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Review

Modeling neuromuscular junctions *in vitro*: A review of the current progress employing human induced pluripotent stem cells

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Abstract: Amyotrophic lateral sclerosis (ALS) is a fatal and progressive neurodegenerative disorder of undetermined etiology with no effective treatments. Motor weakness and bulbar dysfunction lead to premature death, usually resulting from respiratory failure. While much of the research has focused on the role of neuronal dysfunction in ALS etiology, evidence from human patients and animal models indicates that the neuromuscular junction (NMJ) shows significant functional and structural abnormalities prior to the onset of motor neuron degeneration and behavioral symptoms. The development of novel experimental approaches will allow the study and manipulation of human NMJs and significantly contribute to our understanding of ALS pathogenesis, leading to advances in pharmacological treatments for the disease. A novel approach that has been employed in recent years is the use of human induced pluripotent stem cells (iPSCs) to generate cell types contributing to synaptic connectivity at the NMJ. The generation and differentiation of cells derived from ALS patient iPSCs is a promising method for investigating disease mechanisms and drug screening at NMJs *in vitro*. In this review, we cover the theories underlying the mechanisms of ALS pathogenesis at the NMJ, an overview of the recent developments in the generation of functional human neuromuscular connectivity *in vitro*, and the advances in human iPSC programming and differentiation technology.

Keywords: amyotrophic lateral sclerosis; neurodegenerative diseases; neuromuscular junction; induced pluripotent stem cells; tissue engineering; *in vitro* culture; disease modeling; disease-in-a-dish

Abbreviations: AAKG: arginine alpha-ketoglutarate; α-Btx: alpha bungarotoxin; ALS: Amyotrophic lateral sclerosis; ACh: acetylcholine; AChRs: acetylcholine receptors; BDNF: brain-derived neurotrophic factor; BMP: Bone morphogenetic protein; Cdk5: cyclin dependent kinase 5; ChR2: Channelrhodopsin-2; ChAT: choline acetyltransferase; CNTF: ciliary neurotrophic factor; DP: Deanna protocol; DRG: dorsal root ganglion; EB: Embryoid body; EPP: excitatory postsynaptic potential; ESC: embryonic stem cell; GDNF: glial cell-derived neurotrophic factor; GSK-3: glycogen synthase kinase-3; iMPCs: induced myogenic progenitor cells; iPSC: induced pluripotent stem cell; Lrp4: low density lipoprotein receptor-related protein 4; MEA: microelectrode array; mEPP: miniature excitatory postsynaptic potential; MN: motor neuron; MuSK: muscle-specific kinase; NCSCs: neural crest stem cells; NMDA: N-methyl-D-aspartate; NMJ: neuromuscular junction; p75NTR: p75 neurotrophin receptor; PDMS: polydimethylsiloxane; ROS: reactive oxygen species; SCs: Schwann cells; SEMA3A: Semaphorin3A; SHH: sonic hedgehog; SKM: skeletal muscle; SMA: spinal muscular atrophy; SMN: Survival motor neuron; SV2: synaptic vesicle protein 2; TSC: Terminal Schwann cell; TGF-β: Transforming growth factor β; VA: Valproic acid

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal, adult-onset neurodegenerative disease characterized by progressive loss of motor neurons (MNs) in the cortex, brainstem, and spinal cord. MN degeneration results in muscle atrophy and paralysis, ultimately leading to death within 2–5 years of symptom onset [1]. The incidence of ALS is approximately 2 per 100,000 people, with a slightly higher proportion of affected males [2]. ALS can be classified into two different types: sporadic and familial. Approximately 90–95% of ALS patients suffer from a sporadic form of ALS with no clear genetic linkage [3], while the remaining 5–10% of ALS patients exhibit familial inheritance. There is currently no cure for ALS, although there are two FDA-approved drugs, riluzole and edaravone, which may increase ALS patient survival by several months and modestly reduce the rate of motor function decline [4,5].

Over 100 genes have been implicated in ALS pathogenesis [6], a subset of which has been shown to be causative for classical familial ALS, namely: (1) superoxide dismutase SOD1[7]; (2) RNA binding proteins TAR DNA-binding protein 43 (TARDBP/TDP-43) [8,9] and Fused in Sarcoma/Translocated in Liposarcoma (FUS) [10,11]; (3) phosphoinositide phosphatase FIG4 [12]; (4) AAA-ATPase VCP [13]; and (5) DENN-domain protein C9orf72 [14–16]. Despite the varied etiology of the disease, a common pathological finding in many ALS subtypes is the presence of misfolded and ubiquitinated cytoplasmic MN inclusions that contain TDP-43 [17–19]. Notable exceptions include cases caused by mutations in SOD1 [20–21].

Multiple mechanisms have been proposed to contribute to ALS MN pathogenesis, including: (1) excitotoxicity [22–23]; (2) mitochondrial dysfunction and oxidative stress [24–25]; (3) abnormal protein aggregation [26–27]; (4) impairments in cytoskeletal and axonal dynamics [28–30]; (5) altered RNA metabolism [19,31]; (6) aberrant neuronal excitability [32–34]; (7) defects in endosomal trafficking and autophagy [35–36]; and (8) abnormalities in nucleocytoplasmic transport [37–38]. However, there may be some unknown underlying molecular links that connect these disparate mechanisms driving the nerve terminal pathology.

Despite decades of intense research, the precise mechanisms underlying MN disease remain elusive. Most ALS research studies have focused on MN dysfunction as the primary pathology, but

there is evidence suggesting that early pathological changes occur in the most distal part of the lower MN—the neuromuscular junction (NMJ) [39–40]. Additionally, there is evidence that skeletal muscle (SKM) and Schwann cells (SCs) may be critical for the manifestation and progression of the disease. Most of the data regarding ALS NMJ function has been obtained using animal models, which provide critical, but limited, insights into the physiology of human NMJs due to species-specific differences in structure and function [41–43]. Furthermore, the examination of human patient NMJs is particularly difficult due to the absence of predictive and reliable biomarkers for ALS disease onset and the use of highly invasive biopsy procedures for pathological examination. To date, there are no techniques that allow visualization and/or experimental manipulation of human NMJs from ALS patients *in vivo.* Thus, the development of technical approaches that allow the examination of synaptic pathology at the human NMJ are necessary to understand the cellular and molecular underpinnings of ALS. These data are also critically important for the discovery and testing of new therapeutics for the disease. In this review, we examine the literature regarding the mechanisms of NMJ dysfunction in ALS and provide an overview of the recent developments in the generation and specification of functional human neuromuscular connectivity *in vitro*, particularly through the use of induced pluripotent stem cell (iPSC) reprogramming technology.

2. ALS and defects at the neuromuscular junction

In mammals, SKMs are innervated by axons descending from MN cell bodies in the cortex and spinal cord, forming highly specialized nerve-muscle contact sites called neuromuscular junctions (NMJs). The terminal ends of MN axons form small swellings termed ‘boutons’, which contain acetylcholine (ACh)-containing synaptic vesicles (Figure 1). These vesicles cluster and fuse at distinct regions of the presynaptic plasma membrane known as active zones [41,43]. Presynaptically-released neurotransmitters bind to ACh receptors (AChRs) concentrated in the postsynaptic folds of the muscle membrane [44]. ACh binding modulates the gating of ion channels and promotes the generation of excitatory postsynaptic potentials (EPPs) in the muscle. Additionally, terminal Schwann cells (TSCs) play a key role in the formation and stabilization of the NMJ, as well as coordinating regeneration responses post-injury [45–47].

Studies of human ALS patients have shown that axonal denervation, synapse loss, mitochondrial abnormalities, and increases in intracellular Ca^{2+} levels are key pathological events in the progression of the disease [48–52]. Examination of early stage NMJs in muscle biopsies from ALS patients have revealed functional and structural abnormalities in the peripheral nervous system, including (1) decreased neurotransmission [53]; (2) changes in synaptic protein composition [54–55]; (3) an increase in synaptic vesicles at nerve terminals [51,56]; and (4) reductions in choline acetyltransferase (ChAT) activity [57]. These findings provide support for the ‘dying-back’ hypothesis, which posits that NMJ dysfunction precedes the axonal and MN cell body degeneration [39–40].

ALS patients also display neurofilament accumulations and other cytoskeletal abnormalities in proximal axons [58–60]. Cytoskeletal disorganization can lead to the formation of axonal swellings or ‘varicosities’, which accumulate vesicles, mitochondria, and other cargo, leading to impairments in retrograde axonal transport [59,61–62]. A recent study by Lavado et al. (2017) investigated the link between aberrant induction of axonal varicosities and glutamate excitotoxicity in human spinal cord stem cell-derived MNs. While cells treated with varying concentrations of glutamate for 48 hrs

showed no deficits in viability or electrophysiological properties, they did exhibit an increased number of axonal varicosities. These varicosities showed an accumulation of synaptic vesicles (a common cargo of axonal transport), suggesting a glutamate-dependent effect on axonal integrity and function [63]. These results were verified with a human SOD1 patient-derived iPSC line, which showed no functional electrophysiological deficits, but exhibited significant numbers of varicosities in the absence of glutamate exposure. Most importantly, axonal defects were reversed after treatment with a modified Deanna protocol (DP) formulation, which contains arginine alpha-ketoglutarate (AAKG) and a number of dietary supplements reported to preserve metabolic function and prevent glutamate excitotoxicity [64]. These findings provide some evidence for axonal defects preceding functional motor deficits in ALS MNs and for therapeutic reversal of axonal varicosities *in vitro*. Hence, axonal structural abnormalities may represent an early detectable marker and therapeutic target for ALS pathology and drug screening in humans.

Nonetheless, due to the paucity of ALS human patient data at the early stages of the disease, much of the evidence for a dying-back neuropathy has been obtained with ALS animal models, including mice, zebrafish, *C. elegans*, and *Drosophila* [39–40,65–83]. The well-characterized $SOD1^{G93A}$ ALS mouse, which develops clinical symptoms by Day 80–90 (p80–90) and dies by Day 130 (p130) [66], first provided critical insights into the pathological mechanisms at the ALS NMJ [57]. Frey et al. (2000) reported selective loss of fast-type (fast-fatigable) NMJ synapses as early as p50 [67], and Kennel et al. (1996) reported progressive loss of functional motor unit numbers by p47 using electromyographic data [84]. Quantitative pathological analysis in $SOD1^{G93A}$ mice showed that at 47 days, more than a month before onset of motor symptoms, 40% of synapses were denervated. However, there was no evidence of ventral root or cell body loss at this stage. Axonal degeneration occurred between p47–p80, whereas severe loss of MN cell bodies occurred after p80. These findings were corroborated with post-mortem material from an ALS patient who died during the early stages of the disease [68]. Another study found that mice overexpressing mutant TDP-43 showed impaired neurotransmission by age 3 months, which preceded the deficits in motor function and MN loss observed at age 10 months [85]. These mutant mice also displayed morphologic alterations such as anomalous innervation patterns and changes in the distribution of synaptic vesicle–related proteins [85].

Data from other animal models have postulated that the dying-back neuropathy may be associated with defects in mitochondrial function, autophagy, and axonal trafficking [51,70,83,86–87]. NMJs of pre-symptomatic $SOD1^{G93A}$ mice exhibited higher numbers of autophagosomes and degenerated mitochondria compared to controls [83]. Damaged mitochondria can generate elevated levels of reactive oxygen species (ROS), which have been shown to affect NMJ function and contribute to MN degeneration in ALS [78,88–90]. Xie et al. (2015) revealed that $SOD1^{G93A}$ mice show progressive reductions in lysosomal density in spinal MNs starting at p40. Lysosomal deficits resulted in the accumulation of autophagic vacuoles engulfing damaged mitochondria along MN axons [86]. These deficits were attributed to aberrant dynein-mediated retrograde trafficking in axons, a finding that supports earlier studies indicating that pre-symptomatic $SOD1^{G93A}$ mutant mice show impairments in axonal transport [29–30]. As noted previously, this is likely a crucial mechanism in ALS pathology, as axonal defects have been shown to: (1) impair the transport of newly synthesized proteins and lipids; (2) disrupt mitochondrial transport and energy supply to synapses; (3) prevent the clearance of damaged or misfolded proteins and organelles [91]; and (4) promote a switch in retrograde transport from neurotrophic to neurotoxic signaling

factors [92]. The findings also provide a more detailed framework for devising therapeutic strategies to ameliorate axonal defects in ALS patients.

Lastly, ALS SOD1 mutant mice harboring manipulations of apoptosis-related genes (Bcl-2 and Bax) designed to prevent MN death still developed the disease despite showing a slight increase in longevity due to delayed muscle denervation onset [70,93]. Attenuating mitochondrial damage and inhibiting aggregation of misfolded SOD1 also had no effect on disease progression [94]. These findings support the notion that abolishing MN cell death is not sufficient to deter ALS disease progression and that drugs targeting MN death, as opposed to the NMJ, may not have much *in vivo* relevance. These studies also suggest that (1) muscle denervation is driven by separate molecular mechanisms than those involved in MN cell death; and (2) that alterations in other cell types (such as SKM or TSCs) may likely influence axonal degeneration and NMJ denervation. Indeed, there is some evidence that ALS is a non-cell autonomous disease, and numerous studies have shown that non-neuronal cells can contribute to disease progression and severity. For instance, glial cells expressing mutant SOD1 and TDP-43 selectively kill MNs [95–96], and SKM expression of $SOD1^{G93A}$ can induce degeneration of MNs [97]. Furthermore, muscles of pre-symptomatic $SOD1^{G93A}$ mice were shown to exhibit reduced levels of cyclin-dependent kinase 5 (Cdk5) [98], a protein critical for AChR clustering and myogenesis [99–100], as well as histological abnormalities occurring simultaneously with nerve detachment [101]. Additionally, $SOD1^{G93A}$ mice show increases in repellent axon guidance proteins (SEMA3A and Nogo-A) in muscle fibers, leading to the idea that these signaling cues may promote the repulsion of the motor axons from the muscle and lead to the eventual axonal denervation and MN degeneration [102–103]. Similarly, De Winter et al. (2006) found that pre-symptomatic $SOD1^{G93A}$ mice have higher expression levels of SEMA3A in SC terminals, providing evidence for its potential role in nerve terminal retraction at the NMJ [104]. However, expression of a signaling-deficient mutant SEMA3A did not ameliorate motor function in $SOD1^{G93A}$ mice, suggesting that SEMA3A signaling may not be an essential factor compromising the integrity of NMJs in ALS [105]. A potentially more critical signaling mechanism at ALS NMJs was uncovered by a recent study demonstrating that $SOD1^{G93A}$ mice treated with an agonist antibody to MuSK (a muscle receptor tyrosine kinase essential for maintaining NMJs), slowed axonal denervation, promoted MN survival, and modestly extended ALS mice lifespan by 7–10 days. The authors postulated that increased trans-synaptic retrograde signaling was mediated through lipoprotein receptor-related protein 4 (Lrp4), which is critical for differentiation and stabilization of motor nerve terminals [106–107]. Although these studies suggest a non-cell autonomous model of ALS, the exact molecular mechanisms by which SKM and SCs contribute to disease pathology remain largely elusive.

In summary, there is substantial data to support a ‘synaptocentric’ etiology of ALS, where the earliest pathogenic events in the disease process include synaptic dysfunction and the dismantling of the NMJ [68,108]. It is possible that impaired retrograde trafficking due to axonal abnormalities leads to the accumulation of dysfunctional mitochondria at synaptic terminals, which then triggers the release of harmful ROS and the initiation of apoptotic signaling cascades [24]. The reduction in ATP levels may also reduce recruitment and transport of vesicles to synaptic release sites [109–110] and cause impaired neurotransmission. Concomitantly, the aberrant function or expression of axon guidance proteins may contribute to the early pathological changes by influencing NMJ function and stability. Nonetheless, whether trans-synaptic signaling processes contribute to ALS pathology is not

well established and will require further research. Figure 1 provides a general overview of the potential molecular mechanisms underlying the early ALS pathology at the NMJ.

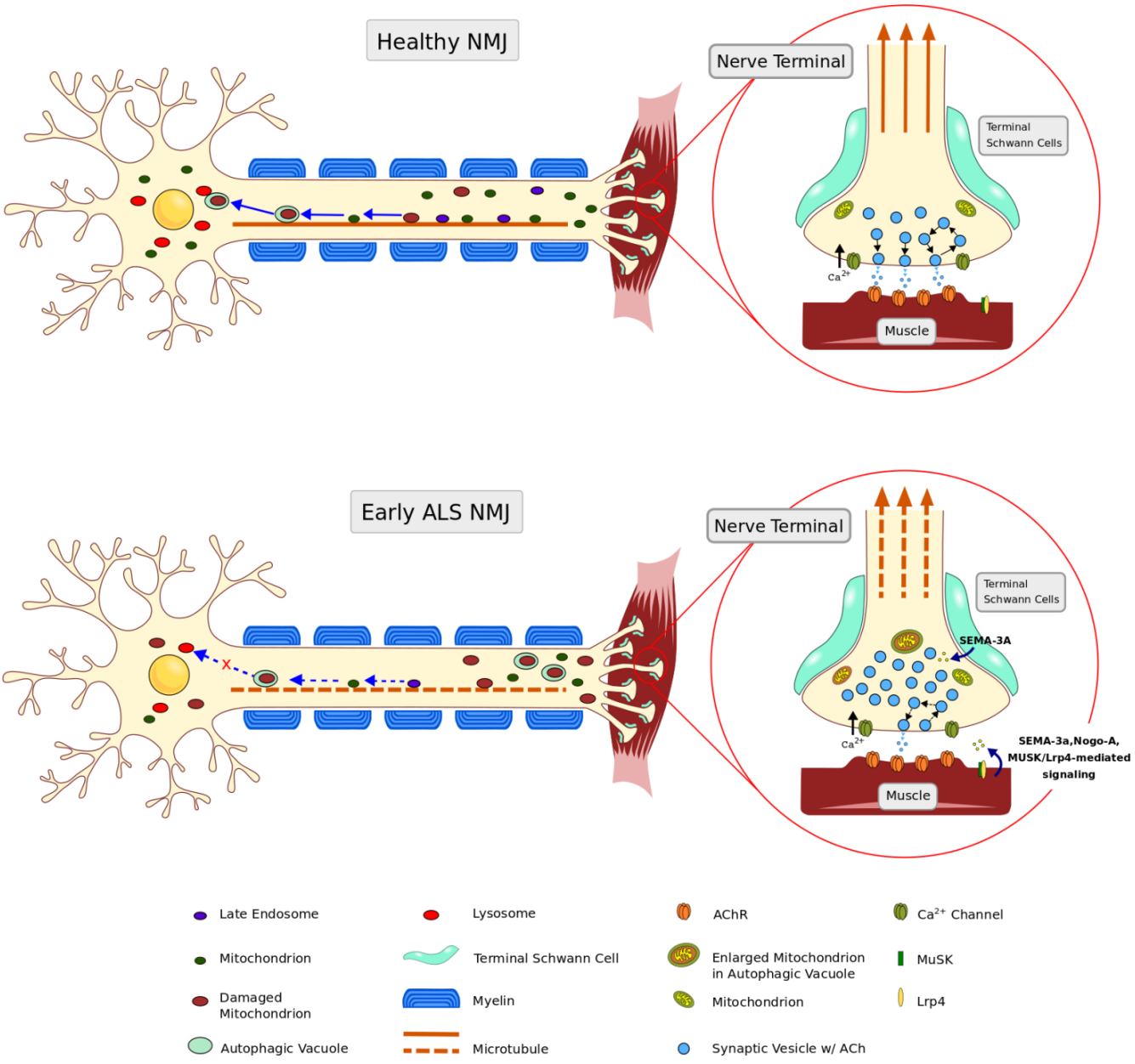

Figure 1. Putative molecular mechanisms underlying early ALS pathology at NMJs.

Note: Neuromuscular junctions (NMJs) are tripartite synapses composed of a motor neuron (MN) terminal synapsing onto a muscle fiber, with terminal Schwann cells (TSCs) enveloping the junction. In comparison to healthy NMJs, at the early stages of ALS, NMJs exhibit impaired neurotransmitter release and abnormal accumulations of enlarged mitochondria and autophagic vacuoles in MN axons and terminals due to impaired microtubule-based retrograde transport (dashed line). Skeletal muscle and TSCs may contribute to ALS progression via the release of repellent guidance cues (SEMA-3A and Nogo-A) and MuSK/Lrp4 muscle-neuron trans-synaptic signaling. These dysfunctional convergent or parallel mechanisms eventually lead to axonal retraction and muscle denervation.

3. Classic *in vitro* NMJ culturing models

While animal models have provided many insights into the pathological and molecular mechanisms occurring at ALS NMJs, there have historically been very limited approaches and techniques available to directly study human NMJ pathology. Early co-culturing experiments of the NMJ were conducted by culturing embryonic spinal cords with intact and attached developing ganglia with muscle from chicks, rodents, or *Xenopus* [111–113]. The development of optimized techniques facilitated the culture of physically separate explants of SKM and spinal cord, which formed robust structural and functional connections *in vitro* [114–116]. Morphological analysis via electron microscopy revealed the formation of immature synaptic connections in culture, including the presence of synaptic vesicles and putative postsynaptic specializations [115–116]. Seecof et al. (1972) later devised techniques to differentiate *Drosophila* embryonic gastrula-stage cells *in vitro* to form morphologically and functionally defined neurons and myotubes [117]. Later, pioneering studies discovered and characterized embryonic stem cells (ESCs), which are derived from the inner cell mass of mammalian blastocysts, and can grow indefinitely, maintain pluripotency, and differentiate into cells of all three germ layers [118–121]. The advent of these sophisticated ESC technologies propelled the development of methods to generate MNs [122–125] and SKM [126–127] *in vitro*.

To date, there have been many ESC-derived and non-ESC-derived NMJ co-culturing studies with *Xenopus* [128–129], chicks [130–132], rodents [133–137], and humans [138–139], as well as cross-species co-cultures [123–124,140–145]. A few seminal studies by Li et al. (2005) and Singh Roy et al. (2005) co-cultured ESC-derived MNs with rodent SKM (myoblast cell line C2C12 and dissociated rat SKM, respectively). Cultures were maintained for several weeks, during which ChAT-positive MNs extended their neurites and formed synapses with myotubes, as assayed by labeling with synaptic vesicle markers and the AChR marker, α-bungarotoxin (α-Btx) [123–124]. The studies revealed that ESC-derived neurons were electrophysiologically active and had functionally intact synaptic transmission. A complementary study by Yohn et al. (2008) showed that mouse ESC-MNs were able to restore contractile force to denervated muscles by forming functional NMJs when transplanted into the distal stump of a transected peripheral nerve [146].

One of the first studies attempting the formation of human ESC-derived NMJs *in vitro* was conducted by Guo et al. (2011). Differentiated MN-SKM co-cultures derived from a human spinal cord stem cell line and primary human SKM progenitor cells formed well-defined and distinguishable NMJs, as synaptophysin-positive terminals colocalized with AChR clusters. MNs and myotubes exhibited normal electrophysiological properties and numerous muscle contractions after 1 week of co-culturing. The silencing of myotube contractions by application of curare confirmed the formation of NMJs [138]. Similarly, Puttonen et al. (2015) reported a successful co-culture of human ESC-derived MNs and SKM. Activation of MNs by the glutamatergic receptor agonist, N-methyl-D-aspartate (NMDA), triggered reproducible action potentials in myotubes and normal miniature excitatory postsynaptic potential (mEPP) amplitudes, suggesting functional presynaptic and postsynaptic mechanisms at the NMJs [147]. Using a more elegant approach, Steinbeck et al. (2016) co-cultured optically excitable Channelrhodopsin-2 (ChR2)-expressing human ESC-derived MNs with human primary myoblasts for 6-8 weeks to form NMJs *in vitro*. Optogenetic stimulation was found to induce muscle contractions, which were subsequently blocked by the addition of an AChR blocker. Quantification of AChR subunits by qRT-PCR revealed that these 6-week co-cultures expressed the fetal gamma subunit, but not the adult epsilon

subunit [148], indicating that their co-culturing method likely only recreated functional fetal-like neuromuscular cholinergic synapses.

In parallel, several labs have also developed SKM engineering platforms to enable the study of NMJ formation and function. Smith et al. (2013) developed a silicon-based cantilever system using primary myotubes and MN co-cultures and analyzed myotube contraction in response to neuronal stimulation via measurement of cantilever deflection using a laser and photo-detector system [149]. Uzel et al. (2016) developed a microfluidic 'NMJ-on-a-chip' platform to co-culture C2C12 myoblast-derived muscle strips and mouse ESC-derived MNs within a 3D hydrogel in continuously perfused chambers. To determine NMJ functionality, they co-cultured ChR2-expressing MNs with C2C12-derived SKM to form NMJs and used optical stimulation to induce muscle contractile activity [150]. Cvetkovic et al. (2017) constructed a cellular system made up of multi-layered tissue rings containing integrated C2C12 SKM and mouse ESC-MNs embedded in an extracellular matrix. The compacted multi-layered rings were placed on hydrogel 'bio-bot skeletons'. Glutamate activation of MNs resulted in contractions of the SKM bio-bots, and addition of the ACh blocker, curare, halted the muscle contractions [151]. Thus, these groups were successful in creating flexible platforms and methods for combining MNs and SKM and obtaining functional NMJ measurements.

In sum, these studies have highly advanced our understanding of *in vitro* NMJ formation and have propelled the development of advanced tissue engineering technologies. However, these studies still have not been able to adequately and convincingly recapitulate the complex NMJ architecture observed *in vivo*, in terms of myotube fusion efficiency, myotube strength, expression of AChRs, and formation of functional synapses. Further research into the basic structural and functional mechanisms involved in MN and SKM development will help us devise differentiation protocols that will yield mature and functional NMJ cell types and lead to the development of more effective bioengineering technologies for NMJ modeling.

4. iPSC reprogramming technology

Due to the ethical and technical issues regarding the use of human ESCs for research, the scientific community geared its efforts to the development of alternative *in vitro* stem cell technologies. In 2007, Takahashi and colleagues successfully reprogrammed human skin fibroblasts into induced pluripotent stem cells (iPSCs), which were found to be indistinguishable from ESCs in terms of morphology, proliferation, and expression of characteristic pluripotency markers. To do this, Takahashi transduced fibroblasts with retroviruses encoding four defined transcription factors: Oct4, Sox2, Klf4, and c-Myc, which function in the maintenance of pluripotency in both early embryos and ESCs [152–154]. iPSCs were shown to have unlimited proliferation capacity *in vitro* and to have the capability to differentiate into multipotent cell lineages.

The successful reprogramming of somatic cells into iPSCs has allowed the possibility for disease modeling, pharmacological screening, and regenerative medicine. iPSCs have provided us with the ability to generate and study cell types from patients and to use gene-editing technologies to determine the effect of specific mutations on disease-relevant phenotypes. CRISPR/CAS9 correction of mutations in an iPSC cell line allows for the development of isogenic controls harboring the same genetic background of the patient, thus removing second-site mutations or other genetic confounders that may affect the study of cellular phenotypes [155]. Additionally, gene editing has also aided in the generation of gene-corrected patient cells. Corrections have been successfully

achieved in iPSCs derived from ALS [156] and spinal muscle atrophy (SMA) patients [157], thereby paving the way for gene therapy applications such as autologous cell transplantation. However, one of the main issues regarding potential treatments with iPSC-derived cells is the possibility of genomic instability and tumorigenesis due to retroviral insertional mutagenesis and transgene reactivation [158]. Numerous strategies have been devised to mitigate these issues, such as the development of integration-free vectors such as Sendai virus vectors [159–161], plasmid vectors [162–163], DNA-free protein-based [164], and mRNA-based methods [165]. Nonetheless, the safety of iPSC-derived cells, as well as their long-term function and survival following autologous transplantation in humans, has not been well-established and will require extensive characterization and validation *in vitro*. Figure 2 outlines a simplified illustration of the use of iPSC reprogramming technology for *in vitro* ALS disease modeling and drug screening.

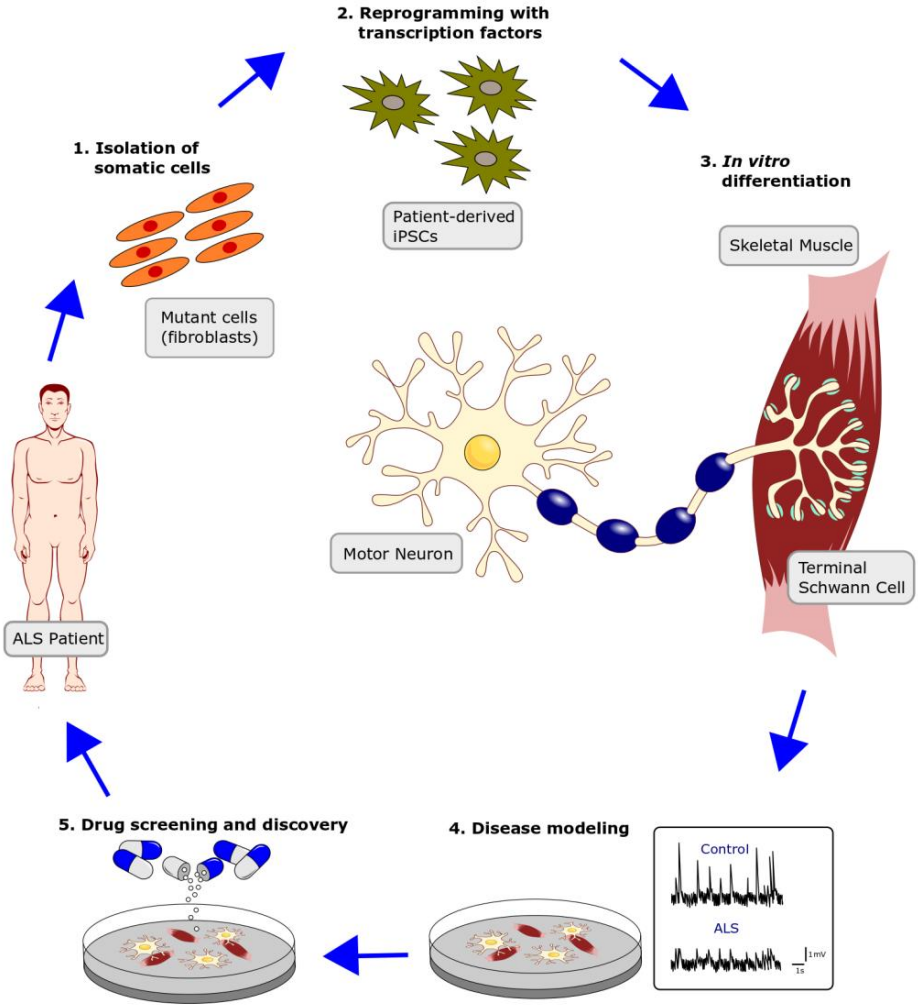

Figure 2. The use of iPSC reprogramming technology for generation of ALS NMJs *in vitro*, disease modeling, and drug screening.

Note: (1) Isolation of patient-specific somatic cells (fibroblasts). (2) Reprogramming of somatic cells using core transcription factors to generate induced pluripotent stem cells (iPSCs). (3) Directed differentiation of iPSCs to form NMJ cell types *in vitro*. (4) Disease modeling and functional characterization of ALS pathophysiology via intracellular muscle recordings in control and ALS patients. (5) Drug screening and discovery for personalized treatments for ALS patients.

4.1. Generation of iPSC-derived motor neurons

To enable the study of neurodegenerative diseases such as ALS, numerous protocols have been devised to differentiate iPSCs to enriched populations of specialized cell types. The most widely used protocols producing enriched MN populations involve (1) neural patterning using small molecule inhibitors of Bone morphogenetic protein (BMP) and Transforming growth factor β (TGF-β) signaling; (2) embryoid body (EB) formation in the presence of retinoic acid and sonic hedgehog (SHH); and (3) MN maturation with neurotrophic factors: including brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF) [166–170]. Another method that has been employed is the transgenic expression of MN-specific transcription factors [87,171] to form induced MNs, which have been shown to: (1) express a MN transcriptional profile; (2) to be electrophysiologically active; (3) to have the capacity to integrate into the developing spinal cord; and (4) to form functional NMJs with chick myotubes *in vitro* [171]. The optimization of directed differentiation of iPSCs into MNs has made it possible to conduct studies modeling various ALS subtypes (SOD1, TDP-43, and C9orf72) *in vitro* [33,87,172–177]. In a groundbreaking study, Egawa et al. (2012) provided the first evidence that patient iPSC MNs could be utilized for drug screening by rescuing ALS-associated phenotypes with a histone acetyltransferase inhibitor [174]. Since then, relevant molecular and cellular phenotypes have been identified in ALS MNs, including mitochondrial and ER stress [177], membrane hyperexcitability [33] and endosomal trafficking/lysosomal dysfunction [87], which have led to the development of promising novel drug therapeutics.

Remarkably, there have also been a few studies showing that human iPSC-derived MNs can be transplanted into ALS animal models and improve disease phenotypes [178–179]. Popescu et al. (2013) transplanted human iPSC - derived neural progenitors into control or ALS $SOD1^{G93A}$ rat spinal cords and reported successful survival and engrafting of the transplanted cells *in vivo*, as well as efficient neural progenitor specification to MNs in both control and ALS recipients [179]. However, the study did not evaluate synaptogenesis of the engrafted cells or the effects of the engraftment on motor function and lifespan of the ALS $SOD1^{G93A}$ rats, so it is difficult to make a conclusive statement about the efficiency of the transplantation method. In a similar vein, Nizzardo et al. (2013) generated and transplanted human iPSC-derived neural stem cells (NSCs) into ALS $SOD1^{G93A}$ mice via intrathecal or intravenous injections. The successful engraftment of the transplanted NSCs into the spinal cord of treated ALS mice resulted in reductions of MN loss, improvements in motor performance, and a slight increase in lifespan. Interestingly, the systematic administration was reported to have a better therapeutic effect than the intrathecal administration [178]. Although more studies are required to understand the long-term functionality and survivability of the engrafted cells, these results provide a promising framework for stem cell-based therapies of ALS.

Despite these great advances in stem cell biology, limitations remain with the current iPSC MN protocols. First, a transcriptome study has revealed that iPSC-derived MNs are closer to embryonic spinal MNs than to their *in vivo* adult counterparts [180]. Unfortunately, it remains unclear whether *in vitro* iPSC-derived MNs, which are produced in several weeks, can recapitulate the decades of *in vivo* events leading to MN degeneration. Several solutions have been employed to further mature and age iPSC-derived MNs, including the use of toxic stressors (pesticides or ROS), chemical manipulations (glutamate-induced excitotoxicity and nutrient deprivation), and the use of

trans-differentiation protocols, which directly convert mature lineages into mature cell types [181–183]. Second, most protocols require expensive and complex media formulations, employ tedious and time-consuming differentiation steps lasting 1–2 months, and have low cell efficiencies. Developing new methods that enable the generation of highly pure and functionally mature MNs in a consistent and time-effective manner will be critical for future research.

4.2. Generation of iPSC-derived skeletal muscle

The use of iPSCs for the generation of SKM has been much more challenging due to our limited knowledge regarding the inductive signals required for SKM formation, as well as the inability to produce large quantities of SKM with high purity. Multiple morphological assessments indicate that iPSC-derived myotubes are more developmentally immature and hypotrophic than primary myotubes. This may be due to: (1) suboptimal extracellular matrix composition for SKM growth [184]; (2) the lack of electrical and mechanical stimulation of SKMs [185]; and (3) the absence of neuronal and glial signals shown to promote the maturation of muscle cells *in vitro*. Most iPSC protocols have used direct reprogramming with muscle-specific transcription factors, such as MyoD and PAX7 [186–188], and/or the induction of SKM using small molecules and cytokines modulating the relevant signaling pathways in myogenesis [189–190]. Various patterned substrates and 3D culture systems have been devised to enhance myotube fusion, differentiation, and AChR formation [150,191–192]. Madden et al. (2016) successfully engineered electrically and chemically responsive, contractile human muscle tissues (‘myobundles’) using primary myogenic cells. The myobundles were fabricated by combining human myogenic precursors with an extracellular matrix in polydimethylsiloxane (PDMS) molds. Myobundles exhibited an aligned architecture, multinucleated and striated myofibers, and a $Pax7^+$ cell pool. Notably, the myobundles displayed normal responses to drugs that modulate muscle activity, demonstrating their utility for pharmacological screening [193]. In a follow-up study, the Bursac lab generated induced myogenic progenitor cells (iMPCs) via the transient overexpression of Pax7 in human iPSC-derived paraxial mesoderm cells. iMPCs readily differentiated into spontaneously contracting multinucleated myotubes expressing AChRs and other developmental muscle proteins. Under 3D culture conditions, multiple iPSC lines reproducibly formed functional SKM tissues (iSKM bundles) containing aligned multinucleated myotubes exhibiting contractile force and robust calcium transients in response to electrical or ACh stimulation. After 1 month in culture, the iSKM bundles underwent further structural maturation, hypertrophy, and the ability to generate force. Most critically, when implanted into the dorsal window chamber or hindlimb muscle in immunocompromised mice, the iSKM bundles vascularized and maintained functionality after 2–3 weeks [188]. This study was the first to demonstrate engineering of 3D contractile SKM tissues derived from human iPSCs.

To advance the translational potential of these studies, a few groups have employed iPSCs to model muscle ALS pathology *in vitro*. Lenzi et al. (2016) generated iPSC-derived control and ALS SKM using an inducible MyoD expression system and found that ALS mutant iPSCs differentiated into mature myotubes expressing functional AChRs displaying ACh-induced ionic currents [194]. Swartz et al. (2016) used a small molecule - based protocol for the generation of multinucleated skeletal myotubes from C9orf72 ALS patient iPSCs and observed no aberrant changes in SKM differentiation efficiencies, TDP-43 localization, and ubiquitin or p62 pathologies specific to C9orf72 ALS patients [195]. These initial studies suggest that muscle function is likely not the

primary cell target in ALS or that the SKMs generated with this specific protocol are missing critical factors essential for eliciting the NMJ pathology. More research is required to understand the role of ALS SKM in the etiology of the disease. Still, these studies serve as proof that diseased iPSCs can be utilized to recapitulate many pathological features of the disease, thus providing a platform for studying the disease *in vitro.*

4.3. Generation of iPSC-derived Schwann cells

Currently, there are no studies that have reported the generation of TSCs, but a few studies have created protocols for generating myelinating Schwann cells (SCs) from human iPSCs. Liu et al. (2012) differentiated neural crest stem cells (NCSCs) from iPSCs using EB formation and PA6 stromal cell line-conditioned medium with FGF2, Rock inhibitor, and ascorbic acid [196]. NCSCs were FACS-sorted using p75 neurotrophin receptor (p75NTR) and subsequently differentiated into SCs by culturing NCSCs in specialized media supplemented with neuregulin-1/heregulin-β1 for 40 days. They demonstrated that the SCs expressed GFAP, S100β, p75NTR, Sox9, and myelin markers such as PMP22 and MBP using immunohistochemistry and gene expression profiling. They validated the functionality of their SC differentiation protocol by demonstrating that human ESC-derived NCSCs were able to myelinate rat dorsal root ganglion (DRG) sensory axons *in vitro* [196]. A similar study by Wang et al. (2011) also devised a protocol to differentiate iPSCs to NCSCs using both EBs and neural rosette formation coupled with FACS-sorting with p75NTR. NCSCs were implanted in a rat sciatic nerve injury model, and after 1 month, the NCSCs formed cells expressing the SC marker S100β, indicating *in vivo* glial differentiation. They demonstrated that the NCSC-engrafted group had regenerated a greater number of axons and a higher number of SCs around individual axons than controls via histological analysis. These results indicated that the transplanted NCSCs had the capacity to enhance regeneration of peripheral nerves by facilitating axon myelination [197]. More recently, Kim et al. (2017) reported a protocol for producing SCs from iPSCs by using a combination of small molecules SB431542 (TGF-β signaling inhibitor), CT99021 (glycogen synthase kinase-3 (GSK-3) inhibitor), and neuregulin-1/heregulin-β1. The SCs were shown to express appropriate developmental and cell-specific markers and were able to myelinate embryonic rat DRG neurons *in vitro*. Additionally, SC transplantation into sciatic-nerve injured mice promoted nerve regeneration presumably through the release of neurotrophic factors that promote axonal growth [198]. Although the myelination capacity of the SCs was reportedly low, the protocol serves as a useful resource for producing functional myelinating SCs.

Despite the major progress in the development of robust iPSC differentiation protocols for NMJ cell types, the immature characteristics of these tissues should be a major consideration when modeling adult-onset neurodegenerative diseases [199–200]. The creation of well-designed medium formulations that accurately reflect temporal developmental processes and the use of biologically relevant extracellular matrices allowing growth, attachment, and spatial orientation of tissues [201–202] is essential to recapitulate anatomically-correct *in vivo* tissues and for more effective and predictive pre-clinical drug screening assays.

5. Generation of iPSC-derived neuromuscular junctions

To date, there is only one published study that has successfully established NMJ co-cultures combining MNs and myotubes from the *same* donor human iPSC cell line. Demestre et al. (2015) demonstrated that 3-week MN-myotube co-cultures formed putative NMJs, expressing relevant pre- and postsynaptic proteins [203]. One week after co-culturing *in vitro*, AChR clusters appeared, and early outgrowing neurites became closely opposed to end-plate regions. At 3 weeks, MNs induced the maturation of muscle cells and AChR aggregation, eventually resulting in the formation of NMJs, which were assessed by the appearance of AChR and MN contact sites. While the authors conducted electrophysiological analysis of SKM to assess functionality, there was no electrophysiological data acquired with the NMJ co-cultures, thus precluding conclusive statements about the functionality of their NMJ model and its use for drug screening and therapeutics.

Because of the technical challenges associated with generating NMJ cell types from the same iPSC line, most groups have opted to form NMJs by co-culturing iPSC-derived MNs and primary muscle cell lines, particularly due to the latter's widespread availability and relatively simple growth conditions. Toma et al. (2015) successfully co-cultured human iPSC-derived MNs with chick myotubes and found that MNs extended their axons along the myofibers. The iPSC-MNs were shown to form stable NMJs with anatomical and physiological features of their *in vivo* counterparts, including the expression of MN glutamate receptors and muscle AChRs and normal electrophysiological properties (as assessed by the synaptic vesicle cycling dye FM4-64 and intracellular electrode recordings at the muscle). Toma et al. also demonstrated that transplanted iPSC-MNs formed anatomically-correct NMJs with previously denervated muscle fibers, restored muscle contractile force, and attenuated denervation-induced atrophy [204]. Most impressively, the iPSC-MNs remained functional several weeks after transplantation, providing support for their use in cell replacement therapies to restore function to denervated muscles after disease or injury.

To identify potential drug therapeutics for neuromuscular diseases, a few studies have reported generating NMJs *in vitro* from diseased patient iPSCs. Shi et al. (2018) investigated the pathogenic mechanisms underlying C9orf72 ALS repeat expansions in MNs. They utilized the forced expression of lineage-specific transcription factors to convert control and C9orf72 ALS patient iPSCs into MNs. To examine whether C9orf72 MNs recapitulated ALS neurodegenerative processes, they performed longitudinal tracking of MN survival *in vitro*. Stress conditions, such as high glutamate stimulation and the removal of neurotrophic factors, caused a rapid degeneration of ALS MNs in culture. They revealed that the C9orf72 MN degeneration was due to deficits in vesicle trafficking and lysosomal biogenesis, resulting in accumulations of glutamate receptors in MN neurites and dendritic spines. The researchers were subsequently able to rescue C9orf72 MN survival and glutamate receptor levels by treatment with Apilimod, a PIKFYVE kinase inhibitor regulating endolysosomal trafficking and kinetics [87]. Moreover, Shi et al. (2018) showed that ChR2-expressing control and ALS MNs co-cultured with primary chick muscle repeatedly induced myotube contraction following depolarization with light, indicating the formation of functional NMJs. Because the co-cultures were maintained under normal medium containing neurotrophic factors, the number and force of the contractions were not significantly different between control and ALS NMJs [87]. Future experiments are required to determine whether there are any differences between control and ALS NMJs under stress-inducing conditions and whether pharmacological agents ameliorate any potential deficits at ALS NMJs.

In addition to ALS iPSC NMJ models, other groups have attempted to establish similar neuromuscular disease models. Yoshida et al. (2015) generated iPSC-derived MNs from patients with spinal muscular atrophy (SMA), a disease characterized by degeneration of lower MNs, leading to progressive paralysis and muscular atrophy [205–206]. The human SMA iPSC-MNs were co-cultured with differentiated mouse C2C12 muscle, and after 40+ days, α-BTX-positive AChRs were found clustered opposed to synaptic vesicle protein 2 (SV2)-positive synaptic nerve terminals. Myotubes innervated by SMA MNs exhibited a reduced number and size of AChR clusters at various differentiation time points relative to control NMJs [206]. These deficits were reminiscent of the early stages of SMA, where NMJ developmental abnormalities precede MN loss. Valproic acid (VA), which has been shown to increase Survival MN (SMN) gene levels, was able to rescue AChR clustering in SMA iPSC MN and myotube co-cultures [206–207]. Yoshida et al. (2015) were able to replicate SMA phenotypes *in vitro* and provide supporting evidence for VA as a promising therapeutic drug candidate for SMA. Whether VA ameliorates any abnormal electrophysiological properties at the NMJ was not assessed in this study but would be a next critical step for the complete validation of this disease model.

In sum, these studies provide a solid framework for the use of diseased iPSCs to form NMJs *in vitro* and to study molecular pathways involved in NMJ dysfunction. Most importantly, the generation of functional iPSC-derived NMJ models provides the possibility for the discovery of drug treatments that may improve neuromuscular function and restore muscle contractility in patients.

6. Challenges with the formation and study of iPSC-derived NMJs *in vitro*

Despite the major advances in the generation of iPSC-derived cell types and co-culturing methods to generate NMJs, iPSC-derived NMJs remain immature and unstable in comparison to NMJs *in vivo*. Co-cultures using iPSC-derived MNs and muscles typically have short lifespans (up to 1 month), likely due to suboptimal culturing conditions lacking the proper biochemical context and microenvironment for each cell type. Recreating the complex cellular milieu at the NMJ has been challenging, as the detailed nature of the trans-synaptic signals and molecular mechanisms regulating NMJ structure and function are not well understood. A major factor that may play a crucial role in the formation of NMJ *in vitro* is the presence of TSCs in co-cultures, as they have been shown to be critical for NMJ development and function. In fact, glial cells at the NMJ have been reported to: (1) regulate glutamate receptor clustering in SKMs via Wnt signaling [208]; (2) coordinate TGF-β-mediated retrograde signaling controlling presynaptic differentiation [209]; (3) modulate synaptic transmission [210]; (4) promote synaptic elimination [211]; (5) control guidance of regenerating axons to muscles post-injury [212–213]; and (6) aid in muscle AChR cluster maturation [47]. Multiple studies have shown the beneficial effect of SCs on neural function in co-culture systems [214–217], however, no study has succeeded in incorporating SCs into an *in vitro* model of NMJs. A neuron-muscle-Schwann cell tri-culture derived from patient iPSCs would produce a more structurally and physiologically accurate NMJ that would address fundamental questions about the roles of each cell type in ALS pathogenesis, as well as provide the ability to effectively screen for drug therapeutics for the disease. More research is required to understand the basic biology underlying TSCs and to generate viable iPSC differentiation protocols for their generation *in vitro*. Additionally, evidence suggests that microglia play a crucial role in the activation of neuroinflammation in ALS. Increasing stress and injury causes ALS MNs to release signals to

microglia, which in turn, release ROS and pro-inflammatory cytokines that promote MN cell death [218]. A few studies have reported the generation of iPSC-derived microglia [219–220] and their inclusion in the NMJ co-cultures may provide a better simulation of ALS phenotypes.

The other challenge is the need for high-throughput screening platforms for ALS drug discovery. Several co-culturing NMJ studies have attempted to set up phenotypic and drug screening models assessing Ca^{2+} kinetics and electrophysiological parameters [138,148–150,221–223]. More recently, several laboratories have employed multi-well microelectrode arrays (MEAs) to characterize MN electrical activity in control and diseased genotypes and after application of drugs or pharmacological agents [33,224]. Most notably, Wainger et al. (2014) utilized MEA recordings to assay hyperexcitability in ALS iPSC-MNs and were able to rescue mutant phenotypes using the Kv7 channel activator retigabine [33], which has been shown to have a modest effect in reducing MN axonal excitability in ALS patients [225]. MEA platforms may be used in conjunction with microchannel structures for cell compartmentalization, thereby providing the opportunity to assay electrophysiological activity in separate pre-and postsynaptic cells and to screen for potential drug therapeutics. Moreover, Kiskinis et al. (2018) developed and optimized an all-optical electrophysiology system 'Optopatch' to study the electrical properties of human ALS iPSC-derived MNs [226–227]. The relative ease of acquiring large quantities of functional data from cellular populations using the Optopatch may be useful for larger scale co-culturing experiments, although the accuracy and temporal resolution lags behind conventional patch clamping methods.

An alternative approach was taken by Santhanam et al. (2018), who generated an NMJ model in which spatially segregated populations of human MNs and myotubes were cultured in a two-chambered construct connected through microtunnels. Electrical stimulation of the human iPSC-derived MNs resulted in robust myotube contractions as assayed by video imaging. The system was validated for its pharmacological and drug screening relevance by measuring the dose-response curves for various NMJ toxins including curare, botulinum toxin, and bungarotoxin [228]. This sensitive screening platform could be highly useful for measuring functionality of ALS iPSC-derived NMJs after drug application.

Hence, in order to facilitate drug discovery, we must be capable of evaluating the functionality of NMJs using automatized, high-throughput systems. The use of optogenetically-controllable MNs for electrical stimulation coupled with sensitive techniques to assay muscle contraction will improve our understanding of NMJ function in both healthy and diseased states and be invaluable for screening novel therapeutic agents for neuromuscular disorders.

7. Concluding remarks

Drug development for the treatment of neurodegenerative diseases is a challenging field. Many drugs tested in animal models fail in human clinical trials due to lack of efficacy or toxicity [229], suggesting that the preclinical animal models are not adequately recapitulating the human disease. These translational failures may be due to inherent biological differences between species or methodological flaws in the animal and human studies, which lead to erroneous data and conclusions about mechanisms underlying the disease and reduced predictive efficacy of treatments on human disease [230]. Neurodegenerative diseases like ALS are complex, multifactorial diseases that are likely caused by several independent mechanisms and may require the simultaneous targeting of several proteins and/or cellular pathways [231]. *In vitro* ALS modeling using iPSC-derived cell

types may facilitate the study of the effects of genetic variants on cellular and molecular phenotypes and lead to the development of pharmacological screens for phenotypic modifiers of the disease.

Lastly, there is a myriad of evidence from animal models and human patients suggesting that the early ALS pathogenesis may occur at NMJs. Using iPSC technology to model ALS NMJs has the potential to provide valuable insights into the basic molecular mechanisms of the synaptic pathology and the contribution of non-neuronal cells (muscles and SCs) to the disease. It is likely that early interventions for synaptic dysfunction are critical for the subsequent irreversible loss of MN axonal and cell body degeneration. Therefore, therapies targeting pre-symptomatic pathological mechanisms (such as defects in axonal integrity and synaptic transmission) could be a powerful way to halt or delay the disease process. As summarized in this review, numerous studies have attempted to recreate neuromuscular connectivity *in vitro*, however, further optimization of co-culturing methods for NMJ cell types is required to yield more physiologically relevant and mature NMJs. Although there are many technical challenges, the rapid developments occurring in iPSC/stem cell biology and biomedical engineering will enable the systematic construction and high-throughput validation of ALS patient NMJs *in vitro*, leading to the discovery of efficacious pharmacological treatments for the disease.

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Conflicts of interest

All authors declare no conflicts of interest in this paper.

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