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Modeling amyotrophic lateral sclerosis (ALS) *in vitro*: from mechanistic studies to translatable drug discovery

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Amyotrophic lateral sclerosis is a rapidly progressing, fatal neurodegenerative disease that causes selective degeneration of the corticomotor system. Currently, ALS remains incurable, and the available treatment options offer little in the way of extending life or improving quality of life. This is due, at least in part, to a lack of representative disease models. *In vitro* modeling offers rapid, experimentally accessible platforms for mechanistic discovery research and drug screening, but modeling the complexity of ALS – a multicellular, multisystem disease – in a dish, is not without its challenges. Here, we review the current landscape of *in vitro* pre-clinical ALS research, with emphasis on the development of compartmentalised culture and the promise this holds for translatable modeling of ALS.

1 Introduction

Motor neuron diseases cause degeneration of the corticomotor system, which consists of the motor cortex in the brain, the brain stem, the ventral horn of the spinal cord and the muscles, together facilitating controlled, complex body movements (Fig. 1). The most common form of motor neuron disease is amyotrophic lateral sclerosis (ALS). There is significant heterogeneity in disease onset, disease progression and responses to treatment in ALS, but symptom progression generally entails a relentless loss of the ability to move, eat, speak, and, ultimately, breathe. On average, patients have a life expectancy of 2–5 years, with a median survival time of 2.5 years after symptom onset.[1,2] Research into ALS aetiology has revealed over 50 causative genetic mutations that can promote a familial form of ALS (fALS). Pathogenic variants in the superoxide dismutase (*SOD1*), fused in sarcoma (*FUS*), TAR DNA-binding protein (*TARDBP*), and chromosome 9 open reading frame 72 (*C9orf72*) genes are the most common cause of fALS, accounting for roughly 70% of fALS cases.[3] However, 90% of all ALS cases arise with no known familial association and are thus currently referred to as sporadic ALS (sALS).[4] It is not yet known what causes sALS, but it is likely that interactions between genetic polymorphisms and environmental and lifestyle factors, including ageing-related factors, contribute.[5,6]

ALS is characterised by involvement of upper motor neurons (UMNs) in the brain, lower motor neurons (LMNs) in the spinal cord, and skeletal and smooth muscle in the periphery (Fig. 1). We still do not understand where the disease begins, how it spreads, or why it selectively attacks UMNs and LMNs. Key pathological processes include circuit dysfunction such as synaptic loss, changes in excitability, and neuroinflammatory processes, as well as cell-centric disturbances such as mitochondrial dysfunction, protein aggregation, impaired RNA processing, and defective autophagy.[7–12] However, it remains unclear which of these is a primary driver *versus* a compensatory mechanism. The involvement of the corticomotor system in its entirety renders a uniquely complex setting for understanding neurodegeneration in ALS. Indeed, dysfunction in this disease can spread through the central nervous system and into the periphery, or *vice versa*, affecting cells in discrete biological compartments.[13] For example, the central nervous system operates in cerebrospinal fluid (CSF), a tightly regulated, immune-privileged environment that is kept separate from systemic circulation by the blood brain barrier. In comparison, muscle tissue resides in an immunologically active environment directly exposed to systemic circulation. In addition, the regenerative capacity of muscle tissue exceeds that of nervous tissue, meaning that it is likely that mechanisms of dysfunction may vary between environments and cell types. Understanding where disease starts, how it spreads and how to treat it in such a complex setting will require accurate preclinical models which best replicate this complexity.[14]

The preclinical landscape has recently changed, with the Food and Drug Administration (FDA) modernisation act 2.0 passing the U.S. senate in late 2022, permitting the FDA to accept *in vitro* evidence as sufficient for progression of novel therapeutics to clinical trials.[15,16] More recently, the NIH made a

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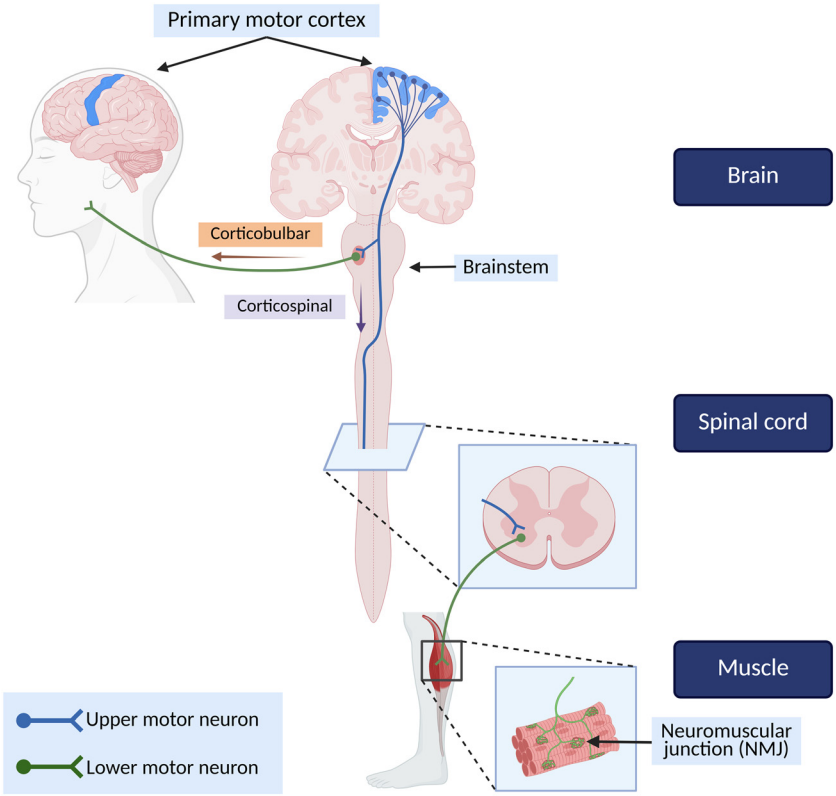

Fig. 1 Schematic of the corticomotor system. The corticomotor system consists of the UMNs (blue) in the brain, the LMNs (green) in the brainstem and spinal cord, and the muscle. Figure created with BioRender.

further decision that grant proposals solely using animal models would no longer be funded.[17] These changes emphasise the value of *in vitro* modeling and create an incentive to further advance our *in vitro* tools and technology in the race against ALS. For a model to be valid for ALS research, be that mechanistic or for drug testing, it must replicate the disease as closely as possible, utilizing cells that are suited to the research question. This review will cover the considerations, regarding cell type and environment, that are necessary to create a model of ALS in a dish. Further, we will reflect on the limitations that persist and the highlights of research breakthroughs that have already come from *in vitro* models.

2 Sourcing selectively vulnerable cell types for modeling ALS

Selective vulnerability of specific neuronal cell types is observed across many neurodegenerative conditions.[18] ALS is characterized by the progressive degeneration of both upper and lower motor neurons, making these the primary cell types of interest for *in vitro* modeling of the disease (Fig. 1). Both these cell types possess structural and functional characteristics that are integral to their identity and likely play a significant role in their selective vulnerability in ALS. UMNs are glutamatergic, multipolar neurons characterised by a large pyramidal cell body that resides in layer V of the motor cortex. They have an apical dendrite extending to higher cortical layers with many branching basolateral dendrites, each covered in dendritic spines, and an axon projecting to the brain stem or spinal cord which synapses onto LMNs, either directly or *via* a spinal interneuron. LMNs are cholinergic multipolar neurons characterised by a large cell body residing in the ventral horn of the spinal cord and a long axon projecting into the periphery to synapse onto smooth or skeletal muscle *via* the highly specialised neuromuscular junction (NMJ). It is becoming apparent that upper and lower motor neurons experience unique types of dysfunctions in ALS and may even be differentially vulnerable to disease mechanisms.[19–21] For example, LMNs with nuclear depletion of the RNA/DNA-binding protein TDP-43 exhibit cytoplasmic aggregates of phosphorylated TDP-43, whereas UMN nuclear loss of TDP-43 does not coincide with any cytoplasmic aggregate formation in human tissue.[19] As such, it is important that *in vitro* models of ALS involving upper and lower motor neurons recapitulate key features of their identity to serve as a useful platform for screening new therapies or understanding disease mechanisms.

2.1 Primary neurons

ALS has been studied *in vitro* using primary cells derived from animal models, such as rats or mice (Fig. 2). While primary cells can be derived from human tissues too, this is impracticable for neuronal tissues. Therefore, the term 'primary neurons' hereafter refers specifically to those obtained from rodent sources. Primary neurons undergo substantial differentiation *in vivo* before becoming post-mitotic, and therefore carry signatures of their unique identities into the dish. LMNs can be

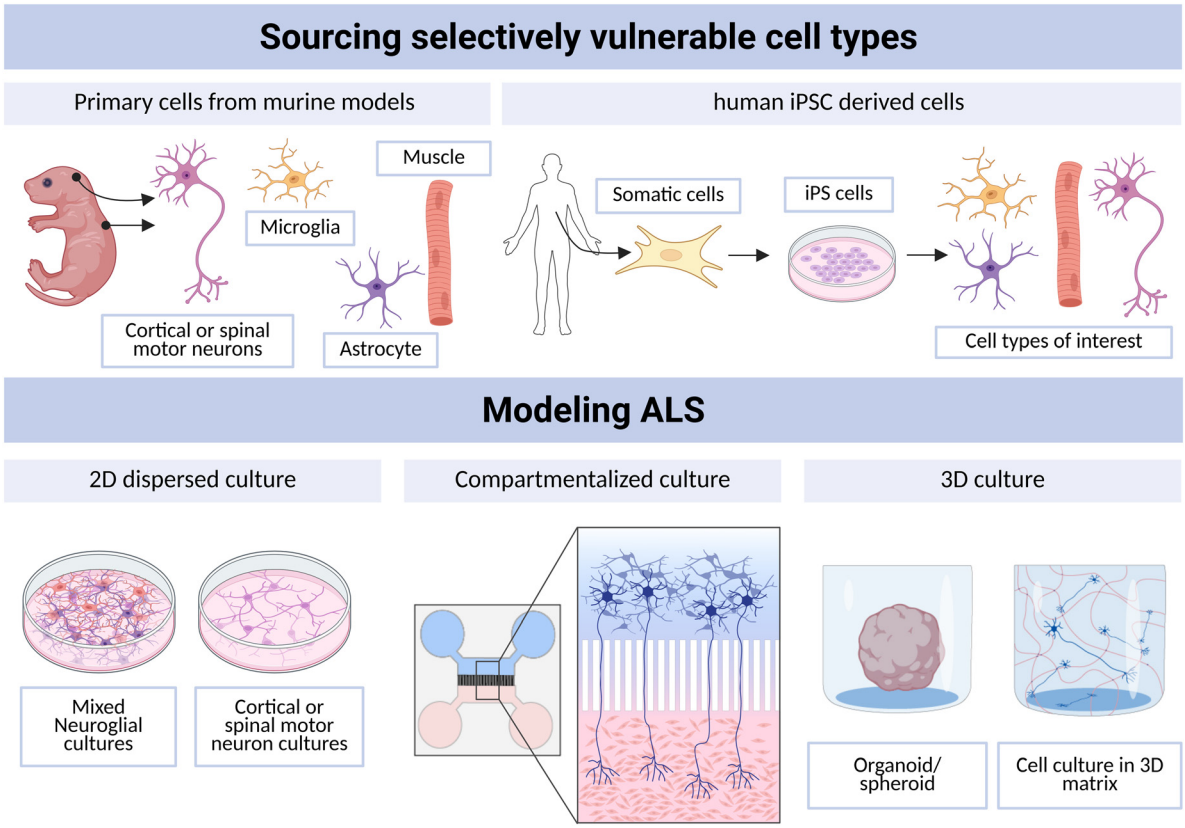

Fig. 2 Current approaches to modeling ALS *in vitro*. Relevant cell types, such as cortical and spinal cord neurons and glia, can be sourced from murine embryos (primary cell culture) or differentiated from iPSCs. To replicate disease, iPSCs can be sourced from ALS patients or otherwise genetically edited to generate cell lines with ALS-associated pathological variants. Cells can then be cultured as a monolayer in a dispersed manner or in a compartmentalised culture device. Cells can also be seeded into 3D culture platforms, such as scaffolded cultures or non-scaffolded (organoid). Figure created with Biorender and Adobe illustrator.

partially isolated from dissociated spinal cord tissue *via* centrifugation due to their large size in comparison to the other cells in the dissociated tissue, enabling production of cultures that are 90% pure for LMNs, as identified by expression of choline acetyl transferase (ChAT) and Islet1 (ISL1).[22–24]

To drive ALS-associated pathological phenotypes in primary neurons, they can be sourced from transgenic animals, transfected with plasmids harbouring pathological genes, or treated with substances that drive ALS-associated pathologies. Primary culture models have been used to investigate the role of TDP-43 pathology in neurons.[25,26] For example, Pisciottani *et al.* generated a model of TDP-43 proteinopathy in mouse cortical neurons by transfecting them with TDP-43 constructs. They found that transfected neurons exhibited characteristics such as cytoplasmic aggregation of TDP-43 (colocalised with stress granules), decreased axonal protein synthesis, oxidative stress, decreased spontaneous bursting activity, decreased synaptic vesical formation and decreased intracellular calcium levels.[27] Importantly, these characteristics reflect those observed in animal models, human cell culture models and human postmortem tissue, suggesting that primary cell culture models can exhibit disease-relevant phenotypes.

A significant limitation of primary culture is the presence of species differences between rodents and humans that may impact the physiological relevance of these models to human disease. In addition, while LMNs can be partially isolated from spinal cord suspensions, some studies have identified that the survival of LMNs in high purity cultures is very low, given the necessity of non-neuronal cells, particularly muscle cells and astrocytes, for LMN identity and survival.[28,29] Regarding isolation of primary UMNs, there are currently no reliable UMN specific markers, so there is no way to identify the UMNs from the other neuronal populations dissociated from brain tissue. This is a significant limitation given the specific vulnerability of UMNs to ALS pathology, and this problem continues to hinder our ability to establish authentic UMNs from stems cells. Neuronal-like cell lines, such as N2a, MN1 and NSC-34, offer the advantage of high throughput, cost effectiveness and lack of immediate ethical impact. However, as an ALS disease model, they possess inherent limitations related to their transformed nature and genetic instability driven by indefinite division. Alternatively, human embryonic stem cells (hESCs) can be differentiated into neurons and other relevant cell types, and further genetically modified to model ALS-associated pathologies or incorporated into complex 3D models of nervous tissue.[30,31] However, their significant ethical implications and limited resourcing renders this option less attractive.[32]

2.2 Induced pluripotent stem cells

The development of induced pluripotent stem cells (iPSCs) was a pivotal breakthrough for *in vitro* studies, improving physiological relevance of cell models and reducing the dependence on animal models.[33,34] In the context of neurodegenerative diseases, iPSC technology allows differentiation of cells from patients with neurodegenerative conditions into disease-relevant cell types (such as UMNs and LMNs) and subsequent comparison to those of unaffected individuals or, ideally, gene-corrected controls (Fig. 2). This allows significant insights into cellular pathological changes that arise from the unique genetic signature carried by individual patients.

2.2.1 Motor neuron differentiation. LMN differentiation protocols generally involve directing development of the neural ectoderm *via* SMAD inhibition, and specification of ventral spinal cord fate by exposure to retinoic acid and finally ventralisation *via* activation of sonic hedgehog.[35–38] Aside from MN-like morphology, electrical activity and synapse formation, expression of some combination of ChAT, HB9, ISL1/2, oligodendrocyte transcription factor 2 (OLIG2) and neurogenin 2 (NEUROG2), βIII-tubulin, neurofilaments (NFs), and microtubule-associated protein 2 (MAP2) detected by immunocytochemistry or RT-qPCR are typically utilised as markers of LMN identity.[39–43] Induced LMNs (iLMNs) have been differentiated from patients with fALS-associated mutations in genes such as *FUS*,[44] *SOD1*,[45] *TARDBP*[46] and *C9orf72*,[46,47] each displaying distinct ALS-related phenotypes such as increased excitability, neurofilament aggregation, stress granules and vulnerability to excitotoxic stress. Generating authentic models of sALS is particularly challenging given that we cannot replicate the causative factors, as they are unknown. As such, generating iPSCs from patients with sALS offers a unique insight into sALS pathology and has shown promising results so far. Indeed, iLMNs generated by Burkhardt *et al.* from a patient with sALS showed spontaneous intranuclear and hyperphosphorylated TDP-43 aggregates, reflective of those observed in the *post mortem* tissue of the patient from whom the iPSCs were derived.[48] Regarding drug screening, iPSC lines developed from sALS patients were recently used to screen a novel therapeutic, ropinirole, which showed protective effects on cell lines generated from FUS-fALS, TARDBP-fALS and sALS patients, as well as identifying responders and non-responders in each group, and has now progressed to a phase I/IIa clinical trial.[42,49] In addition, drug screening on motor neurons derived from 100 sALS patients identified a promising combination therapy with baricitinib, memantine, and riluzole, and accurately predicted failure of other therapeutics that proved unsuccessful in clinical trials.[131] These examples demonstrate the value of iPSC derived models of sALS for both mechanistic and drug screening applications. Differentiation of iPSC's into UMNs has proven more challenging than that of iLMNs. Of the few attempts made, the neuronal cultures differentiated from iPSCs are more generally defined as cortical neurons, or cortical projection neurons, that express markers such as paired box protein 6 (*PAX6*), COUP-TF interacting protein 2 (*CTIP2*), special AT-rich sequence-binding protein 2 (*SATB2*), and vesicular glutamate transporters (*VGluTs*), of which UMNs are a more specific subpopulation.[50–52]

2.2.2 Non-neuronal cell types. In addition to UMNs and LMNs, muscles are also an important element to incorporate into *in vitro* models of ALS. Unlike neurons, skeletal myocytes can be cultured from muscle biopsies of patients with ALS, and can also be generated by differentiation of patient-derived iPSCs, allowing for investigation of pure cultures of myocytes, from myogenesis to functional muscle formation.[53–57] iPSC-derived muscle cells carrying a variant in the *SOD1* gene have shown reduced regenerative capacity, reduced contractility, and metabolic dysfunction relative to muscle cells derived from healthy controls, demonstrating that the SOD1 mutation is toxic to muscle cells independent of any association with LMNs.[58] It is also becoming increasingly obvious that other non-neuronal cells, such as astrocytes, oligodendrocytes, microglia and Schwann cells play potentially pivotal roles in ALS progression, conferring toxicity in some circumstances but offering neuroprotective roles in others.[59] iPSC technology allows multiple cell types to be generated from the same patient, providing the opportunity to model the interactions between genetically-matched cell types. In addition, co-cultures can be generated with a gene variant limited to a specific cell population – thereby allowing investigators to determine cell autonomous from non-cell autonomous effects.

Despite being a pivotal addition to the *in vitro* toolkit, some limitations remain in iPSC technology. Importantly, our current inability to generate authentic iUMNs limits our ability to investigate UMN-specific contributions to disease progression. In addition, variation in iLMN differentiation protocols affects the yield, purity and phenotype of the resultant culture.[60–62] ALS research has also revealed that specific subtypes of LMNs are more vulnerable to degeneration.[63] Indeed, oculomotor neurons are resistant to degeneration, despite ubiquitous expression of pathogenic genetic variants, emphasizing the need to be able to effectively differentiate and identify subtypes of LMNs.[64] In addition, some caveats exist that may be important to consider when interpreting iPSC data for translation. Namely, the retainment and erasure of different epigenetic markers in iPSCs may cause information to be lost regarding the contribution of non-genetic factors to neurodegenerative disease pathogenesis. While some studies show that epigenetic memory can be retained by specific methods of differentiation,[65,66] the extent to which other epigenetic alterations are retained is not yet known. Additionally, a significant issue associated with the application of iPSCs to studying neurodegenerative diseases in general is the cellular rejuvenation that occurs during the iPSC reprogramming process, meaning that the resultant cells are not replicative of the age-affected cells that are so vulnerable in neurodegenerative diseases.[67] Additional cell stressors and environmental factors can be employed to drive some forms of age-associated cellular damage.[68,69] Recently, it has been shown that inhibition of epigenetic modifiers in neural progenitors drives rapid maturation of iPSC-derived neurons, perhaps paving the way to generating more physiologically relevant models for neurodegenerative diseases.[70]

3 Modelling ALS *in vitro*

Accurately modeling complex diseases requires more than the inclusion of relevant cell types, as the interplay between the cell types and their spatial arrangement may play a crucial role in disease pathology. A critical challenge lies in organising the cells into a physiologically relevant and experimentally compatible conformation. Here, we explain the principals of 2D compartmentalised culture and 3D culture systems and then discuss the experimental readouts compatible with these methods. Finally, we highlight recent findings from *in vitro* ALS models that demonstrate their utility in advancing our understanding of the disease, and point out current limitations.

3.1 Compartmentalised 2D cultures

2-Dimensional (2D) culture systems consist of cells grown in a dispersed monolayer and is the simplest and most cost-effective form of *in vitro* research (Fig. 2). Historically, 2D culture has been the principal method of studying cellular mechanisms of disease as the conditions are largely controlled and cells can be examined in an isolated manner. 2D culture has led to significant advances in understanding pathobiological mechanisms of neurodegeneration but has limits to its translatability in complex diseases like ALS. Human neuroanatomy is highly organised, and cellular function and health is likely to be highly dependent on these organised connections. This is particularly true for studies of ALS, in which multiple regions of the nervous system are affected and there appears to be directional spread of pathology through circuits.[71] As such, it is possible that 2D monocultures may provide false positives or false negatives when used to screen novel therapeutic agents. The development of microfluidic platforms for *in vitro* studies has offered immense progress to overcoming this limitation, providing a model for questions pertaining to network dysfunction and spread of disease. Whilst dispersed 2D culture methods are still valid, the explosion of microfluidic culture paradigms and 3D cultures present new opportunities to model ALS in a dish and interrogate disease progression and therapeutic options, with the intention of translation.

3.1.1 Microfluidics. Microfluidic devices offer a miniaturised, compartmentalised environment that can be used to model neural circuits and anatomical relationships, while still maintaining relative ease of experimentation and observation (Fig. 2 and 3). Since the first compartmentalised cultures in the late 1970s,[72] advances in microfabrication technology and methods of *in vitro* experimentation have allowed for the design and use of microfluidic devices in many conformations and research contexts. Notably, the ability to

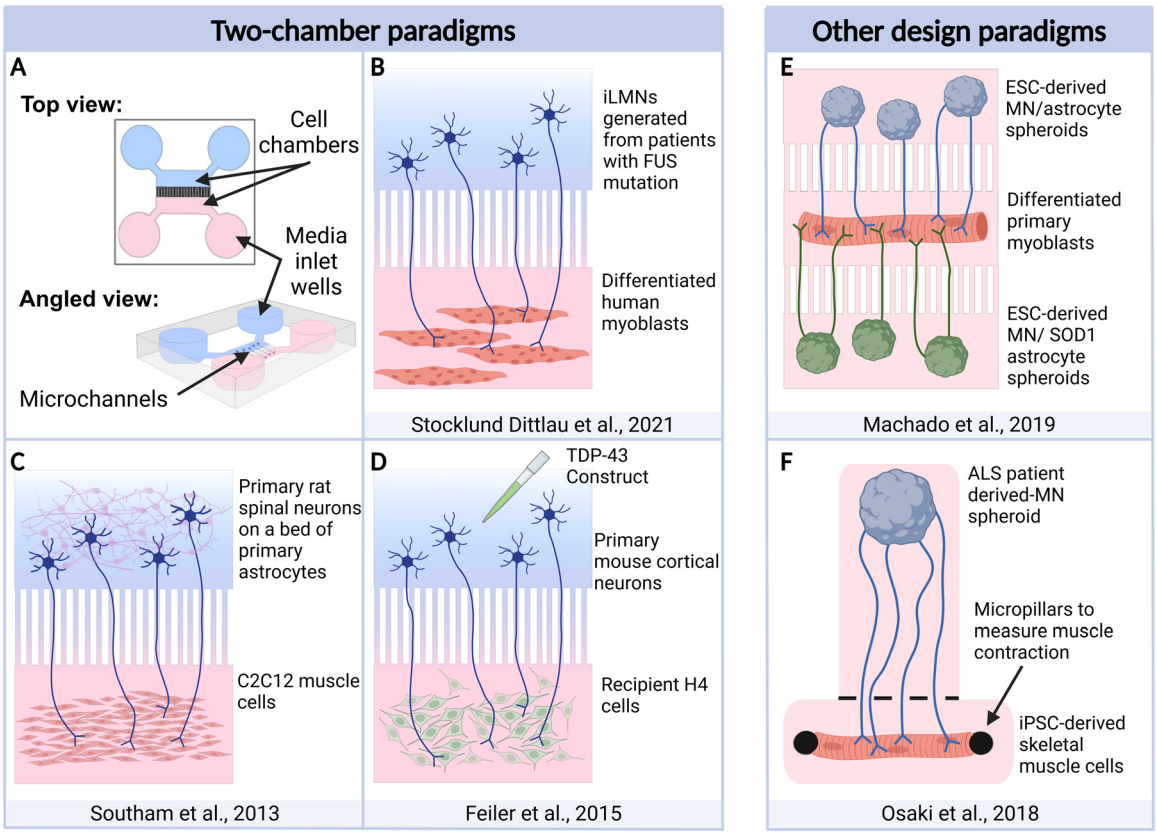

Fig. 3 Microfluidic modeling of ALS. Microfluidic models can be tailored to meet the needs of specific research questions and have been particularly useful in studies of ALS-associated NMJ pathology. (A–F) Graphical depictions of microfluidic-based ALS research that is discussed in this review. Aspects such as the number of chambers, the type/s of cell/s in each chamber and the source of the cells are all important considerations when designing microfluidic-based models. Additional structures can also be fabricated into the devices, such as micropillars to measure muscle contraction (Osaki *et al.* 2018). Figure created with BioRender and Adobe illustrator.

maintain physical and fluidic separation between multiple connected populations of cells has granted the ability to study spread of disease through cell populations and environments in a way that has revolutionised *in vitro* research. This is particularly important for studying ALS, in which progression of disease occurs through multiple regions (brain, spinal cord and the periphery) in a very specific circuit (the corticospinal tract) that includes multiple cell types (upper and lower motor neurons, and muscles). In the context of ALS, integration of iPSC and microfluidic technologies enables the development of ALS-on-a-chip models – powerful platforms for dissecting molecular and pathological mechanisms in a cell-type specific manner. These compartmentalised models allow researchers to interrogate the unique contributions of each cell population to disease progression, acknowledging that the different cell types may not only contribute to, and respond to, ALS-associated pathologies differently, but may require distinct therapeutic strategies. These model systems offer unprecedented opportunity to screen interventions on patient-derived cells in a scalable, physiologically relevant way, paving the way for truly personalised precision therapies in ALS.

3.1.2 Design and fabrication for application to neuronal studies. Microfluidic devices are characterised by etched channels that are generally 500 nm–1 μm in diameter and contain microvolumes of fluid. A key advantage of microfluidic modelling is that the design can be tailored to the specific research question. Experimenters can add cellular compartments to model relationships between more populations of cells, or add additional features such as microchannels to connect cell populations, 'curtains' to restrict cells to a particular chamber upon seeding, or columns to increase surface area inside the chambers.[73,74] Furthermore, biosensors or other analytical features can be integrated into the fabrication, such as multielectrode array (MEA) technology for electrophysiological recordings.[75] Currently, polydimethylsiloxane (PDMS) is a popular choice for fabrication of microfluidic devices because it is optically transparent, hydrophobic, permeable to oxygen and carbon dioxide, affordable and sterilisable.[76,77] Alternatively, microfluidic devices can be produced from materials such as thermoplastics, silicon, or even paper-based materials like cellulose.[76] To decrease manufacturing variability from one device to another, microfluidic devices can be cast from a mould. Fabrication of microfluidic devices is discussed in full by Ching *et al.*,[78] but briefly, PDMS can be poured onto and subsequently peeled away from a mould and bonded to an appropriately prepared glass coverslip, generating channels and chambers between the PDMS and the glass coverslip.

For application to neuronal studies, a microfluidic device generally contains some configuration of 3 main elements: inlet and outlet wells where media/treatments are added, chambers where the cells reside, and microchannels connecting the chambers (Fig. 3A). Cells are deposited into the inlet wells, and capillary action pulls them into the chambers. An important feature of microfluidic models is the ability to promote fluid flow and/or a diffusion gradient through the fluid. Fluid flow can be achieved by manipulation of the volume at the inlets and outlets of the device, which results in passive pumping of fluid by capillary action and hydrostatic pressure, or by active fluid pumping through the device.[79] This continuous fluid perfusion enables circulation of nutrients and removes waste, replicating the role of the fluid flow of cerebrospinal fluid (CSF) *in vivo*.[80] In the context of ALS, circulation of CSF may be important in understanding how pathology and pathological cues travel through the central nervous system, as well as being an important aspect of consideration in therapeutic delivery.[81] In addition, concentration gradients can be established in microfluidic systems by adding a substance (growth factor, solute, drug) at a high concentration to a particular region of the device, causing a diffusion gradient to passively establish through the device. For example, Chennampally *et al.* used a diffusion gradient of rapamycin across a culture of iPSC-derived motor neurons carrying the ALS-associated A315T mutation in the *TARDBP* gene. This enabled them to identify a therapeutic window: rapamycin concentrations between 0.4–1 μM rescued 40.44% of neurons, while lower concentrations were ineffective and higher concentrations were toxic.[82] Low dose rapamycin has now been found to be safe for patients with ALS in a phase 2 clinical trial.[83]

Microfluidic models are a promising culture platform for ALS research and have shown great value already (see applications and outcomes section). However, there is currently significant variability in microfluidic models of ALS, in the specific design of the model, the fabrication process and the cell handling within the model (such as surface pre-treatment, seeding density, the culture medium used for multi-cell type models, and control of media circulation). While flexibility in design is a key strength of microfluidic modelling (particularly for mechanistic studies), a model of ALS for the purpose of therapeutic screening will need to be more consistent to ensure reproducibility. PDMS is also known to absorb small molecules, which is an important consideration for drug screening applications, and solutions for this issue, as explained by Gomez-Sjoberg *et al.*, are yet to be broadly applied.[77] In addition., with the added physiological relevance of compartmentalised cultures, there are some introduced stressors, such as physical sheer stress and altered soluble gas availability, that make culturing cells in microfluidic devices more challenging than in traditional cultureware.[84] Specifically, seeding density, cell survival, media changing schedules and media components will all need to be reconsidered when translating from dispersed 2D culture to microfluidic culturing. Accordingly, microfluidic use can be manually arduous and require technical expertise, as much of the process cannot be, or is yet to be, automated in an academic laboratory setting.

3.2 Modeling ALS in 3D

The most recent advancement made to *in vitro* modeling has been the development of 3-dimensional (3D) culture systems. 3D culture systems include those that are 3D with regards to the cellular environment or 3D with regard to the arrangement of the cells themselves. Cell culture in a 3D

environment refers to the seeding of cells into a 3D extracellular matrix-like scaffolding, within which the cells can move freely and interact with each other and the matrix. 3D tissue culture involves the co-culture of immature cells and depends on their directed self-assembly into 3D clusters (Fig. 2).[85] These clusters (often referred to as organoids or spheroids) can be grown in a matrix or free-floating in culture media.

3.2.1 3D environment. Growing cells in a 3D environment overcomes a limitation of monolayer culture systems by introducing an extracellular matrix within which the cultured cells can interact and communicate in 3 dimensions. It has long been known that the extracellular matrix (ECM), and cell–matrix interactions, are fundamental contributors to cellular health and maturity. In line with this, experiments culturing myocytes in 3D scaffolds show enhanced contractility and maturity compared to cells plated in 2D, making it an important consideration for experiments and disease models relating to muscle function.[86–89] Each tissue type in the body has a distinct extracellular composition of proteins that provide structure, metabolic support and signalling support. The muscle ECM is rich in fibrous collagen and elastin for structural support and elasticity, while neurons require less dense ECMs and contain molecules that support synaptic function, shock absorption and process extension, such as hyaluronan and proteoglycans.[90,91] Manufactured hydrogel scaffolds used in culture generally consist of some combination of culture medium, a basement membrane (Matrigel®, Geltrex® *etc.*) and some combination of glycoproteins such as thrombin, fibrinogen, laminin and/or collagen, but are tailored to the needs of the tissue type. Alternatively, human or rodent tissues can be decellularized and reconstituted into a culture-compatible matrix through a decellularization process that retains important structural, mechanical and biological properties of the native tissue.[92] Indeed, culturing brain organoids in a microfluidic device on human brain-derived extracellular matrix has been shown to improve neurogenesis and maturation compared to Matrigel alone, with similar findings in culturing primary neurons on rodent brain derived ECM.[93,94] Importantly, a compartmentalised system also offers the ability to tailor the scaffold to the cell types that will be growing in each compartment. Modeling ALS with iPSCs in a scaffolded microfluidic platform is a rapidly evolving field of *in vitro* research that has already demonstrated its value, as evidenced by the clinical trials emerging from studies on these models.[83,95,96] In these models, muscle cells were added to the microfluidic device in a scaffold solution of Matrigel and collagen and spontaneously formed muscle bundles. In the future, this technology could be used to generate *in vitro* models of the entire corticospinal tract amenable to screening potential treatments, while still being able to assess outcomes at high cellular resolution.

3.2.2 3D tissue culture. 3D tissue culture refers to the cultivation of cells into 3D clusters, such as organoids or spheroids. Organoids are typically derived from iPSCs and may be embedded into an appropriate 3D extracellular matrix or free-floating in low-adherence culture ware. The cells are provided with biological cues to drive differentiation into the relevant cell types and promote self-organisation into structures resembling miniaturised organs, with polarity, cellular complexity and connectivity.[97] Spheroids, in comparison, lack the complexity and cellular heterogeneity of organoids. They are simple, spherical aggregates of cells grown free-floating in culture media. The application of spheroid and organoid culture to the study of neurodegenerative disease is rapidly evolving, and the appealing factor of organoid culture for neuroscience is in their potential to model structurally complex tissue. Brains are comprised of highly ordered and complex networks of multiple cell types and the ability to model this complex connectivity using human cells would be an invaluable tool for mechanistic research as well as drug discovery.[98] Additionally, organoids can be cultured for extended periods of time, with one group studying maturation and development of a forebrain organoid for 20 months.[99] This offers the potential to observe disease-affected models (patient-derived or *via* introduction of a mutation) for long periods of time. Organoids have also been shown to be amenable to compartmentalisation, providing the opportunity to create miniaturised, compartmentalised, 3D organ systems.[31] These developments could have significant impacts on the study of neurological diseases, potentially enabling the establishment of miniaturised 3D cerebrospinal pathways from ALS patients.

Currently, there are some limitations on the use of 3D organoids and spheroids for the study of neurodegenerative disease. Importantly, recent single-cell transcriptomic characterisation of cerebral organoids suggests that even after being grown for 12 months, only 15% of the excitatory neurons show signatures of early post-natal stages, with most of the nuclei in the organoid aligning with early foetal signatures of human brains.[100] This suggests that cerebral organoids are useful for modeling the developing brain, and thus clearly suited to the study of neurodevelopmental disorders, but their utility for modeling neurodegenerative disease may need further development. In addition, 3D culture methods (including cells grown in 3D matrices) are complex in nature, more expensive than 2D methods, and require technical expertise and large numbers of replicates to account for culture–culture variability.[101] Regardless, organoids introduce an aspect of dimensionality to disease modeling that is otherwise unachievable, giving them the potential to significantly improve translational outcomes.

4 Readouts and experimental compatibility of microfluidic and 3D models of ALS

One of the key strengths of cell culture-based models compared to animal models is their high compatibility with diverse experimental manipulations and analytical readouts. This is essential for validating the models, probing mechanistic pathways and assessing therapeutic outcomes.

Importantly, in culture-based modeling, the cellular environment is readily accessible for addition of experimental manipulations or therapeutics. In compartmentalised on-the-chip models, these treatments can be added in a site-specific manner by establishing fluidic isolation of one region from others. This is achieved through precise control of the volume of media feeding into the two chambers connected by microchannels.[102–104] Further, concentration gradients and continuous fluidic perfusion can be established. Following experimental manipulation, readout methods such as electrophysiology, live/timelapse microscopy, calcium imaging, region-specific-omics studies, reporter assays, optogenetics and immunocytochemistry can all be adapted for microfluidic studies (Table S1).

Electrophysiological outcomes are a central measure of neuronal and circuit health, and it is thus essential that electrophysiological monitoring is compatible with 2D and 3D cell-models. Traditional patch clamp electrophysiology remains a significant challenge in on-the-chip models, because while the media is accessible, the cells generally are not. However, microelectrode array technology has been successfully integrated into microfluidic models, enabling compartment specific measurements of spontaneous and evoked potentials, synaptic function and network function, and is compatible with 2D and 3D cell-models.[75,105,106] In addition, the ease of accessibility means that treatments can be added to modify neuronal activity or mimic excitotoxicity, such as tetrodotoxin or kainic acid, respectively, and the cellular and network electrophysiological outcomes can be monitored in real time.[107–109]

Imaging-based readouts are also compatible with on-the-chip models. For 2D on-the-chip models, live cell microscopy can be used to observe axonal growth and cellular degeneration in real time, and calcium imaging can be used to observe intracellular calcium flux and thus indicate neuronal excitability and network function.[110] Following cell fixation, immunocytochemistry can proceed as usual, allowing for identification of cell types, cell health markers and protein aggregation labelling. For 3D models, live imaging is often used to record the growth of organoids over time.[106] At the end point of an experiment, organoids can be sectioned and fixed, making them compatible with immunohistochemistry. Prior to fixation, the sections, or even the whole organoid, can also be used for calcium imaging or patch clamp electrophysiology.[111]

Compartmentalised on-the-chip models also allow compartment-specific omics studies, allowing comparative omics investigations of the neuronal somatodendritic compartment *versus* axonal compartment, or cortical compartment *versus* spinal compartment.[112] This provides a deep exposition on the gene and protein expression profiles in response to disease processes, potentially revealing mechanistic and compensatory mechanisms of specific cells in the circuit. 3D organoids can also be dissociated into a cell suspension and then used for omics studies.[106]

A shortcoming of many of these options are that these observation methods are, in their nature, end-point observations of the culture. Biosensor integration into on-the-chip models is revolutionising our ability to continuously monitor live culture analytics in real-time.[113] Biosensors, by definition, are analytical tools that use biological molecules (enzymes or antibodies) to detect the presence of chemicals. Biosensor integration, however, more broadly refers to integration of readout methods into microfluidic models to capture complex biological or physiological readouts. For example, incorporation of MEAs into microfluidic devices is an example of biosensor integration. Biosensors have been developed to report (optically, magnetically or electrically) on culture properties such as levels of dissolved gases, pH, nutrients, metabolites and biomarkers, as well as cell-centric read outs such as cell growth and survival, electrical and mechanical properties, nucleocytoplasmic translocation, autophagy, and mitochondrial health.[113–115] In models of ALS, biosensor integration can provide continuous, non-invasive readouts of physiological and pathological changes in the model, providing a dynamic window into ALS progression and therapeutic responses. As an example of biosensor integration into ALS microfluidic models, Uzel *et al.* tailored their on the chip model to have micropillars in the muscle compartment, between which the myofibers formed a muscle bundle, and the pillars were able to record the force of muscle contraction, providing a live readout of circuit function.[116]

Importantly, although many chemical biosensor assays exist, few are useful for long-term, continuous monitoring given their dependence on laser excitation, which causes photobleaching over time. As such, biosensor development that is not dependent on laser excitation for detection is currently an important area of research. For example, Zhang *et al.* developed a reactive oxygen species (ROS)-responsive probe that undergoes a structural change in the presence of ROS, resulting in a significantly enhanced Raman scattering signal detectable *via* surface-enhanced Raman spectroscopy (SERS).[117] This approach demonstrated high specificity and sensitivity for ROS detection *in vivo* and *in vitro*, and its design is well-suited for adaptation to microfluidic platforms, where real-time, spatially resolved monitoring of oxidative stress could greatly enhance mechanistic studies and therapeutic screening.

The readout methods discussed here, while they are not exhaustive, have facilitated highly informative research in the ALS research space.

5 Applications and outcomes of ALS *in vitro* studies

The development of microfluidic techniques has revolutionised our capacity to investigate how pathology spreads through corticofugal tracts in ALS. For example, spread of pathological TAR DNA-binding protein 43 (TDP-43) appears to be an accurate predictor of symptom progression, but whether the spread of TDP-43 pathology is due to direct transmission of protein intercellularly or a consequence of increasing neuronal dysfunction remains unknown.[71,118] 2D dispersed culture investigations into TDP-43 pathology revealed that cytoplasmic

expression of TDP-43 (be that WT or harbouring an fALS associated mutation A315T) is toxic to neurons, and that the amount of TDP-43 in the cytoplasm (but not in the nucleus) is a strong predictor of cell death.[119,120] This provides an example of how simple *in vitro* paradigms can demystify cellular pathological phenomena observed in clinics or *in vivo* settings, but does not allow investigation of spread of pathology, given the disordered connections and lack of directional resolution. It has not been clear, for example, whether spread of TDP-43 requires contact between cells or if its transmission occurs predominantly *via* secretion of extracellular vesicles. To answer this question, Feiler *et al.* cultured primary mouse cortical neurons into one compartment of a microfluidic device and transfected them with a luciferase-tagged TDP-43 construct, or just the luciferase construct, and recipient H4 cells into the other compartment (Fig. 3D).[121] This experiment revealed increased luciferase activity in the cultures treated with the TDP-43 construct compared to the control, demonstrating that TDP-43 can undergo directed anterograde, contact-mediated spread between cells *via* axons. By reversing the paradigm and adding TDP-43 to the dendritic compartment, the authors also identified retrograde spread of TDP-43, indicating bidirectional transmission of TDP-43. This platform of *in vitro* studies presents exciting opportunities to study how ALS pathology spreads through the corticomotor system, how different pathological mechanisms affect different cell types and what mechanisms protect some cell types from degeneration.

Compartmentalised microfluidic platforms are particularly useful for studying neuromuscular junction (NMJ) pathology. The NMJ is a specialised synapse between motor neurons and muscle fibres. An action potential in the LMN leads to release of acetylcholine at the neuromuscular junction, which initiates a post-synaptic current in the muscle that ultimately results in muscle fibre contraction. The NMJ is highly vulnerable in ALS, and it is widely accepted that the disassembly of the NMJ is pivotal to symptom onset and progression.[122] Early attempts at generating an *in vitro* model of the NMJ utilised human iLMNs with C2C12 mouse myoblasts in a 2D culture dish, and demonstrated functional NMJ formation through electrophysiological stimulation and post-synaptic current record.[123] However, this approach lacks the spatial separation and distinct environments that LMNs and muscle cells naturally inhabit, limiting its physiological relevance. We have previously overcome these limitations by generating a microfluidic-based motor unit model by culturing embryonic rat LMNs on a bed of astrocytes in one compartment and rat myoblasts in another[124] (Fig. 3C). With this protocol, motor neurons extended distally and formed neuromuscular junctions with myocytes as evidenced by clustered presynaptic synaptophysin and postsynaptic acetylcholine receptors.[125] Using this model, we showed that rodent LMNs degenerate following somatodendritic, but not axonal, exposure to kainic acid, supporting the die-forward hypothesis of pathological spread in ALS. The addition of iPSC technology to microfluidic devices has improved the translatability of microfluidic motor unit models, combining the benefits of human cells and compartmentalised culture. For example, in 2021, Stoklund Dittlau *et al.* generated a microfluidic model of the human motor unit using iLMNs and human myoblasts (Fig. 3B). NMJ-like connections in the myoblast compartment were quantified in cultures grown from patients harbouring *FUS* mutations and CRISPR-corrected control cultures, and revealed that *FUS* mutant neurons, compared to controls, formed fewer NMJs and those that did form were less likely to have multiple contact points, indicative of defective maturation.[126]

As an example of the value of non-compartmentalised 3D culture, a recent study by Faustino Martins *et al.* showed the generation of a hESC-derived 3D, functional neuromuscular organoid that self-organised into neural and muscular regions. Additionally, the organoid formed functional NMJs that, upon stimulation of the neural region, were able to confer observable contractions in the muscle cells.[106] The group also establish a neuromuscular organoid from iPSCs derived from a patient with spinal muscular atrophy, and the organoid exhibited reduced NMJ formation and contractility.[127] In an effort to integrate a cortical region into a neuromuscular organoid, Andersen *et al.* generated organoids resembling the cerebral cortex, the spinal cord/hindbrain and skeletal muscle from human iPSCs and cultured them together to generate a corticomotor assembloid.[128] Optogenetic stimulation of the cortical organoid was able to translate through the miniaturised circuit and cause recordable muscle contraction in the muscle organoid. Neuromuscular organoids and cerebrospinal-muscular assembloids formed from patient-derived iPSCs could form an invaluable platform to study motor dysfunction in ALS and screen novel therapies. However, these models do not exhibit the compartmentalised nature of the motor system as the neuronal and muscular cell types are co-cultured in the same media with no physical boundary and limited axonal distance between them.

In contrast, Machado *et al.* designed a compartmentalised device capable of housing spheroids (Fig. 3E).[31] This device included three open-top compartments, where spheroids containing ESC-derived motor neurons (MNs) and astrocytes were placed in the outer compartments, while primary myofibers were positioned in the central compartment. The spheroids projected axons through the microchannels connecting the compartments and formed NMJs onto the myofiber. This format allowed the authors to assess the NMJ formation and functionality from different populations – and potentially genotypes – of neural/astrocyte spheroids, onto the same myofiber. They found that when the astrocytes in the MN/astrocyte spheroid were affected by a mutation in the *SOD1* gene, the resultant NMJs exhibited reduced myofiber contractions. In 2018, Osaki *et al.* generated an elaborate human ALS on-a-chip model.[95] iPSCs derived from a patient with sALS were differentiated into 3D skeletal muscle bundles as well as iLMNs and cultured into ECM scaffolded microfluidic devices, forming functional motor units (Fig. 3F). By optogenetically stimulating the iLMNs, the authors observed that the sALS-derived motor unit

model generated fewer muscle contractions than controls, as well as aggregation of TDP-43 in the cytoplasm. Additionally, the LMN degeneration and contraction deficits were rescued with a combination of treatments, bosutinib and rapamycin, which have now been assessed in phase one and phase two clinical trials respectively.[83,96]

These examples present convincing evidence that *in vitro* models of ALS are valuable for questions ranging from simple, single cell mechanistic studies to complex, multicellular 3D modelling of pathological spread. In fact, of the three currently published examples of drug screening in iPSC culture translating into a clinical trial (bosutinib, ropinirole and retigabine), all utilised 2D monoculture of iPSC-derived motor neurons from ALS patients[42,47,129] – confirming that simple monoculture systems can be informative for drug screening trials. Furthermore, these examples provide evidence that they are relevant, in that they reflect complex pathological signatures observed in humans and animal models, and that their translational potential is vast.[49,83,96,130]

6 Future directions and conclusion

In the future, *in vitro* models of ALS are likely to increase in complexity, model currently unknown aspects of disease, and identify additional drugs with translational potential. For example, not only will they have the necessary cell populations in the dish, but each in their own relevant extracellular matrix with associated supporting tissues such as vasculature, glial populations and immune cells. To support data collection from increasingly complex models, multiplexed biosensors—targeting aspects such as protein aggregation, oxidative and mitochondrial stress, and electrophysiological dysfunction—will need to be integrated into microfluidic devices. This integration will enable continuous monitoring of cell health, pathological spread, and therapeutic responses throughout the system. However, this should not be misinterpreted—complex, multi-cellular or multi-tissue models are not necessary for every ALS research question. There is substantial value in cell-autonomous studies, particularly for mechanistic insights. Nonetheless, for translational research, the use of more complex models becomes increasingly important. In the present, it is clear that the issues of clarifying cell identity in iPSC studies, our inability to generate *bona fide* iUMNs, and the limited cellular maturity/ageing associated with iPSC-derived cells continue to impede our ability to faithfully model ALS in a dish. Regarding translatability, the miniaturisation of model systems can lead to challenges in understanding dosage, toxicology, drug availability and delivery upon translation into the clinic. Specifically, models currently do not incorporate the role of the blood brain barrier or liver filtration in therapeutic function, meaning that direct translation of novel therapeutics (rather than repurposed therapeutics) from the dish to the clinic need to be carefully considered.

Here, in the context of the FDA no longer necessitating animal model experiments to progress a drug to clinical trials, we have discussed the ways that *in vitro* models are being used to model and to study ALS. The purpose of this reflection is to identify the strengths and the limitations of our current *in vitro* models that may hinder their ability to be standalone preclinical platforms of drug discovery and mechanistic research. Promisingly, microfluidic devices and iPSC technology have revolutionised our ability to model human diseases in a compartmentalised way that is reflective of anatomical organisation. This has been particularly useful in the study of ALS given that it is a multi-compartment, multicellular disease that affects a very specific circuit. Furthermore, microfluidic models are inherently scalable, allowing researchers to run high-throughput, parallel experiments with precise control over cell types and conditions, making them ideal for drug screening, mechanistic studies, and personalised medicine. Indeed, new advances in *in vitro* technologies have enabled significant mechanistic discoveries and multiple instances of iPSC-based drug screens that have progressed to clinical trials. In contrast to the resource-intensive and ethically constrained nature of animal studies, *in vitro* models provide a relevant, scalable and ethically favourable alternative that is well-positioned to meet the demands of mechanistic and translational research.

Conflicts of interest

There are no conflicts of interest to declare.

List of common abbreviations

ALS	Amyotrophic lateral sclerosis
fALS	Familial amyotrophic lateral sclerosis
sALS	Sporadic amyotrophic lateral sclerosis
UMN	Upper motor neuron
LMN	Lower motor neuron
ESC	Embryonic stem cell
iPSC	Induced pluripotent stem cell
iLMN	Induced lower motor neuron
iUMN	Induced upper motor neuron
TDP-43	TAR DNA binding protein 43

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review. Supplementary information (SI): supplementary Table 1 details the studies mentioned in section 4 and the readout methods applied. See DOI: https://doi.org/10.1039/d5lc00577a.

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