

Review

A new era in neuromuscular junction research: current advances in self-organized and assembled *in vitro* models

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Understanding the development and function of the human neuromuscular system is crucial for deciphering the mechanisms of neuromuscular disorders and developing effective therapies. However, limitations of animal models necessitate the development of human-specific *in vitro* models to study such complex diseases effectively. Here, we discuss different approaches for *in vitro* neuromuscular junction (NMJ) modeling: complex self-organized models that rely on the inherent abilities of cells to form NMJs based on embryonic developmental principles and assembled models that depend on integrating different cell types for controlled NMJ formation. Finally, we discuss the advantages and limitations of these models and the need for continued advancements enhanced by bioengineering approaches to deepen our understanding of human NMJ biology and pave the way for personalized medicine.

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Introduction

The neuromuscular junction (NMJ), where motor neurons connect with muscle fibers, is the foundation of various physiological functions, such as respiration and movement. A motor neuron axon terminal contacts a skeletal muscle fiber at the NMJ, typically forming a one-to-one connection. This specialized junction is

essential for transmitting signals from the presynaptic motor neuron terminal to postsynaptic skeletal muscle fiber [1]. The neurotransmitter acetylcholine (ACh) plays a central role in this process. ACh is released into the synaptic cleft upon depolarization of the presynaptic membrane and binds to ACh receptors (AChRs) on the postsynaptic muscle membrane, triggering calcium release from the sarcoplasmic reticulum, leading to muscle contraction [2,3].

The molecular architecture of the NMJ involves specialized pathways and proteins that govern synaptic transmission and ensure the precise alignment of pre- and post-synaptic elements. The agrin/lipoprotein receptor-related protein 4 (LRP4)/muscle-specific tyrosine kinase (MuSK) pathway is one of the most extensively studied pathways in vertebrate NMJ development. Agrin, secreted by motor neuron terminals, interacts with specific receptors, including LRP4 and MuSK on the myotube surfaces. This interaction initiates a cascade of signaling events in the myotube that result in the clustering and stabilization of AChRs at the postsynaptic membrane, a process conserved across species, from rodents to humans [4-7]. The molecular integrity of the NMJ relies on a complex network of cytoskeletal proteins, scaffolding molecules, and adhesion molecules. These components contribute to the structural stability of the synapse, facilitate neurotransmitter release, and mediate cell-cell interactions essential for synaptic function [8].

Understanding the mechanisms underlying the communication at the NMJ in both health and disease is crucial for the development of effective therapies targeting neuromuscular disorders such as (1) motor neuron diseases including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) [9,10], (2) specific myopathies such as Becker's and Duchenne's muscular dystrophies [11,12], and (3) auto-immune diseases such as myasthenia gravis (MG) [13]. Directly studying NMJs in human subjects poses significant challenges due to ethical concerns, limited tissue accessibility, and the inability to isolate tissue at different stages of the disease. In some of these disorders, only the muscle or the neural components are initially affected. Still, in many cases, it is difficult to identify the primary cause and cell type that becomes dysfunctional. Understanding which

cell type is affected first and the timing of this dysfunction is crucial for developing preventive strategies and targeted therapies.

This review delves into the current landscape of *in vitro* human NMJ models, specifically focusing on the advantages and disadvantages of assembled and self-organized NMJ models. By elucidating their strengths and limitations, we aim to provide insight into how these models can advance our understanding of NMJ biology and related pathologies.

The critical need for human-specific *in vitro* neuromuscular junction models in neuromuscular research

Although mouse models have significantly contributed to our knowledge of NMJs, exclusively depending on such models presents certain constraints in translational research. Evolutionary divergence has introduced notable differences between human and mouse NMJs, including variations in their structure, function, and response to stimuli [14,15]. Consequently, directly extrapolating findings from mice to humans can result in ambiguous conclusions, thereby hindering the progress toward developing effective therapies for human neuromuscular disorders.

In vitro models of the human neuromuscular junction

In recent years, the development of *in vitro* models derived from human pluripotent stem cells (hPSCs) has significantly advanced our understanding of cellular development and disease pathogenesis, particularly within homogeneous cell populations, such as motor neurons and skeletal muscles. However, studying the interactions between different cell types and tissues *in vitro*, such as the formation of NMJs, presents distinct challenges. These challenges stem from the inherent complexity of the neuromuscular system and the difficulty of generating reproducible *in vitro* models that involve the functional interaction of many different cell types in the dish. Additionally, the contractile properties of muscle cells and the need for continuous activity in both neurons and muscles for proper development make it challenging to culture these cells on stiff surfaces and to image their function during activity. Despite these challenges, several methodologies have emerged for modeling human NMJ formation *in vitro* (Figure 1) [16]. Initially, 2D coculture models of two cell types were established. These models were based on the coculture of hPSC-derived motor neurons with primary skeletal muscles [17,18] or with hPSC-derived skeletal muscles [19–21]. Additional cell types and cutting-edge techniques involving bioengineering and microfluidic devices have been used to enhance the complexity and functionality of NMJ models [22–26]. Most recently, 2D

NMJ models have been described where all different cell types are generated in parallel from hPSCs [27,28]. In addition to 2D models, 3D complex organoid models of more than one tissue have been developed based on self-organization [29,30] and assembly [31].

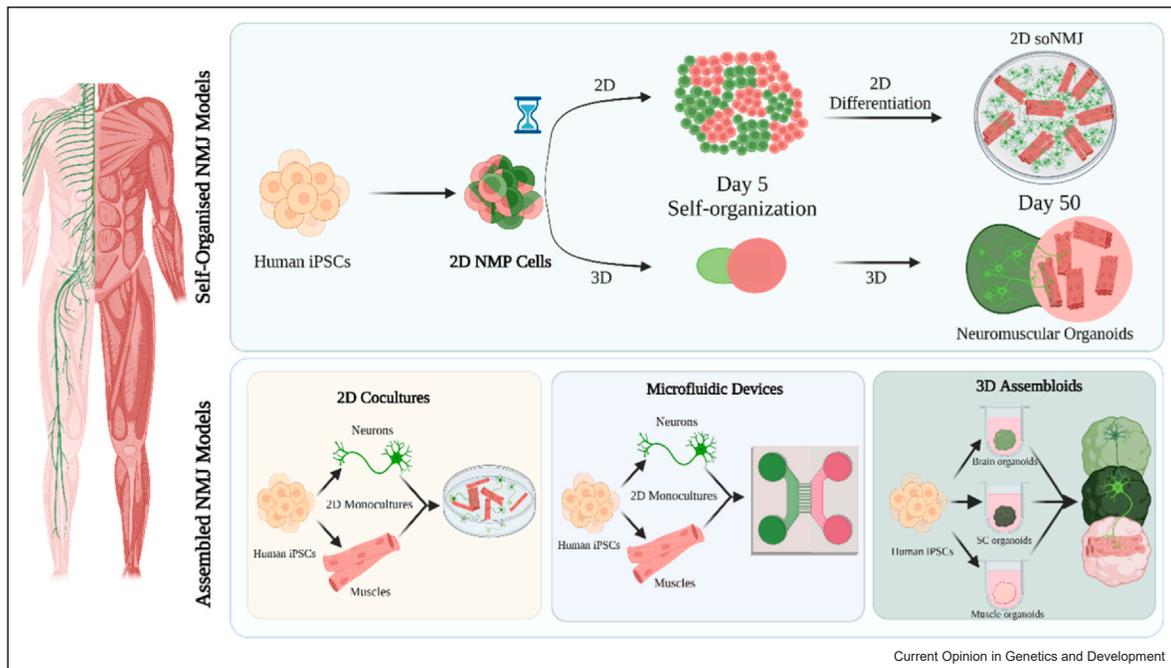
Generation of human pluripotent stem cell-derived neuromuscular junction models based on coculture and assembly

NMJ culture systems employ various methods that combine independently differentiated cell types to guide NMJ formation by manipulating cell interactions and positioning. These methods utilize 2D coculture systems, microfluidic devices, or 3D assembly technologies.

In 2011, Marteyn et al. established the first *in vitro* human-specific NMJ coculture model using hPSC-derived motor neurons with primary human skeletal muscle cells [17]. Building on this, in 2016, Steinbeck et al. used optogenetic activation of motor neurons to control the activity of primary myoblasts and instruct the formation of functional NMJs in a coculture system [18]. These studies demonstrated the potential of such models in understanding neuromuscular disorders. The efficient generation of skeletal muscle tissue from hPSCs opened up new opportunities in the neuromuscular field by establishing human PSC-derived motor neurons and skeletal muscles [19–21,24]. However, a major limitation of coculture models is their inability to fully recapitulate the entire NMJ niche due to the absence of terminal Schwann cells, which are essential for synapse development, maintenance, and plasticity [32]. To overcome this limitation, Hörner et al. established the directed differentiation of hPSCs to Schwann cells and a coculture model comprising hPSC-derived Schwann cells, hPSC-derived motor neurons, and C2C12 myoblast cells. The addition of Schwann cells resulted in an increased size of AChR clusters in myotubes [33]. The development of such a functional framework provided an insightful approach to assessing the impact of Schwann cell addition in coculture models. It also highlighted the ability of coculture systems to investigate a specific aspect of NMJ functionality, thereby enhancing our understanding of neuromuscular disorders.

Apart from cocultures in standard culture dishes, microfluidic devices offer precise control over the spatial and temporal organization of the different cell types. In 2018, Osaki et al., in an elegant study, developed a 3D NMJ model in a microfluidic device using hPSC-derived motor neurons and primary skeletal muscles to control the formation of NMJs through the optogenetic activation of motor neurons [25]. This approach demonstrated significant control over NMJ formation and functionality. Subsequently, in 2019, Bakooshli et al. generated a 3D coculture model of human muscle progenitors with

Figure 1



Schematic overview of self-organized and assembled NMJ models discussed in this review. Created with [BioRender.com](https://www.biorender.com).

hPSC-derived motor neurons and compared it to a 2D coculture model, observing faster maturation of the skeletal muscle in the 3D model [22]. The same year, Bellmann et al. generated a human NMJ model in a customizable microfluidic platform using hPSC-derived motor neurons and skeletal muscles [23] to increase throughput. In 2021, Dittlau et al. described a method for generating human NMJs using hPSC-derived motor neurons and primary mesoangioblast-derived myotubes in commercially available microfluidic devices [34]. In a subsequent study, the system was used to model ALS. They demonstrated reduced NMJ numbers in a *FUS*-ALS model and rescued this effect with HDAC6 inhibition [35]. Recently, the authors established a tri-culture model by incorporating hPSC-derived astrocytes from *FUS*-ALS patients into their previously established coculture system [36]. In 2022, Takahashi et al. established a novel strategy for generating an assembled human NMJ model by coculturing human primary myofibers with hPSC-derived neurons by using micropatterned scaffolds. These micropatterned scaffolds facilitated the growth of aligned myofibers and the addition of hPSC-derived motor neurons enabled the generation of NMJs and the quantification of muscle contraction [37]. Similarly, Massih et al. developed self-fabricated dishes for coculturing 3D muscle tissue and hPSC-derived motor neurons [38].

Another advanced approach involves using 3D assembloids to model the connection between motor neurons

and muscles by combining distinct organoids. Andersen et al. combined a cortical and spinal organoid with a primary muscle spheroid to recapitulate the connection between cortical neurons, motor neurons, and skeletal muscle in a complex 3D model. In this approach, they separately differentiated cortical and spinal organoids from hPSCs and generated a muscle spheroid from primary human skeletal muscle cells. The three organoids were then incubated closely together in transwells for a few days. The development of neuronal projections, forming functional synapses, between the cortical and spinal organoids demonstrated the formation of a primary connection. In addition, the authors observed neuronal projections from the spinal organoids to the skeletal spheroids and the presence of small AChR clusters at NMJ-like structures. Finally, by expressing the light-sensitive channel ChR2 in cortical organoids, the authors could measure a contractile response from skeletal muscles following light stimulation, demonstrating the interconnection between the three organoids [31].

Assembled models allow the generation of specific regions of the brain, spinal cord, and skeletal muscles from diverse genetic backgrounds. This modular approach allows assembling organoids from control and patient-induced pluripotent stem cells (iPSCs) in various combinations. This flexibility offers the unique opportunity to study the effect of specific mutations on certain cell types and their potential to induce specific disease

phenotypes. However, despite their advantages, assembled NMJ models have certain limitations that must be carefully considered. In the assembled models, while the guided placement of different cell types in close proximity can mimic certain aspects of their interaction, such as cell–cell communication and synaptic connectivity, the timing of these interactions during embryonic development might differ. During development, cellular crosstalk is established at early stages of NMJ development, starting around embryonic day 12–14 in mice and 8–9 weeks of gestation in human embryos [3,39]. This crosstalk is essential for the growth, support, and maturation of the respective cell types. These dynamic developmental processes are not fully captured when the different cell types are brought together at later developmental time points. Therefore, such models should be used cautiously when addressing early developmental questions. Additionally, identifying cell culture media that support the proper growth and maturation of all different cell types can be challenging, as well as the long-term maintenance of these cultures.

Instead of coculturing different cell types, Mazaleyra et al. [27] performed multilineage differentiation from the same iPSC culture, generating a 2D NMJ model where skeletal muscle cells and motor neurons connect to form NMJs. At the initial stages, the authors used extracellular signals to induce a mesodermal-specific fate. Surprisingly, Notch inhibition promoted the additional appearance of motor neurons, which formed NMJs with myotubes. By assessing muscle contractions and calcium flux within the postsynaptic compartment, they confirmed the functionality of their neuromuscular system, which could be maintained for several months in the dish. The results showed that the codifferentiation of two cell types reduced the time necessary to form multinucleated mature muscle fibers [27].

Neuromesodermal progenitors and the generation of self-organized neuromuscular junction models

In recent years, neuromesodermal progenitors (NMPs), the building blocks of the posterior neuromuscular system, have opened up new opportunities to generate neuromuscular models that closely follow the *in vivo* sequence of developmental events. NMPs, also known as axial progenitors, are bipotent cells that give rise to spinal cord neural and mesodermal progenitor cells [40]. Based on the potential of NMPs to generate both lineages, Martins et al. pioneered the generation of self-organized neuromuscular organoids (NMOs) from hPSCs. Initial exposure of hPSCs to the canonical Wnt activator (CHIR99021) and basic Fibroblast Growth Factor (bFGF) for 3 days resulted in the efficient generation of NMPs [41]. Subsequently, NMPs were plated in round-bottom low-attachment 96-well plates to

induce the formation of 3D organoids. Strikingly, NMOs self-organized into a spinal cord neural and skeletal muscle compartment. The NMOs started contracting after 40–50 days of culture due to the formation of functional NMJs supported by the presence of terminal Schwann cells. As a proof of concept, NMOs were successfully used to model MG [29]. Recently, a study showed the generation of NMOs from *C9ORF72* ALS patient-derived iPSC lines. The ALS NMOs could model peripheral defects observed in ALS, such as reduced muscle contraction and neural denervation [42].

In 2021, Pereira et al. generated sensorimotor organoids from healthy and ALS patient iPSCs using a combined 3D and 2D approach. They first generated 3D spheres by adding FGF, Wnt, and forskolin on hPSCs to induce an NMP identity. Next, the spheres were plated on matrigel-coated dishes in 2D, resulting in the migration of cells outward to form an adherent organoid culture. Single-cell RNA sequencing demonstrated the generation of various cell types, and α -BTX staining confirmed the presence of AChRs in healthy and ALS-related cultures. They also showed that the NMJs were impaired in sensorimotor organoids from *TDP43* mutant iPSCs [30].

While 3D organoids represent an exciting tool to model and study the mechanisms of human neuromuscular diseases, their large size poses challenges for high-throughput approaches. To address this challenge, Urzi et al. developed a 2D self-organized NMJ (soNMJ) model using a similar NMP-driven approach. By day 50, this model exhibited functional NMJs between spinal cord neurons, skeletal muscle cells, and terminal Schwann cells. Optogenetic activation of neurons and chemical inhibition of NMJs indicated the functional connection between neurons and muscles. The 2D soNMJ models generated from SMA patient iPSC lines showed reduced numbers of NMJs and defective muscle contraction, indicating that this model could efficiently model neuromuscular diseases. This technology allows for high-throughput drug screenings and the design of novel therapeutic strategies, as the cells can be cultured in a multiwell format for extended periods [28].

Self-organizing 2D and 3D neuromuscular models derived from NMPs represent a physiologically more relevant platform for studying NMJ development and related diseases. Despite some inherent variability introduced by their autonomous self-organization, these models reproducibly establish and maintain functional neuromuscular networks, mature over time and can be maintained for extended periods in culture.

Future directions

There has never been a better time to study human neuromuscular diseases with hPSC-derived *in vitro*

Table 1**Table showing comparative advantages and disadvantages of assembled and self-organized NMJ models.**

Advantages/disadvantages	Assembled NMJ models	Self-organized NMJ models
Precise control of cell types	+	-
Allows investigation of certain cell types from different genetic backgrounds	+	-
Combine specific cell types in different proportions	+	-
Resembles natural NMJ development and architecture	-	+
Offers a holistic representation of NMJ physiology	-	+
Challenges with reproducibility and standardization	+	+
May not fully capture the maturation of native NMJs	+	+
Complex assembly	+	-
Suitable for high-throughput drug screening	-	+

models. Many laboratories around the world have described the generation of human-specific NMJ models. In this review, we focused primarily on assembled and self-organized human NMJ models to highlight the advantages and limitations of each approach. Self-organized NMJ models are more complex and ideal for studying overall NMJ development, function and disease modeling, whereas assembled models are better suited for probing interactions between cells from different genetic backgrounds in a controlled environment (Table 1).

The development of new methods that can compensate for the limitations of previous approaches will further the understanding of the development of neuromuscular tissue and related diseases. In the future, hybrid models incorporating elements of both approaches will advance the current state, creating even more sophisticated models. Moreover, enhancing vascularization remains a critical goal, as it would significantly impact the functionality and maturation of these models. Finally, developing novel high-throughput platforms and strategies for scaling up the production of reproducible models is crucial for improving drug screening studies and accelerating the development of novel therapies.

Author contributions

A.N. and M.G. conceived and wrote the manuscript.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

MG has filed patents on the generation of the 2D soNMJ model and the 3D NMO model.

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research and innovation program (101002689), and the European Molecular Biology Organization Young Investigator program award. The figure was created with [BioRender.com](https://www.biorender.com).

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The authors generated patient-specific NMO models using C9orf72 ALS patient-derived iPSC lines. The patient-specific NMOs reflected disease-related defects, including neural denervation, contraction weakness, and loss of Schwann cells. This study further confirmed the applicability of the NMO model in modeling neuromuscular disease phenotypes.