Creating stem cell-derived neuromuscular junctions in vitro

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Abstract
Recent development of novel therapies has improved mobility and quality of life for people suffering from inheritable neuromuscular disorders. Despite this progress, the majority of neuromuscular disorders are still incurable, in part due to a lack of predictive models of neuromuscular junction (NMJ) breakdown. Improvement of predictive models of a human NMJ would be transformative in terms of expanding our understanding of the mechanisms that underpin development, maintenance, and disease, and as a testbed with which to evaluate novel therapeutics. Induced pluripotent stem cells (iPSCs) are emerging as a clinically relevant and non-invasive cell source to create human NMJs to study synaptic development and maturation, as well as disease modeling and drug discovery. This review will highlight the recent advances and remaining challenges to generating an NMJ capable of eliciting contraction of stem cell-derived skeletal muscle in vitro. We explore the advantages and shortcomings of traditional NMJ culturing platforms, as well as the pioneering technologies and novel, biomimetic culturing systems currently in use to guide development and maturation of the neuromuscular synapse and extracellular microenvironment. Then, we will explore how this NMJ-in-a-dish can be used to study normal assembly and function of the efferent portion of the neuromuscular arc, and how neuromuscular disease-causing mutations disrupt structure, signaling, and function.

KEYWORDS
disease modeling, drug discovery, hiPSC, neuromuscular junction, NMJ, stem cells

1 | INTRODUCTION

The motor neuron-muscle synapse is the primary site of neuromuscular interactions and has been studied widely due to its large size and relative accessibility compared to synapses in the central nervous system. The vertebrate neuromuscular junction (NMJ) is composed of the presynaptic lower motor neuron originating from the ventral horn of the spinal cord (Figure 1A), the postsynaptic muscle myofiber (Figure 1B), and terminal Schwann cells associated with the synapse (Figure 1C). Muscle contraction occurs when acetylcholine (ACh) is released from synaptic vesicles in the presynaptic nerve terminal and...
bind to nicotinic acetylcholine receptors (nAChR) in the postsynaptic motor end plate of the muscle fiber (Figure 1C). Interactions at the NMJ are required for normal development and synapse function, and are necessary for NMJ maintenance and survival.\textsuperscript{5-7} Disease pathology in neuromuscular disorders is often coupled with maturation defects in the NMJ, which typically precede the onset of progressive disease symptoms.\textsuperscript{8-15} Abnormalities associated with the NMJ may initiate complete denervation of muscles, escalating subsequent muscle degeneration.\textsuperscript{16} The neuromuscular synapse is therefore an important site to assess early onset of disease pathology.

Several obstacles, however, prevent systematic experimental analysis of NMJs in patients with neuromuscular disorders. The inherent variability in the pathophysiology of many neuromuscular syndromes makes accurate modeling of them difficult and invasive biopsies are required to thoroughly examine disease pathology.\textsuperscript{17} Furthermore, these samples yield NMJs from a specific point in time and do not reveal temporal pathological changes associated with disease progression. Animal models of NMJ disorders have provided valuable insight into disease etiology. For example, Murray et al. used a spinal muscular atrophy (SMA) mouse model to show that denervation of NMJs was present before the onset of detectable disease symptoms.\textsuperscript{13} Similarly, Frey et al. used SOD1\textsuperscript{G93A} mice to show significant denervation in limb muscles occurred about 40 days before progressive muscle weakness was displayed.\textsuperscript{10} A wide range of animal models, from dogs and zebrafish to invertebrate models like Drosophila and C. elegans have been used to elucidate neuromuscular disease pathophysiology.\textsuperscript{18-21} While these models have revealed a wealth of information, they have critical limitations. There is increasing evidence that animal models do not fully recapitulate human disease phenotypes. Numerous clinical trials have failed due to preliminary studies in animal models inadequately predicting human responses to compound exposure.\textsuperscript{22-29} Human NMJs are distinct from murine neuromuscular synapses, both morphologically and molecularly. Human NMJs are smaller and more fragmented with thinner pre-terminal axons.\textsuperscript{30,31} Human NMJs have extensive postsynaptic folds, compared to other vertebrate models, which are thought to amplify transmitter action.\textsuperscript{31} Healthy, adult human NMJs are relatively stable and do not undergo extensive degeneration and remodeling over time as has been shown in murine models.\textsuperscript{32,33} Comparison of the proteome between human and murine NMJs has revealed significant differences in gene expression levels in molecular pathways known to impact NMJ development and function, including synaptic signaling, axonal guidance, and cAMP-response element binding protein (CREB) signaling in neurons.\textsuperscript{30} These differences highlight the importance of using human NMJs to study neuromuscular development and maintenance, as well as pathological mechanisms of human neuromuscular disorders.
Human induced pluripotent stem cells (hiPSCs) are emerging as a promising model with which to study human NMJ dynamics. Patient-derived NMJs would help identify pathological mechanisms in diseases with multifaceted genetics. Furthermore, CRISPR-created variants of patient-derived cell lines could help detect downstream effects of single gene mutations. Ultimately, iPSC-based models would enable analysis of patient-specific responses to therapeutic strategies in vitro, thus validating personalized treatment for people suffering from peripheral neuropathies and ensuring greater confidence that observed responses will translate to human tissues in vivo.\textsuperscript{34,35} Given the value of iPSCs for disease modeling and therapeutic efficacy evaluations, as well as the importance of accurately modeling NMJ development, this review will discuss recent efforts to create stem cell-derived neuromuscular junctions using classical culturing techniques, as well as novel biomimetic platforms. We will highlight strengths and limitations of each approach, emphasizing gaps in our knowledge and specific areas that require further refinement to fully recapitulate and analyze NMJ formation and function in vitro.

2 | MOLECULAR MECHANISMS OF NMJ DEVELOPMENT

Recapitulating the molecular mechanisms of synaptogenesis and NMJ maturation may be key to exploiting stem cell-derived in vitro NMJ models. While this process remains incompletely understood, numerous studies have revealed key pathways modulating synaptic development and communication at the human NMJ.\textsuperscript{2,3,36,37} Neuro-muscular synapse formation and maturation is tightly regulated through a series of complex interactions between motor neurons, muscle fibers, and Schwann cells. Initially, myofibers accumulate AChRs in their centers in a process called prepatternning.\textsuperscript{27-29} The primitive clustering of receptors may serve as guidance cues for migrating axons, but their exact role has yet to be fully elucidated. Receptor density increases in clusters where axons make contact with muscle, while non-innervated AChRs disperse over time. The postsynaptic membrane invaginates forming junctional folds at the primitive NMJ inducing a morphological shift from a plaque-like shape to a mature branching pattern resembling a pretzel.\textsuperscript{1,2} Signaling cues from the pre- and post-synaptic terminals, as well as from Schwann cells, regulate NMJ development, maturation, and maintenance. One of the most well characterized pathways regulating NMJ development is the agrin/LRP4/MuSK pathway. The sulfate proteoglycan protein, Agrin, is synthesized by motor neurons and is essential for stabilizing synaptic AChR clusters.\textsuperscript{40,41} Agrin binds the muscle-bound transmembrane lipoprotein receptor-related protein 4 (LRP4), which triggers dimerization and phosphorylation of muscle-specific tyrosine kinase receptor (MuSK, Figure 2).\textsuperscript{43,44} MuSK appears to be a key postsynaptic organizer and regulator of NMJ development and maturation. MuSK deficient mice do not have prepatterned muscle fibers and they do not form AChR clusters or NMJs in response to innervation.\textsuperscript{38,39,45,46} MuSK interacts with a variety of molecules to regulate

\begin{figure}[h]
\includegraphics[width=\textwidth]{Agrin_LR4 MuSK organizing complex}
\caption{The Agrin/LRP4/MuSK organizing complex. Wnt signaling molecules bind to MuSK, activating Dvl in the cytoplasm. The Dvl protein bridges MuSK with PAK1 to bring about structural changes in the cytoskeleton. Upon innervation, neural agrin binds to LRP4 on the muscle membrane. LRP4 interacts with MuSK causing dimerization and phosphorylation (P). Downstream-of-tyrosine-kinase 7 (Dok7) also binds to MuSK and is required to complete the MuSK multi-protein complex stabilizing AChR clusters at the NMJ. The Agrin/LRP4/MuSK complex mediates interactions with an array of proteins including the tyrosine kinase Abi and the metalloenzyme GGT, as well as Rac and Rho GTPases. The Agrin/LRP4/MuSK complex recruits the adaptor protein Rapsyn to developing AChRs. Rapsyn interacts with both AChRs and the actin crosslinker \(\alpha\)-actinin to anchor the AChR cluster to the cytoskeleton. The MuSK complex recruits heat shock proteins to prevent rapid degradation of Rapsyn and promote long term stabilization of AChRs at the NMJ. Retrograde transport of GDNF from muscle to the motor neuron works to stabilize and mature the pre-synaptic terminal. Figure adapted from Refs. 3,42}
\end{figure}
neuromuscular development. Prior to innervation, Wnt signaling molecules bind to MuSK, which is thought to activate Dishevelled (Dvl) in the cytoplasm. The Dvl protein bridges MuSK with the p21 kinase (PAK1) to bring about structural changes in the cytoskeleton, which allows MuSK to induce AChR clustering by regulating actin dynamics (Figure 2). Downstream-of-tyrosine-kinase 7 (Dok7) also binds to MuSK and is required to complete the MuSK multi-protein complex stabilizing AChR clusters at the NMJ through a series of complex mechanisms (Figure 2). The Agrin/LRP4/MuSK complex mediates interactions with an array of proteins including the tyrosine kinase Abl and the metalloenzyme geranylgeranyl transferase 1 (GGT), as well as Rac and Rho GTPases (Figure 2). GGT facilitates Rho GTPase activation, which controls cytoskeletal changes in actin involved in AChR trafficking, membrane insertion and immobilization. The Agrin/LRP4/MuSK complex leads to the recruitment of the adaptor protein Rapsyn to developing AChRs and promotes stabilization of the receptors at the synapse. Rapsyn interacts with both AChRs and the actin crosslinker α-actinin to anchor the AChR cluster to the cytoskeleton (Figure 2). Rapsyn has an incredibly short half-life, therefore it is thought that the MuSK complex recruits heat shock proteins to prevent rapid degradation of Rapsyn and promote long term stabilization of AChRs at the NMJ (Figure 2). Terminal Schwann cells aid in eliminating redundant nerve terminals by releasing transforming growth factor β (TGFβ), thereby aiding in NMJ maturation. Gli and cell-derived neurotrophic factor (GDNF) from the myofiber also regulates maturation of the presynaptic terminal through retrograde signaling and promotes superfluous synapse elimination (Figure 2). Together, these molecular events work to induce, stabilize, and mature neuromuscular synapses. Exogenous application of small molecules, like neural agrin and growth factors, may promote robust NMJ development and maturation in stem cell-derived NMJ cultures. Agrin has been shown to enhance AChR development in vitro in primary muscle fibers, but has yet to be tested in stem cell derived skeletal muscle co-cultures. Treatment with growth factors can augment the density of axonal branches and induce skeletal muscle hypertrophy. Overexpression of GDNF in vivo triggers hyperinnervation of skeletal muscle in mice. Determining the optimal concentration of GDNF in stem cell co-cultures remains unexplored and may be key to promote normal physiological levels of muscle innervation in vitro. A wide variety of growth factors are commonly used in differentiating both iPSC-derived motor neurons and skeletal muscle, but their precise modulation in developing NMJ cultures has yet to be studied. In fact, neuronal co-culture with skeletal muscle alone may affect intrinsic growth factor production in cultured cells, further hampering our ability to control concentrations to promote synaptogenesis. Systematic experimental analysis of applying small molecules and growth factors will be necessary to create stem cell-derived NMJ models that recapitulate in vivo development. The pathways discussed here are a small fraction of the known molecular cascade of events controlling vertebrate NMJ formation and maturation, however, the complete molecular network is beyond the scope of this review. For detailed and comprehensive reviews on the molecular mechanisms of NMJ development, see Wu et al. and Ferraro et al. Induced pluripotent stem cells are readily becoming an invaluable tool to decipher neuromuscular development and disease mechanisms. Stem cell-derived tissues created from the same individual ensures a complete patient-specific neuromuscular disease profile, thereby eliminating possible cellular mismatch through mechanisms that are not fully understood. A major strength of this approach is the ability to pair normal neurons with diseased muscle and vice versa to figure out where the root of the phenotype lies. Incomplete cellular differentiation and maturation, however, has been a critical roadblock to using iPSCs for studying adult-onset diseases and drug discovery studies. Immature, fetal-like cells may not display advanced or late-onset disease phenotypes and underdeveloped cells may respond differently to pharmacological assays compared to fully mature, neuromuscular synapses in adult tissue. Innovative cell culturing technologies inspired by a greater understanding of native embryogenesis are beginning to produce more mature cellular phenotypes. These recent advancements will enable the de novo creation of functional neuromuscular junctions in vitro that far surpass previous attempts and will undoubtedly change the future of personalized neuromuscular disease therapies. In the past decade, iPSCs have been created by a variety of reprogramming methods and starting cell types, including skin fibroblasts, peripheral blood mononuclear cells (PBMCs), adipose tissue, keratinocytes, and urine cells. Urine-derived iPSCs are gaining in popularity as a new stem cell source due to the safe and non-invasive nature of somatic cell collection. A recent study claims exfoliated renal system epithelial cells are the cell type that is reprogrammed in voided urine, however, these cells have yet to be well characterized. Regardless, the resulting iPSCs possess self-renewal and multilineage differentiation potential. Yi et al. successfully differentiated urine-derived iPSCs into motor neurons. Furthermore, they compared motor neurons differentiated from urine and blood-based iPSCs and found there was no significant difference in differentiation potential. Numerous groups have made iPSC-derived motor neurons, including terminally differentiated cells from patients with neuromuscular diseases. Late-onset disease phenotypes, however, may not be accurately displayed in iPSC-derived motor neurons. Differentiated cells that have been reprogrammed back into a pluripotent state may not retain the epigenetic and phenotypic features of aged cells. Furthermore, iPSC-derived motor neurons are functionally immature and must undergo lengthy culture times to achieve mature cell traits. Direct reprogramming of patient fibroblasts is another approach to generate motor neurons that may be better suited to study late-onset neuromuscular diseases, such as amyotrophic lateral sclerosis (ALS). Transdifferentiated motor neurons retain characteristics of aged cells, including loss of heterochromatin and nuclear organization, extensive DNA damage, and increased senescence-associated beta-galactosidase (SA-β-Gal) activity. Direct reprogramming bypasses
the pluripotent state by using transcription factors and small molecules to direct differentiated cells to neuronal fates.\textsuperscript{100-102} While this strategy yields functional motor neurons with high efficiencies, cells are typically reprogrammed using viral vectors to deliver transcription factors. One potential problem with this approach is the possibility of random integration of viral DNA into the host cell genome. Viral DNA insertional mutagenesis could possibly mask or change patient-specific disease phenotypes. Both direct reprogramming and iPSC-based models have advantages and shortcomings, but overall, both have produced comprehensive and efficient protocols to create high quality motor neurons, a key component of the NMJ. For a review of reprogramming methods, see Morris and Daley.\textsuperscript{103}

Another vital component of the NMJ is skeletal muscle. Methods to generate mature, functional iPSC-derived myotubes have proved to be more challenging, but new protocols are emerging with promising results. Since the advent of pluripotent stem cells, there have been two basic strategies to derive myogenic precursor cells. Most studies have used direct reprogramming via forced expression of myogenic transcription factors, such as MYOD, PAX3, or PAX7, using a viral gene delivery approach.\textsuperscript{104-108} A drawback of direct reprogramming, in addition to the possible integration of viral DNA into the host cell genome, is that expressing one master transcription factor will only turn on a subset of muscle genes. Without the full complement of transcripts critical for myogenesis, the skeletal muscle produced may not fully recapitulate native developmental processes. An alternative method to generate skeletal muscle progenitors from iPSCs avoids these shortcomings by using stepwise induction protocols with small molecules and growth factors that recapitulate embryonic myogenesis. Chal et al. used reporter iPSC lines and gene expression analysis to show that activating the Wnt signaling pathway by using a GSK-3\textsuperscript{b} inhibitor (CHIR-99021) directs cells to paraxial mesoderm fates.\textsuperscript{109} Furthermore, they used a small molecule inhibitor of bone morphogenetic protein 4 (BMP4) (LDN-193189) to prevent cells from becoming lateral plate mesoderm. This method, in combination with growth factors to promote myogenesis, produced striated, spontaneously contracting, skeletal muscle myotubes from iPSCs after about 4 weeks of differentiation in 2D cultures, without using cell sorting or purification techniques. Other groups have used similar strategies, some combined with cell sorting and purification, to obtain myogenic progenitors from human iPSCs.\textsuperscript{110-113} For a comprehensive review of skeletal muscle cell induction from pluripotent stem cells see Kodaka et al. and Miyagoe-Suzuki and Takeda.\textsuperscript{114,115}

Terminal Schwann cells (TSCs) are the third cell type found in normal NMJs. This cell is distinct from myelinating Schwann cells and forms a cap around the nerve-muscle contact (Figure 1C).\textsuperscript{1-3} TSCs have been shown to play critical roles in the development and maintenance of the synapse.\textsuperscript{116-121} While these cells have successfully been differentiated from iPSCs,\textsuperscript{122-124} they have yet to be included into NMJ culturing platforms. Recent studies, however, have shown that TSCs can form spontaneously in vitro from both myogenic cells in 2D cultures and axial stem cells in 3D organoids.\textsuperscript{125,126} Terminal Schwann cells were localized to NMJs, capping neuronal terminals, establishing that all three cell types can be cultured simultaneously. Adding TSCs into NMJ cultures in a controlled and deliberate process will be an exciting avenue for future studies and may help to generate complete neuromuscular synapses, recapitulating in vivo NMJ formation.

4 | 2D IN VITRO NMJ MODELS

Stem cell-derived models of single, mostly homogeneous cell types exist and have been used successfully to reveal a wide range of developmental processes and complex disease mechanisms.\textsuperscript{83,109,127-129} Co-culturing different cell types together to study tissue interactions during development, however, is considerably more challenging to establish and maintain over time. Numerous biological factors control and strictly regulate cell migration and cell–cell interactions. Extracellular guidance cues, including substrate topography and morphogen gradients established by soluble factors can direct cell identity, polarity, and movement.\textsuperscript{130-133} Ligands must also be presented in the right spatial and temporal orientation to engage cell-surface receptors during a specific window of development.\textsuperscript{134-136} Different cell types also have widely divergent metabolic needs, requiring specialized culture media. Further confounding these in vitro models, metabolic needs for each cell type change as the cells proliferate, differentiate and mature over time. All of these factors combined make co-culturing challenging, even for two different cell types. Many of these obstacles, however, are currently being surmounted through the application of innovative technologies that incorporate endogenous molecular and architectural cues to mimic the native microenvironment. These novel, biomimetic platforms are transforming stem cell culturing methods and opening new avenues to study developmental processes, including muscle development and synaptogenesis, synaptic maintenance and repair, and pathological mechanisms associated with neuromuscular disorders. Below, we highlight some of the issues associated with maintaining neuromuscular co-cultures for NMJ studies and provide examples of how innovative culture techniques have been used to address these problems.

Traditional two-dimensional (2D) culture platforms have been used widely for NMJ models and are advantageous for their simplicity (Figure 3A). These systems are typically arranged as two layers, with differentiated myotubes on the bottom and motor neurons uniformly plated on top. Early co-culture models investigated the development, function, and maintenance of NMJs.\textsuperscript{137-141} More recent studies have shown that co-culture actually facilitates myotube development and subsequent NMJ formation.\textsuperscript{142} Myogenic differentiation can be improved further by temporally adjusting delivery of growth factors.\textsuperscript{143}

Stem cell-derived motor neurons were first incorporated into 2D nerve-muscle co-cultures in 2010,\textsuperscript{144} showing that derived cell types could form synapses in vitro. Moreover, stem cell-derived motor neurons can recapitulate disease phenotypes and, notably, can be used to measure the efficacy of pharmacological drugs on disease pathology.\textsuperscript{145} Since then, a variety of studies have used stem cell-derived motor neurons, myotubes, or both to uncover intricate molecular mechanisms of synaptogenesis, synapse functionality, and NMJ...
formation using the relatively simple 2D platform.\textsuperscript{146-148} Notably, it was shown that motor neurons and myotubes could be differentiated from the same donor line and subsequently co-cultured in a common medium for several weeks.\textsuperscript{146-148} Diseased iPSC-derived motor neurons and myotubes from patients with fused in sarcoma (FUS)-ALS have since been used to reveal endplate maturation defects in the NMJ due to toxic localization of FUS in both motor neurons and myotubes affecting NMJ maintenance and stability.\textsuperscript{149} The 2D platform is a straightforward culturing method, capable of exposing disease pathology using relatively simple molecular assays.

One drawback of high density, 2D co-cultures is the difficulty in isolating individual motor neuron-myotube cell pairs for functional analysis. Umbbach et al. found a way around this obstacle by plating motor neurons and myotubes together in low density co-cultures.\textsuperscript{150} Dual patch clamp recordings confirmed both spontaneous and multi-quantal evoked synaptic communication. Functional NMJs were only observed for about a week, however, limiting the efficacy of this model for large scale drug discovery assays using iPS-derived cell types.

Another obstacle in using iPSC-derived cells in 2D cultures is the heterogenous mix of cell types typically seen during differentiation. Puttonen et al. seeded monolayer cultures from hiPSC-derived neurospheres and observed spontaneously contracting, multinucleated myotubes along with motor neurons.\textsuperscript{148} While the authors did not specifically set out to differentiate myogenic cells from hiPSCs, their study demonstrates that myotubes can form spontaneously in heterogenous stem cell cultures and likely communicate with surrounding cells through signaling molecules, paracrine factors, and direct cell–cell junctions. In fact, obtaining highly pure motor neuron or skeletal muscle cell populations from small molecule directed differentiation of hiPSCs without using cell purification strategies is a common obstacle.\textsuperscript{151,152} On the other hand, heterogenous cell types, including glial cells, are involved in vivo NMJ formation, maintenance, and repair.\textsuperscript{1-3} Furthermore, variable cell–cell interactions are often coupled with disease pathology in neuromuscular disorders.\textsuperscript{34,153} Heterogeneous cell populations may provide a more realistic environment for modeling NMJ disorders.\textsuperscript{34,153}

The 2D culture platforms are relatively inexpensive and have been instrumental in developing protocols for motor neuron-muscle co-cultures. They have demonstrated that motor neurons and myotubes can be derived from the same individual and cultured together to form functional NMJs. This is a critical first step to establish parameters and minimum factors necessary to model genetic, neuromuscular diseases in vitro. The primary downside with 2D platforms is the difficulty in tracking and interrogating individual cell pairs with high confidence in densely populated cultures. Moreover, standard cell culture dishes typically have flat, rigid substrates, which restricts cellular migration and alters cell morphology and possibly gene expression. The extreme disparity between 2D culture conditions and the in vivo microenvironment likely contributes to underdeveloped myotube formation and weak synaptogenesis. Many 2D studies report randomly oriented myotubes, fetal-like hiPSC-derived myofibers, and asynchronous myotube contractions.\textsuperscript{143,144,150} Altering the topography of the 2D substrate is one strategy researchers are using to more accurately replicate in vivo extracellular architecture to control myotube alignment and maturation.
Myotubes align with remarkable precision in living systems, creating structurally ordered, anisotropic muscular tissue. This highly organized morphology is absolutely critical to achieve coordinated, force generating muscle contraction. The native extracellular microenvironment plays a critical role in this process, providing directional cues to dictate cell alignment. The extracellular matrix (ECM) is composed of various protein fibers that have nanoscale features that connect to and interact with muscle cells. The quantity and composition of individual fibers within the ECM specifies the rigidity and topography of the microenvironment. Numerous studies have shown that nanoscale topographical cues augment myogenic cell alignment when compared to unpatterned surfaces. Moreover, surface patterning enhances myotube development by producing wider muscle fibers with increased numbers of nuclei per myotube. ACHRs are also larger, suggesting greater receptor clustering. This is supported at the transcriptional level through upregulation of LRPs and MuSK, thereby promoting improved levels of myotube prepatterning. A main drawback of this platform, like other 2D systems, is that myotubes tend to detach from the substrate after about a week of differentiation. This has been observed in other studies using patterned and unpatterned substrates and is likely due to a combination of forceful myotube contraction, which increases with greater maturation, and increasing passive tension as cells develop in vitro.

**TABLE 1** Summary of in vitro NMJ maturation enhancers

| Application          | Advantage                                                                 | Disadvantage                                                                                     | References |
|----------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|------------|
| Surface patterning   | • Increased cell alignment/anisotropy                                      | • Increased time and cost to manufacture                                                         | 160        |
|                      | • Increased cell fusion                                                   | • Size and width of ridges and grooves in pattern must be optimized for each cell type          |            |
|                      | • Larger myofiber bundles                                                 | • Orientation of pattern relative to cell alignment and axonal pathfinding trajectories must be optimized |            |
|                      | • Directed axon pathfinding                                               |                                                                                                 |            |
| Electrical conditioning| • Simulates exercise or work leading to larger myotubes and increased hypertrophy | • Over stimulation can damage or kill cells                                                     | 168,171    |
|                      | • Increased Sarcomerogenesis and contractile force                         |                                                                                                 |            |
|                      | • Increased Ca²⁺ handling                                                 |                                                                                                 |            |
| Optogenetics         | • Precise, controlled stimulation over duration and amplitude              | • Cell transfection and transgenic cell lines might alter disease phenotypes or behavior       | 176        |
|                      | • Fine temporal resolution of cellular activation and inhibition          | • May require implantation of an optical fiber in 3D or thick constructs                        |            |
|                      | • Highly reproducible and stable responses                                 | • Increased cost                                                                                 |            |
|                      | • Increased myotube hypertrophy                                           |                                                                                                 |            |

Optogenetic technology allows for light-mediated activation of genetically engineered cells, including neurons and myotubes. Ion channels in these cells induce action potentials when stimulated with specific wavelengths of light, which allows for precise stimulation of cells in heterogenous cell cultures. Steinbeck and colleagues plated purified, Channelrhodopsin2 engineered human embryonic stem cell (ESC) -derived motor neurons under control of the human synapsin promoter onto human fetal or adult primary myoblast-derived skeletal muscle and observed muscle contraction in response to light after 6–8 weeks of co-culture. This method of neuronal stimulation ensures that muscle contraction is being modulated through bona fide NMJ signaling as there is no artificial electrical stimulation in the system. Another advantage of this approach is that chronic stimulation regimens can be used, simulating exercise programs to induce greater synapse, as well as myotube, maturation. Another benefit might be introducing other cell types, such as terminal Schwann cells, to more electrically excitable and artificial electrical stimulation has been shown to promote maturation in individual cultures of both cell types. NMJ development in co-cultures is also enhanced, giving rise to more mature phenotypes and increased neuronal and myogenic gene expression. The exact mechanisms that facilitate this process are largely unknown, but studies have shown that electrical stimulation fosters NMJ stability and accelerates NMJ development through activation of ADAM19/neuregulin/ErbB signaling pathways. Biomimetic techniques that recapitulate in vivo processes are essential to produce normal and disease tissues that progress through all developmental stages, particularly late stages, when certain disease phenotypes are established. Careful experimental analysis is necessary, however, to optimize electrical stimulation parameters that induce maximum cell maturation without damaging sensitive myotube and neuronal developmental mechanisms.
accurately mimic the in vivo NMJ. Table 1 compares the advantages and disadvantages of NMJ developmental and maturation enhancers.

Recent 2D models have provided an enormous amount of insight into the mechanisms that promote NMJ formation in vitro. Their relative simplicity makes for an attractive platform to quickly assess the viability of novel NMJ formation and maturation technologies. 2D systems, however, do not fully recapitulate in vivo architecture, likely impeding long-term culture. This impacts the ability of cells to achieve mature phenotypes and limits the ability to study NMJ maintenance and survival over time. New models are beginning to address this problem by creating matrices that support NMJ culture in three dimensions.

8 | 3D NMJ MODELS

The native NMJ microenvironment provides critical chemical and mechanical cues to cells that facilitate cell migration, differentiation and maturation. Surrounding cells with ECM permits unobstructed movement, which facilitates the natural mechanics and shape of muscle cells.177 Furthermore, axons are able to migrate freely in response to chemical cues from myogenic cells and the ECM. The 2D systems limit cell migration and overall development by prohibiting movement and contact with neighboring cells in all directions.

Models in 3D can be relatively simple, using ECM only to separate motor neurons and myotubes, essentially creating a sandwich. This method has been shown to promote mature pretzel-like clustering of AChRs at the NMJ.178 Time lapse photography is possible in this system and can be used to visualize cell migration in real time. Layers of Matrigel, a proprietary blend of proteins found in ECM that support myogenic differentiation,179,180 above and below contracting muscle fibers may serve to stabilize them, helping to prevent delamination from the substrate.

More complex 3D systems can incorporate mechanical cues to mimic in vivo processes. Stretching cells through mechanical loading has been shown to significantly increase muscle specific proteins that facilitate development of contractile machinery.181,182 Stretching regimens in both 2D and 3D systems emulate exercise induced muscle development, which stimulates cell alignment cues. Stretching muscle in 2D, however, may not be informative for the purposes of NMJ modeling as fibers may detach from the substrate with increased tension. Mechanical stretch in 3D systems can be achieved by applying tension to the matrix surrounding the cells and muscle fibers align with the direction of strain (Figure 3C).183

Culturing neurons and myotubes together in 3D enables cells to receive extracellular cues from all membrane surfaces, more faithfully reproducing the in vivo environment. Matrigel and fibronectin are popular 3D matrices; however, collagen has also been shown to promote neuromuscular interactions and facilitate NMJ formation.184 This is not surprising considering the high concentration of collagen in the ECM of native skeletal muscle.185 This highlights the importance of experimenting with different matrices to optimize 3D scaffold parameters for specific cell types. Compliant ECM allows cells to freely migrate, while providing structural support to establish cell-cell signaling, fusion, and anisotropic alignment. This enhances cell differentiation and maturation, leading to larger myotubes and enhanced NMJ development. Long culture times are also possible with 3D systems, enabling access to diverse cell types. Moreover, cells can be evaluated at different stages of development, elucidating temporal changes in disease phenotypes. On the other hand, thick ECM and high cell density in 3D cultures can lead to visualization and imaging difficulties, as well as unequal oxygen and nutrient distribution, causing cell death. Furthermore, patch clamp and electrophysiological evaluation is more challenging and often impossible in 3D systems.

9 | CANTILEVER NMJ MODELS

Microscale cantilevers have conventionally been used to investigate muscle contractile function.186–188 Generally, cantilevers are composed of a rigid post or beam anchored at one end to a supporting structure. Bending of the post can then be used to derive the amount of force acting on the cantilever. A novel study in 2013 reported an automated system to evaluate NMJ functionality using silicon cantilevers (Figure 3B).189 A custom-designed scanning laser and photodetector system analyzed 32 individual cantilevers simultaneously in real time under automated control. Cantilever deflection in response to muscle contraction was used to measure the effect of controlled motor neuron stimulation providing functional force assessments in an automated, multiplexing, high-throughput platform. There are several advantages to this system over other NMJ models. Automated scanning of 32 isolated cantilevers provides significant statistical power. Moreover, muscle contractile force changes, especially in diseased NMJ systems, indicate the level of functional rescue in response to drug treatment. Additionally, this system facilitates analysis of individual NMJs, as opposed to multiple NMJs acting in concert.

Cantilevers are useful in a variety of different configurations and can be used in single or multi-post arrays. They can be used in 3D systems and combined with anchoring points to induce strain on skeletal muscle (Figure 3D).190 Anchoring points have been shown to promote anisotropy because cells align in the direction of strain.74,170 Cantilevers can be used to apply tension to cells in 3D hydrogels, inducing mechanical stress and enhancing cell alignment and myotube maturation.191 A primary benefit of using cantilevers is that it provides a method to quantitatively measure the contractile force of myotubes in culture. This readout delivers a diagnostic tool to gauge the effect of pharmacological drugs on synaptic transmission, maintenance and repair in healthy and diseased NMJs. Furthermore, these systems are capable of multiplexing and automation, which is advantageous for high throughput or semi-high throughout pharmacological assays.

10 | BIOMEMS NMJ MODELS

Biological microelectromechanical systems (BioMEMS), sometimes referred to as “lab on a chip” are miniature, compartmentalized systems, normally used to study neuronal based co-cultures. Two
chambers are separated with a physical barrier and contain numerous microchannels for axonal growth. They are usually composed of polydimethylsiloxane (PDMS) and are typically the size of a microscope slide to facilitate optical examination. These systems can be used with broad field electrical stimulation to excite motor neurons for functional assays, including drug efficacy and toxicity trials in diseased NMJ constructs (Figure 4A).\textsuperscript{192,193} Furthermore, BioMEMS are ideal systems to examine disease pathology in the pre- and post-synaptic junction. Compartmentalization allows for temporal and spatial interrogation of each cell type and is useful when applying therapeutics targeting only one component of the NMJ. The use of PDMS could potentially be problematic in these platforms, however, as PDMS has been associated with leaching of uncured oligomers and absorption of small, hydrophobic molecules.\textsuperscript{194}

Microfluidic technology has become a widely popular platform to create stem cell NMJ models due to the ability to construct a microenvironment that resembles in vivo design. These devices are oxygen permeable, enabling long-term culture of cells. They are compartmentalized systems, maintaining physical and fluidic isolation of different cell populations, enabling different tissue specific media applications. They can be manufactured quickly and arranged in various geometries, including multi-chambered arrays. Individual compartments and channels are amendable to surface modification, such as microgrooves, to augment cell differentiation and axonal migration (Figure 4B).\textsuperscript{195-197} Microfluidic platforms can help distinguish between proximal and distal effects of applied factors.

**FIGURE 4** BioMEM and microfluidic NMJ culturing platforms. A, BioMEMS showing the setup and microscope view with motor neurons (MN) and skeletal muscle (SM) in separate chambers. Axons migrate through an array of micro-tunnels to innervate SM. Electrodes in the motor neuron chamber induce action potentials that stimulate muscle contractions. Figure from 192. Reprinted from biomaterials, Vol 166, Santhanam N, Kumanchik L, Guo X, Sommerhage F, Cali Y, Jackson M, et al. Stem cell derived phenotypic human neuromuscular junction model for dose response evaluation of therapeutics, pg 64–78 (2018) with permission from Elsevier, license #5075181320930. B, Microfluidic device with separate chambers for neurospheres (green) and muscle strips (red) embedded in a 3D hydrogel. Micro-tunnels for axonal migration connect the two chambers. Figure from 201. Reprinted through the creative commons attribution license 4.0 (CC BY). DOI: 10.1126/sciadv.1501429
revealing key mechanisms of NMJ function, cell signaling, and transport processes."^{198-200}

A key advantage of microfluidics is the ability to incorporate multiple technologies and cell types into one system. Optogenetically engineered motor neurons enable controlled neuronal stimulation, while culturing 3D skeletal muscle tissues on compliant pillars provides a way to quantitatively measure contractile force (Figure 4B).^201,202 These technologies, when used together, provide a system to functionally and systematically compare healthy and diseased cell types to create a platform for disease modeling, as well as, neuromuscular drug screening trials.

Microfluidic technology is similar to BioMEMS and both offer many of the same advantages and disadvantages over other platforms. They both use extremely small cell and fluid volumes, minimizing reagent cost and reducing waste. They are both compartmentalized, allowing separation of cell types and subsequent individual interrogation of pre- and post-synaptic mechanisms. Both systems can be integrated with electric and mechanical stimulation and miniaturized biosensors for automation, multiplexing, and real-time data analysis. Both systems are high throughput capable, making them appealing models for hiPSC-derived NMJ disease modeling and drug discovery. Small cell volumes, however, can be difficult to seed. Furthermore, microscale fluid volumes are affected by capillary forces and surface topography, limiting the experimental reproducibility between different devices. Novel, complex chip designs will also likely require skilled production personnel and significant protocol validation to improve repeatability among different labs and individual operators. Table 2 compares the advantages and disadvantages of current NMJ culture platforms.

| Platform                        | Advantage                                                                                       | Disadvantage                                                                                      | References               |
|--------------------------------|------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|--------------------------|
| 2D                             | • Simplicity<br>• Inexpensive<br>• Well established protocols<br>• Easily reproducible<br>• Easy to observe and image<br>• Relatively homogenous cultures<br>• Easy to scale up | • Flat, rigid substrates<br>• Fixed device architecture<br>• Does not mimic the native environment<br>• Limited cell-cell, cell-ECM interactions<br>• High reagent consumption<br>• Cells anchored to plate, which alters morphology and possibly gene expression<br>• Chemical gradients are difficult to achieve<br>• Typically end point analysis only | 142-145,147,148,150    |
| 3D                             | • ECM surrounds cells on all sides<br>• More similar to native architecture<br>• Complex cell-cell interactions<br>• Increased cell migration<br>• Cells retain natural morphology | • Can be difficult to image<br>• Not compatible with patch clamp technology<br>• Unequal distribution of nutrients and oxygen leading to cell death<br>• May require additional equipment and handling expertise<br>• Reduced reproducibility<br>• Scaffolds may not be compatible with downstream applications | 178,183,190              |
| Cantilever                      | • Functional readouts possible<br>• Can be automated<br>• Capable of multiplexing<br>• Real-time analysis | • Increased fabrication time and expense<br>• Specialized equipment required | 189,191                  |
| Microfluidics, BioMEMs/lab on a Chip | • Low fluid volumes reduce waste and reagent cost<br>• Easily integrated with imaging platforms<br>• Compartmentalized, allowing separation of cell types<br>• Flexibility of device design<br>• Chemical concentration gradients possible<br>• Real-time and direct downstream analysis<br>• Short diffusion distances result in faster response times and analysis<br>• Integrated with electric and mechanical stimulation and miniaturized biosensors<br>• Automation<br>• High throughput capable<br>• Multiplexing | • Small cell volumes can be difficult to seed<br>• Leaching of surface material (e.g. PDMS)<br>• Complex chip design<br>• Increased fabrication time and labor intensive<br>• Specialized manufacturing equipment and skilled production personnel required<br>• Novel devices lack significant protocol validation<br>• Microliter scale fluid volumes are affected by surface topography and capillary forces reducing experimental reproducibility<br>• Increased manufacturing cost | 192,193,195-198,201 |
12 | CONCLUSION AND FUTURE PERSPECTIVES

Very few in vitro NMJ models have been composed entirely of human cell types and even fewer have used hiPSC-derived motor neurons and myotubes. This is likely due to the challenges of differentiating mature, hiPSC-derived skeletal muscle. Moreover, limited cell viability post differentiation in stem cell co-cultures restricts the amount of time NMJs have to fully develop. Methods that incorporate environmental cues native to the physiological NMJ niche, like electrical conditioning, topographical patterning, mechanical stretching regimens, compliant substrates, 3D technology, and anchoring points, are significantly improving hiPSC developmental phenotypes. Given that many of these technologies are being incorporated into NMJ systems, often in combinatorial approaches, it is likely that all hiPSC-based co-cultures will be used more widely in the near future.

New technologies will also enable improved assessment of NMJ function, which has been a major shortcoming of many in vitro models. The majority of NMJ studies have relied on visual inspection, using immunofluorescence and brightfield imaging, and electrophysiological recordings, using dual patch clamp technology, to assess NMJ formation. Few of these methods produce functional data in terms of muscle contractile force in response to motor neuron stimulation. Functional readouts are ideal to compare potential rescue of pharmacological drugs on pre- and post-synaptic mechanisms in neuromuscular diseases. Several studies have been successful in quantitatively measuring NMJ-induced muscle contraction and preliminary NMJ drug response testing. The platforms reviewed here, as well as new and improved strategies, will be instrumental in creating models to generate all human based stem cell-derived neuromuscular junctions for in vitro disease modeling and drug discovery. Moreover, this would enable a method to bin heterogeneous diseases involving NMJ dysfunction into subclasses for clinical trial inclusion/exclusion criteria to better predict drug efficacy.

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ETHICAL STATEMENT

We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

CONFLICTS OF INTEREST

The authors declare no financial holdings or conflicts of interest for all work presented in this Review.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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