypes, and potentially a way to address the difficult problem of modelling sporadic disease. With increased knowledge of neurodevelopment, hiPSCs will provide access to the full repertoire of ALS-relevant human cell types at scale, enabling high-throughput assays such as genetic and chemical screens (Fujimori et al., 2018; Imamura et al., 2017). An up-to-date list of ALS models using hiPSC technology as well as their main findings is provided in Table 5.

Motor neuron models

i) C9orf72—HRE mutations in the first intron of *C9orf72* can be associated with pure ALS, pure FTD or ALS/FTD within the same pedigree, suggesting that the mutation acts on cortical neurons or spinal MNs in concert with complex genetic interactors and age-related cellular events. Given the challenges in generating rodent models of *C9orf72*-related neurodegeneration (Mordes et al., 2020), hiPSCs represent a unique tool to study the differential effect of the mutation on neuron subtypes and the identification of potential modifiers.

MNs generated from *C9orf72* hiPSCs consistently exhibit the characteristic RNA foci and dipeptide repeat-associated non-AUG (RAN) pathology of *C9orf72*-ALS/FTD (Almeida et al., 2019; Donnelly et al., 2013; Gendron et al., 2017; Sareen et al., 2013), and variable degrees of reduced C9orf72 protein expression (haploinsufficiency) (Donnelly et al., 2013) (Sareen et al., 2013). Although TDP-43 mislocalisation and aggregation is a core feature of *C9orf72*-ALS/FTD at post-mortem, few studies to date have convincingly demonstrated this in hiPSC-MNs. Whether this means that TDP-43 dysregulation in *C9orf72*-ALS can be dissociated from other aspects of pathophysiology, or whether further development in cell maturation is required for full pathological processing of TDP-43 is unknown. However, a range of phenotypes reported in *C9orf72* lines suggest that the mutation is associated with reduced firing capacity, sensitivity to glutamate toxicity, impaired nucleocytoplasmic transport and altered vesicular and synaptic trafficking (Dafinca et al., 2016; Donnelly et al., 2013; Freibaum et al., 2015; Sareen et al., 2013; Zhang et al., 2015a). The use of hiPSC-MNs has been an important pre-clinical tool in demonstrating the effectiveness of ASO therapy currently in clinical trials (Sareen et al., 2013). *C9orf72* hiPSC models

therefore remain an important tool for investigating pathophysiology and for validation of drugs targeting key aspects of *C9orf72*-ALS pathogenesis.

ii) SOD1—*SOD1* was the first gene in which mutations were found in ALS, and it is therefore not surprising that *SOD1* models have dominated translational research for the last 20 years. However, with the exception of ASO therapy now in Phase 2 clinical trials, the results have been disappointing (Miller et al., 2020). Rodent models tended to emphasize the effects of high levels of misfolded mutant *SOD1* on neurons and the contribution of glia and neuroinflammation, but despite a vast literature on work from transgenic mouse models, the mechanism whereby *SOD1* mutations cause ALS is still unclear. hiPSC models showed that MNs in monoculture display phenotypes which can be disease relevant and independent of other cells types such as astrocytes. For instance, increased apoptosis, fewer neurites, reduced soma size, and neurite length specifically in MNs derived from a patient carrying the severe *SOD1A4V* mutant were rescued with genetic correction using a two-step, zinc-finger nuclease (ZFN)-mediated gene targeting strategy (Kiskinis et al., 2014). Other studies also confirmed the effect of mutant *SOD1* on neurofilament dynamics, axonal growth and function (Chen et al., 2014; Kim et al., 2020) and that misfolded mutant SOD1 is present in basal culture conditions. However, direct non-cell autonomous toxic effects of mutant SOD1-expressing astrocytes on MN survival have also been demonstrated and are discussed in the section on “non-neuronal cell types” below. Overall, *SOD1* mutations have been less extensively studied in hiPSCs compared to *C9orf72* or *TARDBP.*

iii) TARDBP—Given that the majority of ALS cases show TDP-43 pathology at autopsy, hiPSC-MNs carrying mutations in its gene *TARDBP* are of particular interest in potentially revealing a mechanism for loss of TDP-43 regulation in ALS. Studies have consistently shown that detergent insoluble TDP-43 levels are increased in the cytoplasm, though not generally in the form of clear aggregates. MNs showed reduced growth and survival (e.g. (Serio et al., 2013)), alterations in neurofilament organization and axonal vesicular trafficking (e.g. (Kreiter et al., 2018)), and a range of alterations in mitochondrial and other sub-cellular organelle dysfunction (e.g. (Dafinca et al., 2020)). Without genetic manipulation to specifically label TDP-43, which could promote artefacts, it is not possible to study its dynamic movement between nucleus and cytoplasm and in phase transition to form membraneless organelles which may be critical to the initiation of the pathological cascades driving observed phenotypes.

Of particular interest for its clinical potential, a study from Fang and colleagues performed a high-content screen of compounds to alter stress granule properties and TDP-43 aggregate-like structures in hiPSC-MNs carrying mutations in *TARDBP* or *FUS* (Fang et al., 2019). Interestingly, compounds containing extended planar aromatic moieties decreased the recruitment of RNA-binding proteins such as TDP-43 into stress granules leading to reduced formation of TDP-43 aggregate-like structures, suggesting that such compounds could be used to treat ALS patients with TDP-43 pathology.

iv) FUS—Mutations in *FUS* account for a small minority of familial cases (<5%) and are associated with characteristic TDP-43 negative neuropathological changes at autopsy. Therefore, although an RNA binding protein, FUS may exert its pathological effects through

distinct mechanisms from most ALS cases. In accordance with postmortem tissue, FUS cytoplasmic mislocalization and accumulation has been recapitulated across multiple studies of (*FUS*) mutant hiPSC-MNs(Guo et al., 2017b; Higelin et al., 2016; Ichiyanagi et al., 2016; Marrone et al., 2018; Naumann et al., 2018). In addition to alterations in splicing (Ichiyanagi et al., 2016), FUS has been implicated in altered DNA damage repair (Higelin et al., 2016) and, of particular interest given the lower MN predominant pattern typical of *FUS*-ALS, in axonal RNA transport (Guo et al., 2017b) and NMJ stability (Picchiarelli et al., 2019).

v) Studies directly comparing different ALS-causing genes and sALS—One caveat to hiPSC studies comparing phenotypes across genotypes is that often such studies are done independently, thus, variability from cell purity, maturation, and the type of MNs generated from the differentiation protocol, as noted later, all act as confounding variables. A study that directly compared electrophysiological properties across hiPSC-MNs derived from *SOD1A4V, FUSM511FS, FUSH517Q,* and *C9orf72* demonstrated that despite differences in firing patterns, all genotypes exhibited hyperexcitability that correlated with reduced cell survival and could be reversed with ezogabine (retigabine), a Kv7 or KCNQ voltage-gated potassium channel activator (Wainger et al., 2014). These data contrasted with studies demonstrating hypoexcitability in ALS hiPSC-MN cultures (Dafinca et al., 2020; Devlin et al., 2015). Longitudinal measurements of excitability in both *TDP43M337V* and *C9orf72* hiPSC-MNs have shown that MNs initially exhibit hyperexcitability which converts to hypoexcitability upon prolonged culture (Dafinca et al., 2020).

Pathological phenotypes and sensitivity to stressors highlight both the overlap and heterogeneity captured by hiPSC models of ALS. Mitochondrial dysfunction and oxidative stress in hiPSC-MNs seem to be shared across multiple genotypes including *SOD1A4V, C9orf72, TDP-43*and sALS (Alves et al., 2015; Dafinca et al., 2016; Egawa et al., 2012; Kiskinis et al., 2014). However, transcriptomic and proteomic comparisons of *C9orf72* and *SOD1* hiPSC-MNs revealed differentially altered pathways, also reflected in expression profiles from MNs isolated from post-mortem spinal cord (Kiskinis et al., 2014; Wong and Venkatachalam, 2019). Multiple *SOD1* mutant hiPSC-MNs have been linked with ER stress; however, *TDP-43* MNs exhibit sensitivity to phophoinositide 3-kinase but not ER stress (Bhinge et al., 2017; Bilican et al., 2012). Conversely, nucleocytoplasmic transport defects have been detected in *C9orf72* and *TDP43* patient hiPSC-MNs but not with *SOD1* mutants, although mislocalization of nuclear pore complex proteins has been reported in *SOD1* patients (Chou et al., 2018; Freibaum et al., 2015; Ortega et al., 2020; Wong and Venkatachalam, 2019; Zhang et al., 2015a). Together these data suggest that different genotypes may achieve MN degeneration via divergent mechanisms.

Studies from sALS hiPSCs particularly highlight the ability of hiPSCs to recapitulate the heterogeneous nature of ALS without known genetic origin. Burkhardt et al. ((Burkhardt et al., 2013)) showed that forebrain cortical neurons and spinal MNs derived from three of 16 sALS patient hiPSCs exhibited TDP-43 aggregate-like structures. These aggregate-like structures seemed to recapitulate the pathology in post-mortem tissue samples from one of the same patients from which the hiPSC were derived. This was important as it led to a high-content chemical screen in both forebrain cortical neurons and spinal MNs which identified FDA-approved drugs (i.e. the cardiac glycoside Digoxin) as potential inhibitors

of TDP-43 aggregate-like structures, demonstrating the feasibility of patient-derived hiPSC-based disease for drug screening. However, it is noteworthy that this is the only study that has shown TDP-43 aggregate-like structures in iPSC models and that the prototypical ALS neuropathology of TDP-43 aggregation has not been convincingly recapitulated in any other hiPSC-based study to-date. An extensive comparison of hiPSC-MNs derived from fALS patients with *SOD1, FUS,* and *TDP43* mutations as well as 32 sALS patients across multiple cellular phenotypes, including neurite length, cell death, and abnormal protein aggregation, demonstrated variability across these *in vitro* phenotypes that correlated with patient clinical heterogeneity (Fujimori et al., 2018).

In a study analyzing the transcriptome of hiPSC-MNs from patients carrying mutations in the valosin containing protein (*VCP*) gene increased intron retention (IR) was identified as a dominant feature of the splicing program during early MN differentiation, which was also observed in independent RNAseq data sets from *SOD1* and *FUS* MNs, with the most significant increase of IR in the Splicing Factor Proline and Glutamine rich (SFPQ) transcript (Luisier et al., 2018). As a result, the SFPQ protein binds extensively to its retained intron and is lost from the nuclei of *VCP, FUS* and *SOD1* MNs. This was also observed in mouse transgenic ALS models and human postmortem tissue from sALS cases, identifying nuclear loss of SFPQ as a unifying hallmark across fALS and sALS.

In a study from 2018, Shi and colleagues found that C9orf72 interacted with endosomes and was required for normal vesicle trafficking and lysosomal biogenesis in hiPSC-MNs. HRE-mediated C9orf72 haploinsufficiency caused neurodegeneration through i) accumulation of glutamate receptors and excitotoxicity and ii) impaired clearance of neurotoxic DPRs (Shi et al., 2018). In 2019, the same group found that iMNs from *C9orf72* and several sALS patients showed impaired autophagosome formation and aberrant accumulation of glutamate receptors (Shi et al., 2019). Moreover, treatment with the anticoagulation-deficient form of activated protein C (3K3A-APC) was able to rescue these defects in both *C9orf72* and sALS iMNs, decreased DPRs and restored TDP-43 localization. Of note, 3K3A-APC also decreased glutamate receptor expression and proteostasis in vivo in *C9orf72* gain- and loss-of-function mouse models. Particularly important for its clinical potential, this study also identified that the ability of 3K3A-APC to rescue ALS iMN survival was dependent on its ability to activate protease-activated receptor 1 (PAR1), which identifies PAR1 as a therapeutic target for both *C9orf72* and sALS (Shi et al., 2019).

Non-neuronal cell types

Numerous studies reported activation of astrocytes and microglia in post-mortem tissue samples from ALS patients and in murine models of ALS, implicating neuroinflammation as an important feature of ALS (Vahsen et al., 2021). Murine and human astrocytes differ in their size and complexity (Oberheim et al., 2009) and microglia exhibit important species-specific differences in receptor expression and response to stimuli (Smith and Dragunow, 2014). Thus, hiPSC models have a potentially important role to play in dissecting the interaction of these cells with MNs in ALS pathophysiology, and to identify effective therapies that target astrocytes and microglia and therefore neuroinflammation.

Astrocytes expressing mutant SOD1 killed spinal primary and embryonic mouse stemcell derived MNs through soluble factors (Nagai et al., 2007). Similarly, MNs derived from mouse ESCs carrying the mutant *SOD1*^{G93A} allele showed neurodegenerative properties when co-cultured with *SOD1*^{G93A} glial cells (Di Giorgio et al., 2007). Astrocytes isolated from mutant SOD1-overexpressing mice or from postmortem brain tissue of sALS patients displayed toxicity to mouse- and human PSC-derived MNs *in vitro* (Brites and Vaz, 2014; Haidet-Phillips et al., 2011; Marchetto et al., 2008). Interestingly, this toxicity is specific for MNs as other neuronal subtypes in the same dish remained unaffected (Di Giorgio et al., 2008). Studies on *C9orf72* hiPSC astrocytes showed that the *C9orf72* mutation leads to both cell- autonomous astrocyte pathology and non- cell autonomous effects on MN pathophysiology. *C9orf72* astrocytes co- cultured with MNs caused MNs to undergo a progressive loss of action potential output due to decreases in the magnitude of voltage-activated $Na^+$and K^+ currents. Notably, this phenotype was reversed by CRISPR/Cas- 9 mediated excision of the *C9orf72* repeat expansion (Zhao et al., 2020). Media conditioned by *C9orf72* hiPSC astrocytes increased oxidative stress in wild type MNs, suggesting this contributes to MN degeneration in *C9orf72* ALS, although it is noteworthy that this study was conducted in *C9orf72* hiPSC astrocytes and hiPSC astrocytes from non-affected donors that were not genetically matched (Birger et al., 2019).

Studies on *TARDBP* hiPSC astrocytes are scarce in comparison, but TARDBP hiPSC astrocytes have increased *TARDBP* expression, higher levels of cytoplasmic TDP-43 aggregation, and decreased cell survival compared to wild type controls, but co-culture with MNs did not induce MN cell death (Serio et al., 2013). In addition, a recent study showed that hiPSC MNs are more vulnerable to TDP-43 aggregation and toxicity compared with their astrocyte counterparts, and that astrocytes provide protection from seeded aggregation within MNs by reducing (mislocalized) cytoplasmic TDP-43, TDP-43 aggregation and cell toxicity (Smethurst et al., 2020).

To date, only one study has investigated hiPSC microglia derived from an ALS patient. *C9orf72* hiPSC-derived microglia displayed a heightened immune response as well as altered expression of endosomal marker early endosome antigen 1 and lysosomal associated membrane protein 1, which was also confirmed in patient post-mortem tissue samples (Lorenzini et al., 2020). Investigating hiPSC-derived microglia in the context of ALS is crucial, considering the growing evidence for the involvement of microglia in ALS pathophysiology. Importantly, hiPSC-derived microglia express ALS-relevant genes, including *C9orf72, SOD1,* and *TDP-43* (Haenseler et al., 2017), increasing the likelihood of identifying phenotypes in hiPSC-derived microglia from ALS patients carrying mutations in these genes. It will be of particular interest to investigate hiPSC-derived microglia in co-culture paradigms in order to further elucidate their putative toxic properties to MNs. Interestingly, co-culture studies demonstrated that astrocytes derived from *C9orf72* but not *TDP43* M337 hiPSCs reduced hiPSC-derived MN functional output and survival (Birger et al., 2019; Madill et al., 2017; Serio et al., 2013; Zhao et al., 2020). Systematic investigation of glia across different genotypes in parallel will be essential in future studies to exclude the confounding effects of technical variation.

5. Challenges, possible solutions, and emerging technologies in hiPSC research

Despite the potential of hiPSCs to model ALS *in vitro,* several challenges remain. These, together with possible solutions and emerging technologies in hiPSC research, are summarized in Figure 2.

Multicellular and 3D culture models to study non-cell autonomous effects in ALS

While simplified hiPSC-derived neuronal, astrocytic, and microglial monoculture paradigms allow the study of cell-autonomous effects of disease-causing mutations, modelling crosstalk between neurons and glia is crucial to understanding their non-cell-autonomous interaction in disease. Co-culture with hiPSC-derived neuronal cells facilitates glial maturation *in vitro,* as this supports, for example, the ramified homeostatic state of microglia (Haenseler et al., 2017) and the highly branched homeostatic state of astrocytes (Enright et al., 2020). Astrocyte/MN co-cultures from hiPSCs have already been harnessed to study ALS patient-derived cells (Smethurst et al., 2020), and protocols for hiPSC-derived MN/microglia co-culture are in development. However, even more complex *in vitro* paradigms that incorporate and model the physiological interactions between all three cell types would be ideal. A triculture model of hiPSC-derived cortical neurons, astrocytes, and microglia has been recently described in the context of Alzheimer Disease (AD) (Guttikonda et al., 2021). A similar approach to study ALS will require adaptations to ensure hiPSC-derived MNs, microglia, and astrocytes reflect the *spinal* subtypes of these cells. Interestingly, recent evidence suggests the existence of multiple different and diverse subtypes of MNs in the mouse spinal cord (Alkaslasi et al., 2021; Blum et al., 2021). It would be intriguing to analyze how the transcriptome of iPSC-derived MNs in monoculture and co-culture corresponds to individual MN subtypes seen in vivo, and whether modifications to existing protocols could lead to an enrichment of different subtypes.

In the CNS, signaling from oligodendrocytes and infiltrating peripheral immune cells, such as macrophages, T-cells and NK cells, also contribute to ALS (Vahsen et al., 2021). In the peripheral nervous system (PNS), skeletal muscle (SkMs) and Schwann cell dysfunction might also play a role in ALS disease onset and progression (Arbour et al., 2017). A ‘motor unit-on-a-chip’ system (Osaki et al., 2020) has been used to test the effect of ALS candidate compounds on MN survival in a microenvironment that recapitulates the crosstalk between MNs and SkMs through the neuromuscular junction (NMJ).

An even more ambitious approach to the investigation of a large range of cell types in 3D is to combine multiple organoids in so-called ‘assembloids’ to model aspects of cellular crosstalk that occur between different tissues or organs (Andersen et al., 2020). Although variability between multiple organoids/assembloids remains a challenge, this technology may be important in understanding more complex, connectivity-dependent mechanisms across neuropsychiatric and neurodegenerative disorders including ALS (Marton and Pasca, 2020).

Overcoming sources of variability in hiPSC production

The emergence of hiPSC-derived models of ALS has coincided with widespread adoption of high-throughput technologies for the unbiased appraisal of the genome, transcriptome, proteome, and epigenome, allowing the in-depth characterization of hiPSC-derived neurons and glia. However, such studies have also demonstrated considerable variability even for cells from the same individual, across independent iPSC inductions and subsequent differentiations.

An extensive study of undifferentiated hiPSCs by the “Human-Induced Pluripotent Stem Cells Initiative” has shown that the primary cause of variability between cell lines is due to genetic variation between individuals, which explains 5.2–26.3% of methylation, gene expression and RNA sequencing differences as well as 21.4–45.8% of differences found in protein immunostaining (Kilpinen et al., 2017). The same study also found significant effects of cell culture conditions and copy number alterations acquired during reprogramming but only minor effects of passage number and biological sex. Other studies have focused on differences between hiPSC lines and established hESC lines, and have identified differences in regional methylation patterns and regulatory gene expression, suggesting an epigenetic memory of the somatic cell of origin (Ohi et al., 2011). However, the degree of variability from such bias appears significantly smaller than the genetic background of the donor, and likely abates after passaging (Bock et al., 2011; Burrows et al., 2016; Nishino et al., 2011). Concerns about reproducibility have therefore accompanied hiPSC-derived disease models including ALS.

One approach to address donor variability has been to greatly increase the number of control and patient replicates, as has been done in the “NeuroLINCS” consortium in ALS (Keenan et al., 2018). The availability of well-characterized cell lines, through international consortia like “Answer ALS” (https://www.answerals.org/) represents an opportunity to overcome the limitations of hiPSC donor variability by analyzing data from MNs differentiated from more than 1,000 patients and control hiPSC lines. In addition to the increased cost associated with scaling up hiPSC production, the challenge of such large, often multicenter, efforts is the introduction of additional sources of variation, related to technical differences in experimental methods, operator-driven differences and sequencing batch effects, which together can obscure the biology of interest (Volpato et al., 2018). On the other hand, the design of such studies enables the identification of these biases, which may otherwise pass by unnoticed in smaller scale experimental designs, and can allow unwanted variation to be regressed out of datasets using appropriate bioinformatic and statistical methods. However, there is a risk that disease-relevant variation may be lost in this process leading to the generation of an ‘idealized dataset’ (Risso et al., 2014).

Advances in genomic engineering have significantly improved the ability to control for genetic variability between individuals by allowing the correction of disease-causing mutations in hiPSCs to generate isogenic controls. (Figure 3). Isogenic hiPSCs lines have been developed using CRISPR/Cas9 technology for several genetic mutations in ALS: *SOD1* (Bhinge et al., 2017; Bursch et al., 2019; Imamura et al., 2017; Kiskinis et al., 2014; Wang et al., 2017) *C9orf72* hexanucleotide repeat expansions (Abo-Rady et al., 2020; Bursch et al., 2019; Dafinca et al., 2020; Lopez-Gonzalez et al., 2019; Selvaraj et al.,

2018), *TARDBP* (Tann et al., 2019),and *FUS* (R521C) (Bursch et al., 2019; Guo et al., 2017a; Wang et al., 2017).

Of note, the correction of HRE in *C9orf72* resulted in three non-overlapping datasets between three laboratories (Dafinca et al., 2020). The lack of reproducibility may stem from differences in the CRISPR/Cas9 strategy design between the three experiments but may also reflect the variability of MN differentiations or even be a product of CRISPR/Cas9 off-target effects, which remain poorly characterized and difficult to detect (Zhang et al., 2015b). Furthermore, there is increasing appreciation of deleterious on-target effects after CRISPR/Cas9 editing, for instance mono-allelic deletions or loss of heterozygosity (Weisheit et al., 2020) . Improvements are continuously taking place in the development of this technology and better tools are already available (i.e. CRISPR/Cpf1, Hi-Fi Cas9) to circumvent some of the previous problems in terms of targeting accuracy and efficiency of homology-directed repair.

In the context of isogenic lines, the NIH Intramural Center for Alzheimer's and Related Dementias recently initiated the largest iPSC genome engineering project to date, termed "iPSC Neurodegenerative Disease Initiative" (iNDI) (Ramos et al., 2021). This project will generate a large series of iPSC lines by engineering a number of disease-causing mutations into deeply characterized parental lines derived from control individuals to model AD, dementia with Lewy bodies/Parkinson's disease dementia (DLB/PDD), ALS and FTD, and other adult-onset neurodegenerative disorders.

Overcoming sources of variability in hiPSC differentiation

In addition to intrinsic differences between parent lines due to genetics, the differentiation process gives rise to heterogeneous cell populations which differ in both number and composition, with significant amounts of interneurons and microglia reported in culture in some studies (Ho et al., 2021; Thiry et al., 2020). Although the identity of MNs in culture typically corresponds to hindbrain and rostral cord, the relative composition of bulk cultures may also obscure important differences in the relative maturity and subtype of cell populations. Single cell characterization (using transcriptomics and cell sorting) will become an essential technique to dissect MN biology in heterogenous monocultures, and, importantly, will enable appraisal of co-cultures and multicellular model systems, both in 2D and 3D. Sorting of hiPSC-derived cells based on their expressed markers (Haenseler et al., 2017) or exogenously introduced transgenic markers (Kiskinis et al., 2014; Shi et al., 2018), while allowing an assessment of overall subtype composition in bulk cultures, introduces stress through a necessary dissociation step, with cells exhibiting significant rate of cell death, which may bias analysis and subsequent culture.

Another purification technique, which may complement the search for cell specific outputs from hiPSC-derived monocultures and co-cultures, is translating ribosome affinity purification (TRAP), which uses tagged ribosomal proteins under cell-type-specific promoters and which has been successfully applied to capture translated RNA from cell populations of interest in mouse brains. The feasibility of this technique in hiPSC-derived cortical neurons has recently been established, although this experiment was performed using a ubiquitous promoter requiring enrichment using MACS (Rodrigues et al., 2020).

The significant variation in protocols for the differentiation of hiPSC-derived cells with critical roles in ALS argues for harmonization of the criteria used to obtain and characterize the differentiated cell types. An example of such an approach, which might be applied to other cell types relevant to ALS, has recently helped to clarify reactive astrocyte nomenclature, phenotype and markers with the goal to reduce astrocyte heterogeneity and promote the development of universal astrocyte-based biomarkers and therapies (Escartin et al., 2021).

Overcoming immaturity of hiPSC derivatives

ALS is an age-related neurodegenerative disorder. Because hiPSC derivatives more closely resemble a fetal than an adult stage, the biological landscape of these cells may reflect developmental pathways or the pre-symptomatic phase of ALS and may fail to capture ALS disease phenotypes (reviewed in (Guo et al., 2017a)). A detailed overview on promoting aging in hiPSC models of ALS can be found elsewhere (Ziff and Patani, 2019); therefore, we focus on key principles here. Electrical stimulation, including using optogenetics and chemogenetics, metabolic stimulation and multicellular and 3D culture approaches, may all promote maturation. Promoting aging through overexpression of progerin, a truncated form of lamin A associated with premature aging, has been shown to augment disease phenotypes in a model of PD (Miller et al., 2013). However, whether this approach is physiologically relevant to mimic ‘normal’ aging in an iPSC-derived model is uncertain.

Because cellular rejuvenation occurs during hiPSC reprogramming, potentially repairing the macromolecular damage that could contribute to neurodegenerative phenotypes (Studer et al., 2015), another strategy is to bypass the PSC stage by direct conversion of fibroblasts into neurons or glia (Hautbergue et al., 2017; Mertens et al., 2015; Meyer et al., 2014; Varcianna et al., 2019). Directly reprogrammed i-astrocytes (iNPC- As), for example, retain the age-related features of the donor fibroblasts and thus have the potential to capture more relevant disease phenotypes (Gatto et al., 2021). Epigenetic changes are reset during reprogramming, which may reduce the utility of hiPSC-derived cells to model responses to environmental stimuli relevant to ALS, while direct lineage reprogramming approaches more faithfully preserve epigenetic memory (Meyer et al., 2014). However, the cell fate stability of directly reprogrammed cells is unclear, and because fibroblasts have limited self-renewal, reprogrammed cells are often non expandable (Chambers and Studer, 2011).

Overcoming ALS heterogeneity with multiplexed, pooled approaches and genetic screens

The variable penetrance of some ALS-determining mutations and the complex genetic contribution to sporadic ALS, based on a combination of common variants of small effect and rarer variants of stronger effect, suggests that there remain many genetic factors to be uncovered. Multiplexed or novel pooled approaches have the potential to identify ALS-relevant mechanisms that are masked in conventional smaller scale experimental setups. One possible approach is the usage of high-content image-based assays, which have been successfully used to distinguish fibroblasts derived from 12 spinal muscular atrophy (SMA) patients and 12 healthy control individuals based on high-throughput imaging and machine learning analysis of morphological criteria (Yang et al., 2019). A complimentary technique, PRobe-based Imaging for Sequential Multiplexing (PRISM) (Tomov et al., 2021), which

permits the simultaneous imaging of 10 or more molecular targets using fluorescent DNA-conjugated antibody probes, has been applied to evaluate cell identity and population composition of iPSC-derived cortical neuron and MN cultures. The combined application of these or similar multiplexed techniques to ALS-relevant cell types has the potential to characterize disease signals and multiple contributing gene and protein targets at large-scale.

A different option is pooled technology, such as a 'village-in-a-dish' in which cells from up to 100 donors are mixed together, fed, passaged, stimulated in a shared environment and scored for phenotypes (Mitchell et al., 2020). After sorting or selecting for the phenotype of interest, the genomic DNA is sequenced in the resulting village, and computational analysis reveals the proportion of cells from each donor contributing to phenotypic variation which can then be mapped back to genomic variation. This permits hypothesis-driven testing of multiple disease phenotypes across many disease-associated mutations, allowing for the simultaneous increase in throughput in both the number of genotypes and phenotypes that can be studied and decreasing inter-line assay variability. Pooled approaches could be employed to uncover phenotypes across large sALS cohorts or to compare pathomechanisms in cells differentiated from individuals carrying ALS-causing mutations in different genes.

A novel approach to studying the role of genetic factors in ALS is to use CRISPR-interference (CRISPR-i)-based platforms for genetic screens combined with single-cell RNA-sequencing technologies, as shown by Tian and colleagues (Tian et al., 2019). CRISPR-i uses a catalytic version of Cas9 (dCas9) which has no DNA cutting activity but is fused to a transcriptional repressor domain that suppresses the expression of human genes. Tian et al. implemented this technique in hiPSCs and hiPSC-derived neurons to reveal neuron-specific genes that are essential for neurons but not for hiPSCs by single-cell RNA-sequencing. The identification of genes which selectively promote neuronal survival in hiPSC-derived neurons may give important insights into neurodegenerative diseases such as ALS.

Translation of promising drug candidates from hiPSC models into human ALS in current clinical trials

Despite the challenges that remain to be overcome, hiPSC modeling approaches have the potential to accelerate translation of drugs into clinical trials by demonstrating target engagement, notably with ASO treatment, and by providing a screening platform for ALS-relevant phenotypes. *In vitro* disease modeling using hiPSC technology has led to at least three clinical trials of drug candidates for ALS.

In *SOD1* and *C9orf72* hiPSC models, ezogabine, a potassium Kv7 channel activator approved by the US Food and Drug Administration for epilepsy, reduced neuronal excitability (Wainger et al., 2014). Based on the *in vitro* data, the investigators moved directly from hiPSC modeling into a multicenter phase 2 randomized clinical trial of 2 doses of ezogabine in patients with ALS and found that ezogabine decreased cortical and spinal MN excitability, though the study was not powered to detect an effect on disease progression (Wainger et al., 2021). Of note, ezogabine has been previously used in humans and has an

established safety profile, which made it substantially more straightforward to move directly into phase 2 studies based on the hiPSC-related work from (Wainger et al., 2014).

Imamura et al. identified src/c-Abl inhibitors as therapeutic targets in a high throughput screen assessing survival of *SOD1* hiPSC-MNs. The most promising src/c-Abl inhibitor bosutinib further rescued viability of hiPSC-MNs derived from patients with *TDP43* and *C9orf72* mutations and two of three people with sALS, suggesting a common target across fALS genotypes and between fALS and sALS. Furthermore, a 3D neuromuscular junction (NMJ) organ-on-a-chip model using hiPSC-MNs derived from an sALS patient co-cultured with muscle skeletal bundles verified that bosutinib co-treated with rapamycin rescued the reduced muscle contractions in sALS cultures (Osaki et al., 2018). Bosutinib is currently in clinical trials to test efficacy in ALS patients.

Ropinirole, a non-ergoline dopamine agonist used to treat the symptoms of PD, showed positive effects on suppression of neurite retraction and cell death in hiPSC-MNs from patients with *TARDBP* or *FUS* mutations, and notably also on cells from patients with sALS. Putative mechanisms of action of ropinirole are 1) suppression of oxidative stress; 2) inhibition of TDP-43 and FUS aggregation; 3) improvement of mitochondrial function, (Okano et al., 2020). A randomized, double-blind, placebo-controlled, single-center, and open-label continuation phase I/IIa clinical trial, was designed to explore the safety, tolerability and efficacy of ropinirole hydrochloride as an ALS treatment (Morimoto et al., 2019).

In further developing iPSCs as validation tool for disease modelling and drug discovery it will be crucial to demonstrate that drugs which successfully rescue phenotypes in iPSC-derived models predict therapeutic efficacy in patients.

Conclusion

hiPSC technology permits the generation of ALS-relevant cell types, both in the CNS and PNS, allowing for the creation of highly relevant disease models that capture the specific genetic signature of a given patient. Using hiPSC -MNs, a variety of cell-autonomous mechanisms have been elucidated, which are thought to be shared between fALS and sALS, such as the dysfunction of nucleocytoplasmic transport, stress granule processing, and autophagy. hiPSC -derived astrocytes from ALS patients have been used to investigate the role of these cells in ALS pathophysiology through their effect on MNs. Differentiation protocols for hiPSC-derived microglia have also recently become available, but research on their role in ALS is still emerging. More complex, multicellular and 3D cultures systems can be used to investigate cell-cell interactions *in vitro* that were not previously feasible in monotypic cell configurations. Protocols to differentiate hiPSCs into other cell types potentially contributing to the disease pathogenesis such as skeletal muscle, or cells that are relatively resistant to degeneration in ALS, such as oculomotor neurons, will additionally increase the battery of tools to investigate ALS.

Several technical caveats of hiPSC-derived models remain to be resolved. The variety of differentiation protocols in use for the different ALS relevant cell types, with resultant

inconsistency between the identity of differentiated cells as well as the results obtained by different laboratories, may be overcome by the use of isogenic controls and enrichment of pure cell fractions. Additional important aspects of hiPSC biology require investigation to clarify their utility for the study of an age-related neurodegenerative disease like ALS. The relative immaturity of hiPSC-derived cells may require more sophisticated approaches to *in vitro* maturation to reveal how the cellular tolerance to genetic mutations present from conception breaks down with aging.

While many important questions remain, clinical trials based on hiPSC-disease modeling studies are now emerging. The ability of hiPSC models to reflect unique aspects of disease can also be expected to contribute to more informed patient stratification in trials, and to the identification of which cell type is most likely to be the target of treatments.

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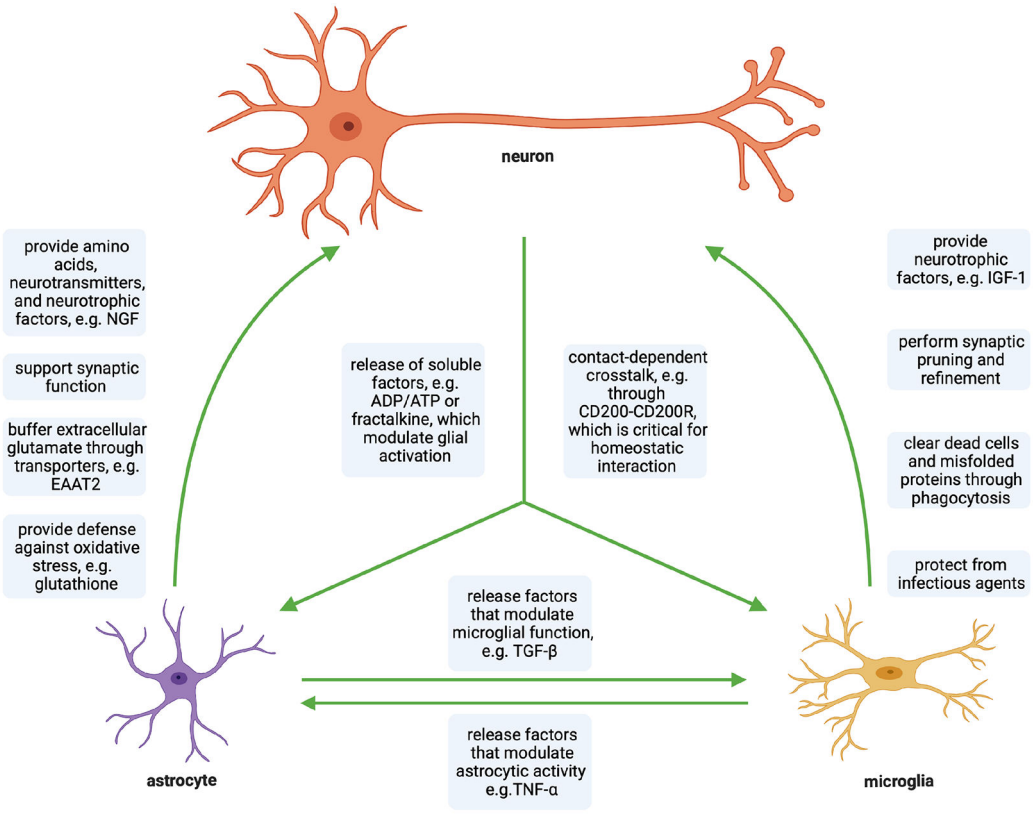

Figure 1. Physiological interactions between neurons, microglia and astrocytes in the brain and spinal cord:

Neuronal and glial cells interact through various contact-dependent and -independent mechanisms under homeostatic conditions.

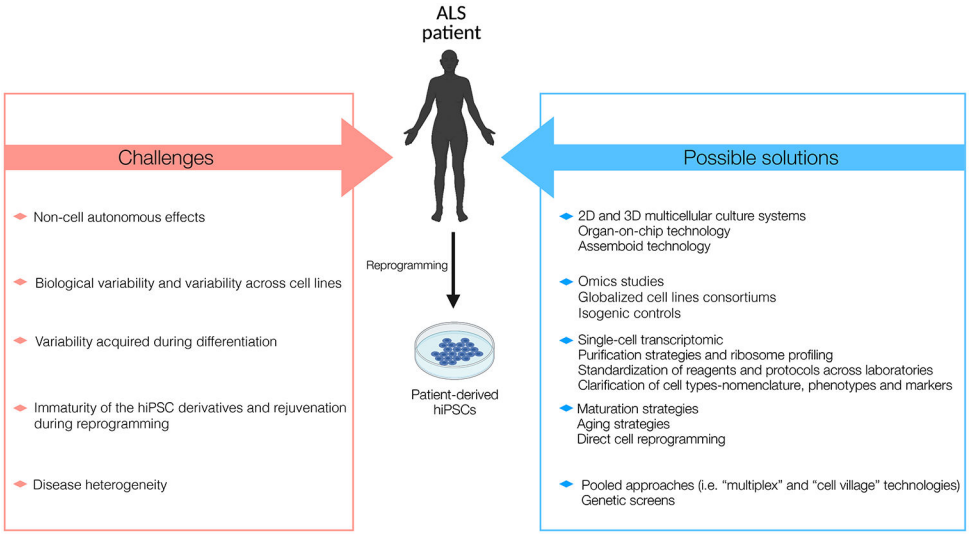

Figure 2. Challenges and possible solutions in modeling ALS using hiPSC technology.

Several challenges, such as weak technical reproducibility and lack of modelling inter-cellular crosstalk, remain to be resolved to improve disease modelling with hiPSC models. Amongst others, possible solutions include cell purification strategies and the development of more complex, multicellular and three-dimensional culture systems.

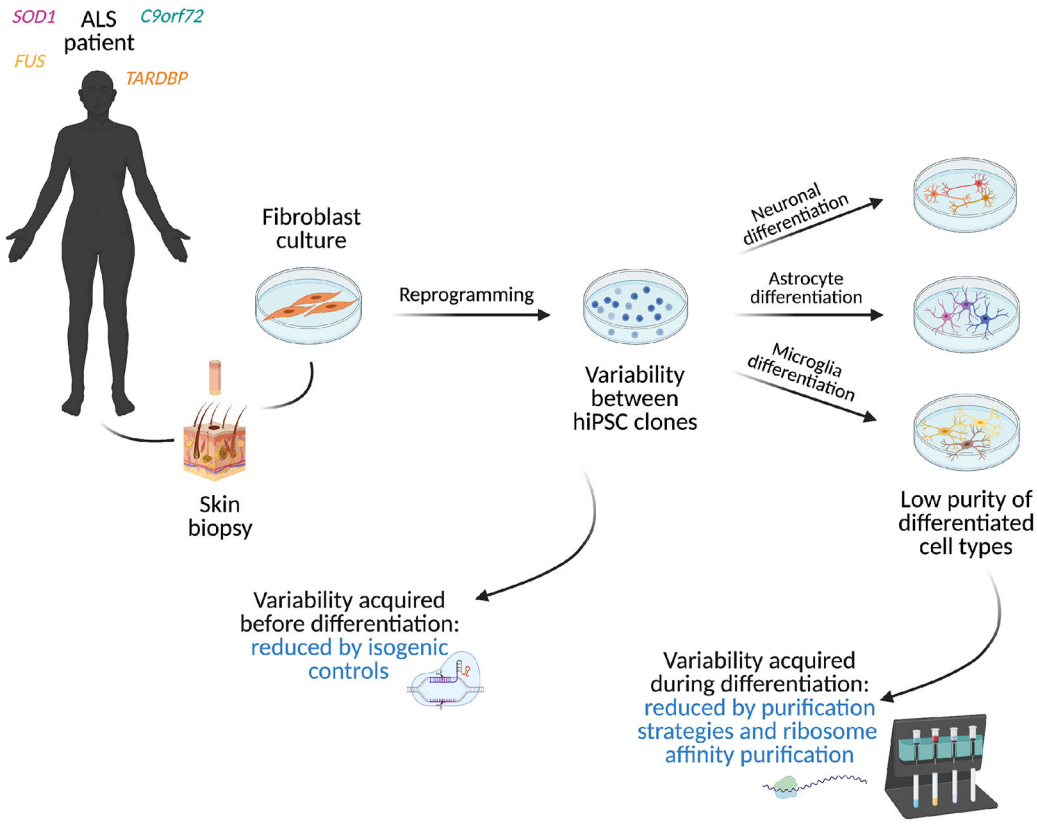

Figure 3. Overcoming biological variability through isogenic controls and cell purification strategies.

hiPSCs are typically generated by reprogramming skin biopsy-derived fibroblasts into pluripotency. Reprogramming-induced variability between different hiPSC clones can be reduced by generating isogenic control lines, where the mutant allele is corrected through gene-editing. Variability acquired during differentiation can be reduced by cell purification and ribosome affinity purification strategies.

Table 1:

Spinal MN differentiation protocols used in ALS studies

Reference	Induction compounds							Protocol length (days)	Culture Purity (%)		
	SMAD inhibition				Patterning						
	Chir	LDN	SB	Compound C	RA	SHH	SAG		HB9	Tuj1	ChAT
(Dimos et al., 2008)					✓	✓		45	20	-	-
(Hu and Zhang, 2009)					✓	✓		56	>40	30-40	-
(Karumbayaram et al., 2009)					✓	✓		90-95	-	-	-
(Patani et al., 2011)			✓					55	-	-	-
(Amoroso et al., 2013)		✓	✓		✓	✓		35	-	-	-
(Sareen et al., 2013)					✓			75	-	-	-
(Maury et al., 2015)	✓	✓	✓		✓		✓	35	-	-	-
(Qu et al., 2014)				✓	✓			30	-	70	-
(Kiskinis et al., 2014)			✓	✓	✓		✓	55	-	-	-
(Devlin et al., 2015)				✓	✓		✓	130	44	81	-
(Du et al., 2015)	✓		✓		✓			28	-	-	-
(Calder et al., 2015)		✓	✓		✓			20	40	-	-
(Bursch et al., 2019)	✓		✓	✓	✓		✓	>42	-	89	27
(Dafinca et al., 2020)	✓			✓	✓		✓	30	-	90	90
(Ababneh et al., 2020)	✓			✓	✓		✓	30	35	80	80
(Mehta et al., 2021)	✓	✓	✓		✓		✓	35	-	-	-

Table 2:

Cortical neuron differentiation protocols used in *C9orf72*-ALS/FTD studies

Reference	Induction compounds	Expansion phase	Final maturation factors	Protocol length (days)
(Shi et al., 2012) Used later in (Dafinca et al., 2016)	Dorsomorphin (10 uM) SB (10 uM) N2, B27	Neurospheres	-	80
(Almeida et al., 2013) Used later in (Lopez-Gonzalez et al., 2016; Lopez-Gonzalez et al., 2019; Maor-Nof et al., 2021)	bFGF, N2, B27	Neurospheres	BDNF, GDNF	72
(Bilican et al., 2012) (Livesey et al., 2014) Used later in (Selvaraj et al., 2018)	N2, B27, Forskolin (10 μM)	Monolayer	BDNF, GDNF	56

Table 3.
Existing astrocyte differentiation protocols from hiPSCs.

Protocols used in non-ALS studies are indicated in black, while protocols in ALS studies are indicated in blue.

Study	Critical factors for terminal differentiation	Length	Presence of serum in terminal differentiation media
(Hu et al., 2010)	cAMP (1 uM)	> 3 months	No
(Krencik and Zhang, 2011)	CNTF (10 ng/ml)	> 3 months	No
(Emdad et al., 2012)	CNTF (20 ng/ml)	~ 5 weeks	No
(Juopperi et al., 2012)	Chemically not defined commercial media (ScienCell)	> 3 months	Yes
(Lafaille et al., 2012)	5% FBS	2–3 months	Yes
(Serio et al., 2013) (Birger et al., 2019) (Zhao et al., 2020)	CNTF (10 ug/ml)	~ 2 months	No
(Shaltouki et al., 2013)	CNTF (5ng/ml) BMP (10ng/ml) FGF2 (8ng/ml) 1% FBS Activin A (10ng/ml) Heregulin1b (10ng/ml) IGFI analog (200ng/ml)	~ 5 weeks from the NSC stage	Yes
(Roybon et al., 2013)	hrFGF1 (0.1 to 100ng/ml, then 50ng/ml) hrFGF2 (0.1 to 100ng/ml, then 50ng/ml)	> 80 days	Yes
(Sareen et al., 2014)	EGF (100ng/ml) FGF2 (100ng/ml) CNTF (−)	> 2 months	No
(Mormone et al., 2014)	CNTF (20 ng/ml)	28-35 days	No
(Meyer et al., 2014) (Hautbergue et al., 2017) (Varcianna et al., 2019)	10% FBS	> 7 days from induced NPCs (iNPCs)	Yes
(Holmqvist et al., 2015)	FGF2 (20 ng/ml) EGF (100 ng/ml)	~ 45 days	No
(Pasca et al., 2015; Sloan et al., 2017)	-	From day 100	No
(Du et al., 2015) (Madill et al., 2017) (NB: Based on (Krencik and Zhang, 2011))	10% FBS	> 3 months	Yes
(Zhou et al., 2016)	Ascorbic Acid (0.2mM)	> 28 days	No
(Yasui et al., 2017)	LIF (1/1.000, Wako) 1% and 5% FBS	~ 28 days from hNPCs	Yes
(Tew et al., 2017) and (Soubannier et al., 2020)	Chemically not defined commercial media (ScienCell)	~ 30 days from hNPCs	Yes
(Hall et al., 2017) (Smethurst et al.,2020)	BMP4 (10 ng/ml) LIF (10 ng/ml)	~ 3 months	No
(Perriot et al., 2018)	CNTF (20 ng/ml)	2–3 months	No
(Canals et al., 2018)	dbcAMP (500 mg/ml) heparin-binding EGF-like growth factor (5 ng/ml) BMP4 (10 ng/ml) CNTF (10 ng/ml)	< 28 days	No
(Lundin et al., 2018)	FGF2 (8 ng/mL) heregulin 1β (10 ng/mL)	> 28 days	No

Study	Critical factors for terminal differentiation	Length	Presence of serum in terminal differentiation media
	IGF1 (200 ng/mL) ActivinA (10 ng/mL)		
(Li et al., 2018)	BMP4 (10 ng/mL) CNTF (10 ng/mL)	4-7 weeks	No
(Rosati et al., 2018)	2% FBS	> 5 weeks from neurospheres	Yes
(Tchieu et al., 2019)	HB-EGF (10ng/ml) CNTF (10 ng/mL) LIF: 10ng/ml	> 4 weeks	No
(Bradley et al., 2019)	BMP4 (10 ng/ml) CNTF (10 ng/ml	~ 3 months	No
(Raman et al., 2020)	BMP4 (10 ng/mL) heregulin-β (10 ng/mL) CNTF (10 ng/mL)	> 50 days	No
(Barbar et al., 2020)	T3 (60ng/mL) cAMP (1 μM) Ascorbic Acid (20 μg/mL)	> 2 months	No
(Leventoux et al., 2020)	BDNF (10 ng/ml) GDNF (10 ng/ml)	From day 48	No
(Peteri et al., 2021)	CNTF (20 ng/mL)	> 75 days	No

Table 4.
Existing microglia differentiation protocols from hiPSCs.

Protocols used in non-ALS studies are indicated in black. One protocol used in ALS studies is indicated in blue.

Study	Critical factors for terminal differentiation	Length	Compatibility with other cell types demonstrated?	Presence of serum in terminal media
(Muffat et al., 2016)	M-CSF (5 ng/mL) IL-34 (100 ng/mL)	~74 d	hiPSC-derived neuroglial cultures (2D and 3D)	no
(Haenseler et al., 2017)	IL-34 (100 ng/mL) *optional* GM-CSF (10 ng/mL)	~45 d	hiPSC-derived cortical neurons	no
(Abud et al., 2017)	M-CSF (25 ng/mL) IL-34 (100 ng/mL) TGFβ-1 (50 ng/mL) CD200 (100 ng/mL) CX3CL1 (100 ng/mL)	~38 d	rat hippocampal neurons, hiPSC-derived brain organoids (3D); transplantation into the cortex of MITRG mice and the hippocampi of AD mice	no
(Douvaras et al., 2017)	IL-34 (100 ng/mL) GM-CSF (10 ng/mL)	~45-60 d	no	no
(Pandya et al., 2017)	dependent on co-culture with human astrocytes IL-3 (20 ng/mL) GM-CSF (20 ng/mL) M-CSF (20 ng/mL) FBS (10%)	~29 d	only for murine iPSC-derived microglia differentiated using this protocol	yes
(Takata et al., 2017)	M-CSF (50 ng/mL)	~46 d	co-culture with hiPSC-derived neurons	no
(Amos et al., 2017)	ScienCell Research Microglia Medium (undefined) mixed 1:1 with media containing M-CSF (10 ng/mL) GM-CSF (10 ng/mL) IL-34 (10 ng/mL) TGFβ-1 (2 ng/mL) FBS (1%)	~40 d	no	yes
(McQuade et al., 2018) (NB: simplified protocol based on (Abud et al., 2017); used in (Lorenzini et al., 2020))	M-CSF (25 ng/mL) IL-34 (100 ng/mL) TGFβ-1 (50 ng/mL) CD200 (100 ng/mL) CX3CL1 (100 ng/mL)	~38 d	transplantation into the cortex and hippocampi of MITRG mice	no
(Brownjohn et al., 2018) (NB: based on (Haenseler et al., 2017))	IL-34 (100 ng/mL) GM-CSF (10 ng/mL) FBS (10%)	~24-39 d	hiPSC-derived brain organoids (3D)	yes
(Konttinen et al., 2019)	M-CSF (10 or 5 ng/mL) IL-34 (10 or 100 ng/mL) FBS (10%)	~24-42 d	hiPSC-derived neurons (3D) and cerebral brain organoids	yes
(Xu et al., 2019) (NB: based on (Abud et al., 2017))	ScienCell Research Microglia Medium (undefined) M-CSF (25 ng/mL) GM-CSF (25 ng/mL) IL-34 (50 ng/mL) TGF~-1 (50 ng/mL) IGF-1 (25 ng/mL)	~37 d	no	yes
(Guttikonda et al., 2021)	IL-34 (100 ng/mL) M-CSF (20 ng/mL) FBS (10%)	~24 d	tri-culture with hiPSC-derived astrocytes and cortical neurons	yes
(Reich et al., 2020) (NB: based on (Haenseler et al., 2017))	IL-34 (100 ng/mL) M-CSF (25 ng/mL) TGFβ-1 (50 ng/mL)	~28-32 d	hiPSC-derived neurons	no

Table 5.

ALS models for *C9orf72, SOD1, TARDBP* and *FUS* mutations using hiPSC technology

Mutation	Cell types	Purpose and application	Key findings/take home messages	Reference
SOD1	MNs	Defining phenotypes directly linked to *SOD1A4V* mutation	• *SOD1A4V* hiPSC-MNs exhibit decreased cell survival that is rescued by gene correction • *SOD1A4V* MNs show mitochondrial dysfunction and evidence of oxidative and ER stress	(Kiskinis et al., 2014)
SOD1	MNs	Studying role of protein inclusions	• *SOD1* hiPSC-MNs display neurofilament aggregation followed by neurite degeneration in the absence of glia • Mutant *SOD1* binds to 3'UTR of NF-L mRNA and changes proportion of NF subunitsn	(Chen et al., 2014)
SOD1; C9orf72; FUS	MNs	Electrophysiological properties of MNs	• Hyperexcitabilty of ALS hiPSC-MNs	(Wainger et al., 2014)
SOD1; FUS	MNs	Determining whether divergent causal mutations exhibit common dysfunctional pathways	• *SOD1* induces degeneration via ERK and JNK signaling in hiPSC-MNs, which is reverted by gene correction • *FUS* hiPSC-MNs exhibit activated p38 and ERK indicating overlapping pathway	(Bhinge et al., 2017)
SOD1; TARDBP; C9orf72	MNs	Screen to repurpose existing drugs for ALS	• Src/c-Abl inhibition increased survival of ALS MNs • One inhibitor (Bosutinib) increased autophagy and decreased misfolded *SOD1* load	(Imamura et al., 2017)
C9orf72	MNs	Studying mechanisms of C9 HRE pathology	• *C9orf72* hiPSC-MNs exhibit RNA foci, differential expression of genes-related to membrane excitability, and decreased firing capacity that were reversed by antisense oligonucleotides targeting *C9orf72*	(Sareen et al., 2013)
C9orf72 (FTD)	Neurons	Understanding C9orf72 pathology in FTD	• *C9orf72* hiPSC-neurons display RNA foci and RAN protein expression as well as sensitivity towards autophagy inhibition	(Almeida et al., 2013)
C9orf72	MNs	Studying the toxic gain-of-function role of *C9orf72* in ALS	• *C9orf72* hiPSC MNs show RNA foci and repeat RNA sequesters ADARB2 • *C9orf72* hiPSC-MNs have increased susceptibility to excitotoxic stress	(Donnelly et al., 2013)
C9orf72	Neurons	Investigating HRE RNA-induced toxicity	• RanGAP1 interacts with HRE RNA and suppresses neurodegeneration • *C9orf72* hiPSC neurons show RanGAP1 mislocalization and impaired nuclear import	(Zhang et al., 2015a)
C9orf72	Neurons	Studying RAN mediated toxicity	• *C9orf72* hiPSC neurons display dysfunctional RNA export out of nucleus	(Freibaum et al., 2015)

Mutation	Cell types	Purpose and application	Key findings/take home messages	Reference
C9orf72; TARDBP	MNs	Characterization of electrophysiological properties of ALS MNs	• Comparison of electrophysiological properties of hiPSC MNs shows a transition from initial hyperexcitability to hypoexcitability after prolonged culture	(Devlin et al., 2015)
C9orf72	Cortical neurons, *MNs*	Phenotypic characterization of cortical and spinal MNs	• Both *C9orf72* hiPSC cortical neurons and MNs exhibit stress granule formation and abnormal protein aggregation • Decreased survival of *C9orf72* hiPSC-MNs is associated with Ca^{2+} dysregulation and ER stress	(Dafinca et al., 2016)
C9orf72	MNs; Neurons	Understanding C9ORF72 HRE-mediated toxicity	• *C9orf72* hiPSC-MNs and hiPSC neurons ectopically expressing dipeptide repeat (DPR) protein (GR)80 have increased oxidative stress and DNA damage • GR(80) preferentially binds to mitochondrial ribosomal proteins and induces mitochondrial dysfunction	(Lopez-Gonzalez et al., 2016)
C9orf72; sALS	Astrocytes, MNs	Investigating mechanisms of impaired proteostasis in MNs	• ALS hiPSC astrocytes impair autophagy in MNs via soluble factors	(Madill et al., 2017)
C9orf72	Astrocytes, MNs	Role of astrocytes in humanized system	• hiPSC *C9orf72* astrocytes show RNA foci and poly-GP DPRs • Co-culture with MNs reduces MN functional output	(Zhao et al., 2020)
C9orf72	Astrocytes, MNs	Studying astrocyte-mediated toxicity	• *C9orf72* hiPSCs display toxicity to MNs via soluble factor • Astrocyte conditioned media induces oxidative stress in MNs	(Birger et al., 2019)
C9orf72	MNs	Investigating proteins with altered nucleocytoplasmic distribution in C9ORF72 HRE-ALS	• *C9orf72* hiPSC-MNs show redistribution of eRF1 in nuclear invaginations • *C9orf72* hiPSC-MNs show reduced translation and hyperactive nonsense-mediated decay	(Ortega et al., 2020)
C9orf72; SOD1; sALS	MNs	Cross comparison of multiOMIC datasets in hiPSC-MNs and patient tissues	• Transcriptomic and proteomic signatures of *C9orf72* hiPSC-MNs corroborated in MNs from *C9orf72* and sALS patients • *SOD1* hiPSC-MNs have different transcriptomic signature	(Wong and Venkatachalam, 2019)
TARDBP	MNs, neurons	Investigating cell-autonomous mechanisms of *TARDBP* pathology	• *TARDBP* hiPSC-MNs show increased soluble and detergent-resistant TDP-43 and selective degeneration over time that can be reversed by PI3K inhibition	(Bilican et al., 2012)
TARDBP	MNs	Developing platform for disease modeling and screening	• *TARDBP* hiPSC-MNs display cytosolic TDP-43 aggregates and fewer neurites • TDP-43 in detergent insoluble fraction interacts with SNRPB2 • Histone acetyltransferase inhibitor anacardic acid reversed the phenotype	(Egawa et al., 2012)

Mutation	Cell types	Purpose and application	Key findings/take home messages	Reference
TARDBP	astrocytes, *MNs*	Investigating non-cell autonomous contribution to *TARDBP* ALS	• *TARDBP* hiPSC-astrocytes have increased *TARDBP* expression and TDP-43 aggregation and decreased cell survival • Co-culture with MNs does not induce MN cell death	(Serio et al., 2013)
TARDBP	MNs	Investigate composition and role of TDP-43 aggregates in ALS	• TDP-43 aggregates sequester nuclear pore proteins and affect nucleocytoplasmic transport	(Chou et al., 2018)
TARDBP	MNs	Studying mechanisms of *TARDBP* pathology	• *TARDBP* hiPSC-MNs display neurofilament abnormalities along with mitochondrial and lysosomal dysfunction and neuron loss independent of TDP-43 mislocalization or aggregation	(Kreiter et al., 2018)
TARDBP	MNs	Comparing calcium dysregulation in C9orf72 and TDP-43 iPS-derived MNs	• Glutamate stimulation leads to high recovery times of normal cytosolic calcium levels in TDP-43 MNs • Calcium-permeable receptors are upregulated in TDP-43 MNs • Reduced mitochondrial calcium uptake is observed in TDP-43 MNs • TDP-43 MNs have low ER calcium release and high levels of Bcl-2	(Dafinca et al., 2020)
TARDBP	MNs	Gaining insights into the role of SGs in pathophysiology	• 20 planar compounds decreased stress granule formation by preventing TDP-43 from entering the stress granules • Generated MNs from *TARDBP* ALS patients and control family members. • After stimulating MNs with toxins, stress granule formation increased and TDP-43 entered the stress granules • TDP-43 formed more permanent clumps in *TARDBP* MNs compared to controls. • Planar compounds reduced the formation of TDP-43 permanent clumps and increased *TARDBP* MN survival	(Fang et al., 2019)
FUS	MNs	Studying FUS-induced pathology	• Compared mild and severe *FUS* mutations at baseline or under stress conditions • Under stress conditions, *FUS* hiPSC-MNs show FUS mislocalization to cytoplasm and larger FUS^+ stress granules • At baseline, *FUS* hiPSC-MNs show DNA damage • Degree of FUS mislocalization correlated with disease onset	(Higelin et al., 2016)
FUS	MNs	Mechanisms of *FUS* pathology	• Mutant *FUS* hiPSC-MNs exhibit increased FUS mislocalization to cytoplasm and in stress granules under stress conditions compared to isogenic counterparts • MN precursor cells display differential splicing and gene expression	(Ichiyanagi et al., 2016)

Mutation	Cell types	Purpose and application	Key findings/take home messages	Reference
FUS	MNs	Mechanisms of *FUS* pathology	• Mutant *FUS* hiPSC-MNs show FUS mislocalization, hypoexcitability, and axonal transport deficits • Treatment of *FUS* hiPSC-MNs with HDAC6 inhibitors and ASOs rescues axonal transport deficits	(Guo et al., 2017b)
FUS	MNs; Neurons	Investigating the role of stress granules in ALS	• Generated wild type- and P525L-*FUS*-GFP isogenic hiPSC lines • P525L mutation alters dynamics of *FUS*-GFP incorporation in stress granules • Screen reveals inhibition of PI3K/AKT/mTOR promotes autophagy and reduces P525L-FUS recruitment to stress granules	(Marrone et al., 2018)
FUS	hiPSCs; MNs	Understanding nuclear-cytoplasmic transport defects due to FUS mutations	• In hiPSC-MNs, FUS mutations in the nuclear localization sequence cause dysfunction of poly(ADP-ribose) polymerase (PARP)-dependent DNA damage response (DDR) signaling This causes FUS mislocalization and subsequent FUS aggregation and cell death	(Naumann et al., 2018)
FUS	MNs	Understanding the effect of *FUS* mutations on neuromuscular junction	• Co-cultures of FUS patient-derived hiPSC-MNs and myotubes show endplate maturation defects	(Picchiarelli et al., 2019)
sALS	MNs	Modeling sALS	• sALS hiPSC MNs exhibit TDP-43 aggregation • Identified Digoxin modulates TDP-43 aggregation in high content chemical screen	(Burkhardt et al., 2013)
sALS	astrocytes, MNs	Investigating astrocyte-mediated toxicity to MNs in human system	• sALS patient postmortem-derived astrocytes induce death of hiPSC-MNs via necroptosis	(Re et al., 2014)
sALS	MNs	Studying non-genetic forms of ALS	• sALS hiPSC-MNs gene expression signature reveals mitochondrial dysfunction and degeneration	(Alves et al., 2015)
sALS	MN; SkM	Developing platform for NMJ studies	• Developed 3D skeletal muscle/MN "ALS-on-a-chip technology" • ALS motor unit showed fewer SkM contractions, MN degeneration, and SkM apoptosis	(Osaki et al., 2018)
sALS; FUS; *TARDBP; SOD1*	MNs	Develop multi-phenotypic screen to cluster heterogeneous sALS lines	• Stratified sALS hiPSC-MNs Identified ropinirole as therapeutic target for non-*SOD1* ALS	(Fujimori et al., 2018)