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REVIEW

Building neuromuscular junctions *in vitro*

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ABSTRACT

The neuromuscular junction (NMJ) has been the model of choice to understand the principles of communication at chemical synapses. Following groundbreaking experiments carried out over 60 years ago, many studies have focused on the molecular mechanisms underlying the development and physiology of these synapses. This Review summarizes the progress made to date towards obtaining faithful models of NMJs *in vitro*. We provide a historical approach discussing initial experiments investigating NMJ development and function from *Xenopus* to mice, the creation of chimeric co-cultures, *in vivo* approaches and co-culture methods from *ex vivo* and *in vitro* derived cells, as well as the most recent developments to generate human NMJs. We discuss the benefits of these techniques and the challenges to be addressed in the future for promoting our understanding of development and human disease.

KEY WORDS: Neuromuscular junctions, *In vitro* models, Human pluripotent stem cells

Introduction

At the beginning of the last century the 'neuron doctrine' proposed by Ramón y Cajal revolutionized the emerging field of neuroscience. The doctrine firmly established individual neurons as the basic anatomical and physiological unit of the nervous system, and prompted many questions regarding how neurons communicate with one another and how the space between them is crossed by the signals responsible for that communication. We now know that neurons make complex structures called synapses that allow chemical transmission of signals among cells. The neuromuscular junction (NMJ) is a tripartite chemical synapse of the peripheral nervous system. It allows the transmission of motor information that governs muscle contraction from the central nervous system to the muscle fibers. The NMJ comprises a motoneuron on the presynaptic side, a muscle cell on the post-synaptic side and a terminal Schwann cell (tSC) wrapping the NMJ structure. A fourth cell type, a kranocyte, which caps the NMJ and plays a role in synapse regeneration, has recently been identified in rodents (Court et al., 2008) (Fig. 1). Fatt and Katz showed that end-plate potentials (EPPs), which correspond to the chemically induced change in electric potential of the motor end plate, originate from the spontaneous or electrically evoked release of acetylcholine (ACh) from the nerves (Fatt and Katz, 1951). Furthermore, Katz and colleagues proposed the 'quantal hypothesis', stating that ACh is released from the nerve terminal in packets or 'quanta'. The spontaneous release of one quantum results in a miniature EPP (mEPP) in the post-synaptic cell, whereas the synchronized release of multiple ACh quanta leads to EPPs. Synaptic vesicles accumulate at discrete zones of the nerve terminal called 'active zones' (Couteaux and Pecot-Dechavassine, 1970). A functional definition of active zones came a few years later, when it was shown that vesicles dock and fuse at the membrane, and their contents are exocytosed in response to nerve depolarization (Heuser et al., 1979). Taken together, these pioneering studies on NMJs provided the basis for our current knowledge of the intricate mechanisms underlying synaptic transmission.

Neuromuscular junctions have since been the most intensively studied synapses, because of their comparatively large size, relative simplicity and accessibility. Indeed, among specific features, neurotransmitter release at this synapse is mediated by multiple docking sites present in long active zones in frogs or several active zones in mammals. In contrast, synaptic transmission in the brain is associated with a single active zone per synapse (Biro et al., 2005; Silver et al., 2003). Another important distinction between NMJs and central synapses is that the post-synaptic element at an NMJ only receives cholinergic inputs, whereas post-synaptic neurons receive a large variety of inputs, which makes the analysis of the response to a single neurotransmitter more difficult to interpret. Therefore, the NMJ remains an excellent model for studying the mechanisms of neurotransmission, and is well suited to study synaptic formation, maintenance and function. Furthermore, the NMJ is at the core of a number of human pathologies that all have the perturbation of neurotransmission in common (Box 1).

Here, we provide an update on the different *in vitro* models that have been investigated to increase our understanding of the normal and pathological development of NMJ. We present a focus on the recent findings on humanized *in vitro* models, which have been made possible using human pluripotent stem cells, and discuss the promises and challenges of these new models.

The neuromuscular junction: a model system for studying synaptic development and function

A great deal of information on how synapses form has been obtained through studying muscle cell lines (mostly the murine C2C12 cell line) that form spontaneous acetylcholine receptor (AChR) aggregates, and co-cultures of muscle cells and motoneurons (Sanes and Lichtman, 2001). Early studies were carried out in cultures of neurons and muscles from *Xenopus laevis* (Anderson et al., 1977) or from chick (Frank and Fischbach, 1979), laying the groundwork for understanding the mechanisms of *in vitro* NMJ formation and the study of NMJ disorders (discussed below). These datasets have been extended *in vivo* with the emergence of transgenic mice, along with the use of non-mammalian animal models (Box 2). Here, we briefly describe the steps in NMJ formation as well as the essential molecular and structural markers of NMJ formation and maturation (Figs 1 and 2). For more detailed discussion of the mechanisms of synapse formation, we refer the reader to recent reviews (Burden et al., 2018; Li et al., 2018).

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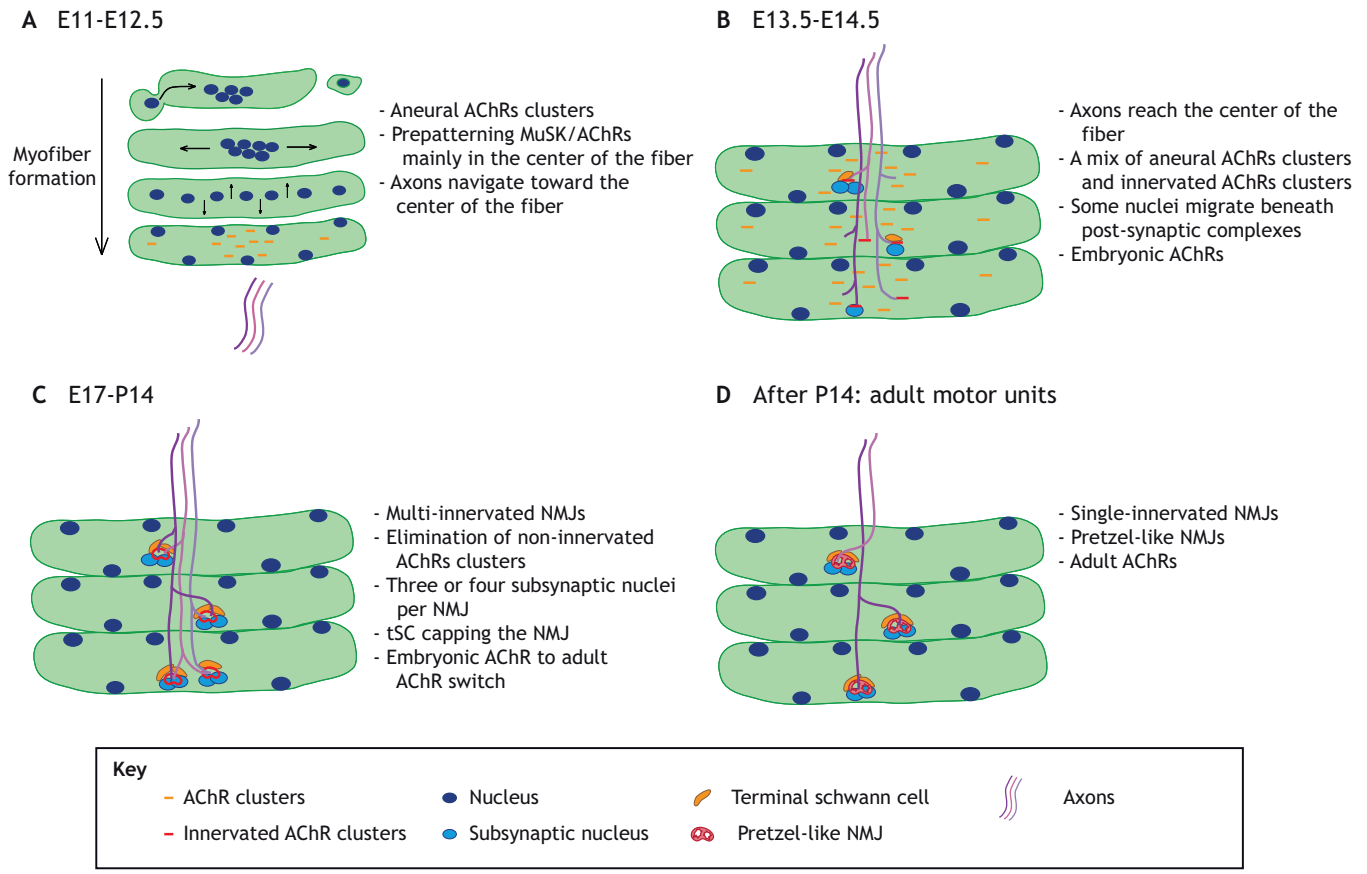

Fig. 1. The stages of NMJ formation in mice. (A) During development, prior to and independent of innervation, AChRs are clustered in the prospective region of muscle. Thus, the muscle cell plays an instructive role from a very early stage of development, in positioning and restricting synapses to a central band in the muscle, and building a post-synaptic density prior to axonal contact. (B) Axons do not contact muscle fibers at random locations, but rather in a narrow central region of the muscle fiber characterized by the presence of aneural 'pre-patterned' AChR clusters (Lin et al., 2001; Yang et al., 2001). Axons then become apposed to AChR clusters. (C) Additionally three or four nuclei accumulate under the post-synaptic membrane in the muscle cells (which are defined as multinucleated syncytia formed during development by fusion of mononucleated precursor cells) and become specialized for transcription of synaptic proteins. Concomitantly, folds form in the muscle membrane increasing the area of contact between the post-synaptic membrane and the extracellular matrix (Guarino et al., 2019). (D) Muscle fibers are poly-innervated until the end of the second week after birth in rodents. This is when the multiple innervations of individual post-synaptic densities are reduced to a single innervating motor axon through a process called synapse elimination (Sanes and Lichtman, 2001). AChR, acetylcholine receptor; adult AChR, $\alpha 2\beta\varepsilon\delta$; E, embryonic day; embryonic AChR, $\alpha 2\beta\gamma\delta$; MuSK, muscle-specific kinase; P, post-natal day; tSC, terminal Schwann cell.

Cellular components in synapse formation

NMJ development requires extensive communication among the three key components of the tripartite synapse: presynaptic motoneurons, post-synaptic muscle fibers and tSCs. NMJ formation is closely intertwined with myogenesis and innervation occurs between embryonic days (E) 12.5 and E14 in mice, immediately after the first wave of myotube formation. This timing varies slightly among specific muscles and their locations (Fig. 1).

The composition of AChRs changes with development as follows: nicotinic AChRs are pentamers composed of five subunits in a stoichiometry of two α-, one β-, one δ- and one γ-subunit in the embryo. These are replaced by a ε-subunit at the postnatal age (Changeux et al., 1992). Within 3 weeks of birth, AChR clusters adopt a complex, pretzel-like configuration in mice (Sanes and Lichtman, 2001). The maturation of the NMJ leads to an increase in number, stability, density and clustering of AChRs in the post-synaptic compartment, as well as the differentiation of the presynaptic membrane into active zones with proper synaptic proteins and associated synaptic vesicles (Burden et al., 2018). The overall process reflects strong reciprocal signaling between pre- and postsynaptic structures.

Another crucial component involved in the formation of the NMJ is the tSC. Although the very first steps in synapse formation do not require the presence of tSCs, these cells are necessary for maintenance and maturation of NMJs (Ko and Robitaille, 2015). Ablation of tSCs after NMJ maturation at P30 in mice, leads to NMJ fragmentation and neuromuscular transmission deficits (Barik et al., 2016).

Molecular components of NMJ development and function

Over the past decade, extensive progress has been made in understanding the molecular mechanisms involved in NMJ formation. Here, we summarize the current knowledge on some of the key molecules and signaling pathways that are indispensable for NMJ formation and maintenance (Figs 1 and 2).

MuSK-Lrp4-agrin signaling

MuSK (muscle-specific kinase), a transmembrane receptor tyrosine kinase and its co-receptor LRP4 (low-density lipoprotein receptor-related protein 4) are crucial for the aggregation of AChRs before and after innervation. Prior to innervation, the MuSK/LRP4 complex accumulates in the muscle central region where it induces AChR

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Box 1. Neuromuscular junction disorders

Neuromuscular diseases (NMD) refer to a vast group of conditions that perturb or even progressively block the control of muscles. NMDs can originate from motoneuron defects, e.g. as in amyotrophic lateral sclerosis (ALS) and in spinal muscular atrophy (SMA), or from a defect of the neuromuscular junction itself, e.g. as in congenital myasthenic syndromes (CMS), and in autoimmune diseases such as myasthenia gravis (MG). NMJ diseases have a wide range of origins, including genetic mutations, antibodies or even toxins that interfere with the control of signaling between the presynaptic nerve ending and the post-synaptic muscle membrane, and/or the formation of the synapse.

Myasthenia gravis (MG) is one of the most common diseases of the NMJ. This autoimmune disorder of the neuromuscular transmission is increasingly considered as a syndrome more than as a single disease. At least three different antibodies to AChRs, MuSK or LRP4 have been identified as the cause of the typical weakness and fatigability associated with MG (Higuchi et al., 2011; Hoch et al., 2001; Lindstrom et al., 1976). In addition, Lambert-Eaton myasthenic syndrome (LEMS) is caused by auto-antibodies against voltage-gated calcium channels (Lennon et al., 1995).

As well as these autoimmune diseases, there is also a large family of genetic conditions that affect the functioning of the NMJ. Remarkable progress in molecular genetics during recent decades has allowed the identification of an increasing number of mutations in genes encoding proteins of the NMJ that drive pathogenesis of several inherited diseases. Mutations in key human genes, such as *DOK7*, *RAPSN*, *MUSK*, *LAMB2*, *COLQ*, *AGRN* and genes encoding AChRs, can cause different forms of CMS (Beeson et al., 2006; Engel et al., 2015; Hantai et al., 2013; Ohkawara et al., 2014; Verschuuren et al., 2016).

clustering. With innervation, LRP4 binds a neural isoform of agrin (AGRN), a heparan sulfate proteoglycan secreted by the axon terminal (Ferns et al., 1993; Kim et al., 2008; Ruegg and Bixby, 1998; Zhang et al., 2008). Agrin interaction with LRP4 results in the formation of tetrameric agrin-LRP4 complexes that promote activation and transphosphorylation of MuSK, leading to AChR clustering and NMJ formation (Zong et al., 2012). AChR clusters that are apposed to nerve terminals persist and grow, while the non-apposed ones disassemble (Wallace, 1989). Mutations in the human *AGRN* gene cause congenital myasthenic syndrome (CMS), with distal muscle weakness and atrophy (Nicole et al., 2014).

More recently, two studies have independently revealed that the muscle LRP4 also acts as a retrograde signal for presynaptic differentiation (Wu et al., 2012; Yumoto et al., 2012). MuSK activation requires Dok7 (docking protein 7; an adaptor protein expressed by the muscle), which is indispensable for MuSK activity and therefore AChR clustering. The binding of Dok7 to the cytoplasmic juxtamembrane region of MuSK strongly enhances the tyrosine kinase activity of MuSK, leading to its activation (Hamuro et al., 2008; Inoue et al., 2009). MuSK-deficient mice show neither muscle pre-patterning of AChRs nor NMJ formation, and die perinatally from respiratory failure (Okada et al., 2006). More recently, it has been shown that the postnatal knockdown of *Dok7* gene in mice causes structural defects in NMJs and motor dysfunction, supporting a role for Dok7 in NMJ maintenance (Eguchi et al., 2016).

AChR-rapsyn

Beginning around the time of birth, fetal AChRs ($\alpha2\beta\gamma\delta$) are gradually replaced by the adult subtype of AChRs ($\alpha2\beta\varepsilon\delta$). Although the physiological significance of this developmental switch remains to be fully understood, different studies have suggested a role of the γ-subunit during the NMJ formation and more specifically in innervation patterning (Koenen et al., 2005; Takahashi et al., 2002). For example, deletion of the mouse AChR γ-subunit leads to perinatal lethality associated with an absence of pre-patterned AChR clusters during initial stages of neuromuscular synaptogenesis (Koenen et al., 2005; Takahashi et al., 2002). A key effector in AChR clustering is the protein rapsyn, a cytoplasmic scaffolding protein expressed in the muscle, which binds directly to AChR and anchors AChR in the post-synaptic membrane (LaRochelle et al., 1990; Sobel et al., 1978). Rapsyn-deficient mice fail to develop post-synaptic specializations (Gautam et al., 1995), indicating that rapsyn is essential for the tethering of AChRs to the post-synaptic apparatus. Although rapsyn assembles with AChRs in the secretory pathway (Marchand et al., 2002; Moransard et al., 2003), the exact mechanisms downstream of MuSK/Dok7 that stimulate rapsyn-induced AChR clustering remain to be determined.

Extracellular components

In addition to these core molecules that are essential for NMJ formation, a number of important regulators also participate in this process. Wnts are a group of glycoproteins secreted at the NMJ by the muscle fiber, motoneurons and tSCs. Several Wnt ligands bind to the cysteine-rich domain (CRD) in MuSK (Barik et al., 2016), and play a role in axon extension and branching, as well as in AChR prepatterning (Li et al., 2018). Both canonical and non-canonical planar cell polarity (PCP) Wnt pathways are affected in transgenic mice in which the CRD in MuSK is deleted (Messeant et al., 2017).

Dystroglycan (DG) has a dual function: it organizes extracellular matrix (ECM) scaffolds and stabilizes AChR clusters. DG is formed of two subunits that are generated by proteolytic cleavage of a single precursor protein. The extracellular subunit is necessary to organize a functional scaffold in the basal lamina including perlecan, acetylcholinesterase/ColQ and laminin (Jacobson et al., 2001; Peng

Box 2. Contributions of non-mammalian model organisms for NMJ understanding

In parallel to the *in vitro* models for NMJ described in this Review, non-mammalian model organisms such as *Drosophila* and zebrafish have also been instrumental in identifying key components present at the pre- and post-synaptic levels, and understanding the mechanisms that underpin NMJ assembly. These organisms provide experimental advantages, including genetic and molecular traceability, amenability to functional studies, and increased experimental throughput compared with traditional mammalian models.

Although the NMJ of *Drosophila* has provided insights into the development, function and plasticity of synapses, the molecular composition and physiology of this NMJ is most similar to mammalian glutamatergic excitatory synapses and no orthologues of key molecules involved in the mammalian NMJ have been identified in *Drosophila*. In contrast, the use of zebrafish NMJ has continuously increased in popularity over recent decades, mainly because of the transparency of the embryonic and larval stages. This renders zebrafish particularly amenable to optical time-lapse studies *in vivo*. In combination with large-scale genetic screens, in the late 1990s Nüsslein-Vohlhard and colleagues identified genetic mutants based on morphological abnormalities and abnormal behaviors (Granato et al., 1996; Haffter et al., 1996). Subsequent identification of the genes shed light on the molecular mechanisms underpinning the motor system; many of the locomotion mutations mapped to genes coding for synaptic proteins in the NMJs that had also been identified in mammals, such as MuSK, Dok7, Lrp4, Rapsyn or specific subunits of AChR (Marangi et al., 2001; Ono et al., 2004). These examples illustrate how these approaches complement the advances obtained using human patients or mammalian animal models, although divergence in some pathways were observed.

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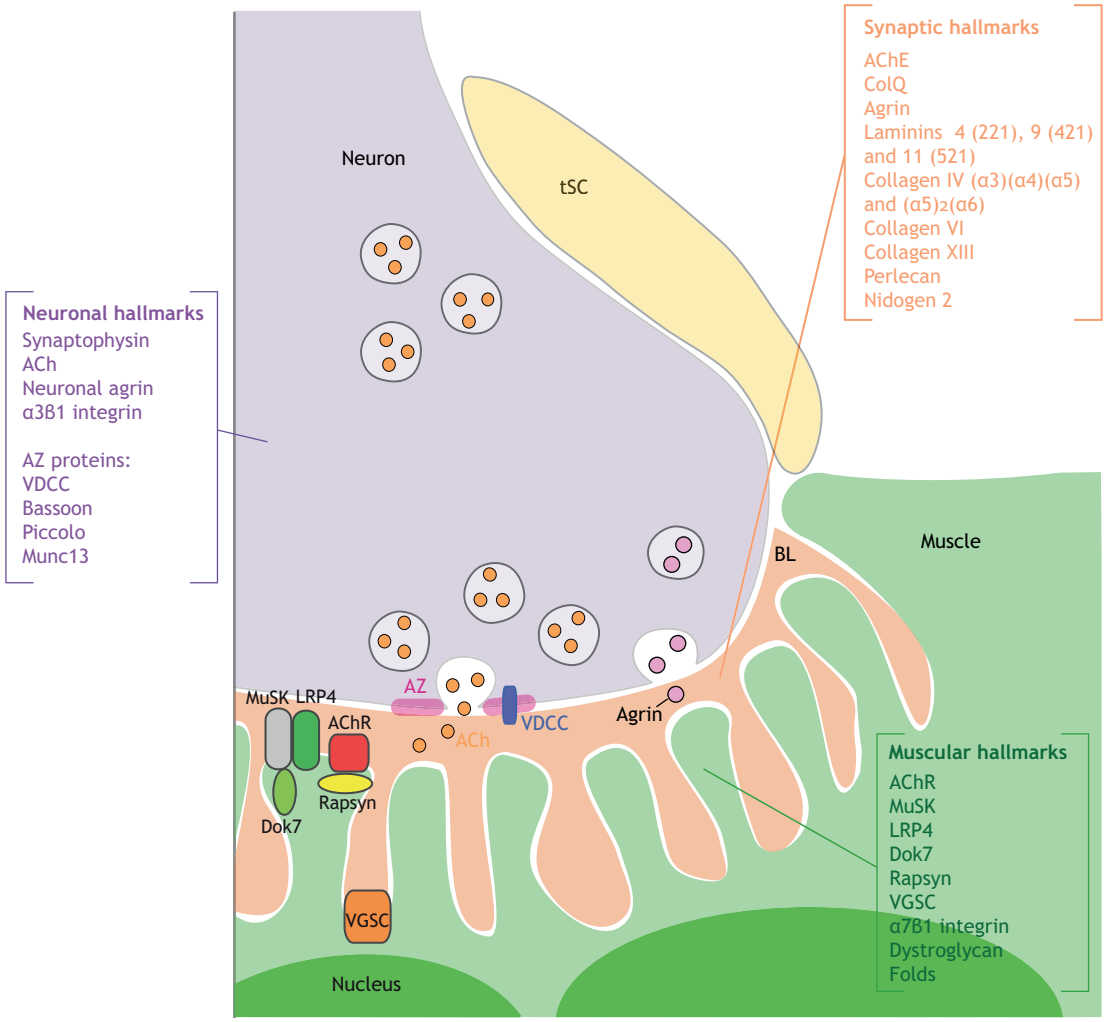

Fig. 2. Muscle, neuronal and synaptic hallmarks of the mature NMJ. The main markers that can be used to characterize the mature NMJ are shown. ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; AZ, active zone; BL, basal lamina; ColQ, collagen Q; Dok7, downstream of tyrosine kinase 7; LRP4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; tSC, terminal Schwann cell; VDCC, voltage-dependent Ca^{2+} channel; VGSC, voltage-gated Na^{+} channel.

et al., 1998). DG is also a receptor for agrin (Gee et al., 1994), although – unlike Lrp4 – it does not activate MuSK.

The synaptic basal lamina contains many components that have important regulatory roles in myogenesis and synaptogenesis. ECM molecules help guide the process of innervation by motor neurons and are crucial to formation of the post-synaptic density as well as organizing and maintaining functional apposition of the pre- and post-synaptic elements (Maselli et al., 2012; Rogers and Nishimune, 2017). Key junctional basal lamina components include collagens [such as collagen IV (α3)(α4)(α5) and $(α5)_2(α6)$ trimers, collagen XIII and ColQ), glycoproteins (such as laminin isoforms α4β2γ1, α5β2γ1 and α2β2γ1, and nidogen 2) and heparan sulfate proteoglycans (HSPGs), such as perlecan and agrin (Fox et al., 2007). The laminin β2 chain is considered as an active zone organizer and binds to the voltage-dependent calcium channels (VDCC) located in the active zones (Nishimune et al., 2004). The interaction between laminin β2 and VDCCs leads to VDCC clustering, which is the first step toward a mature active zone. In laminin β2 knockout mice, the number of active zones and synaptic vesicles are reduced along with a reduction in neurotransmitter release. Mice lacking ColQ, collagen XIII, collagen IV (α3)(α4)(α5), $(α5)_2(α6)$ trimers or collagen VI also show immature nerve terminals and/or NMJs (Cescon et al., 2018; Sigoillot et al., 2016; Zainul et al., 2018). Networks of laminins and collagens are connected to one another by nidogen and are anchored to the sarcolemma and intracellular cytoskeleton by binding to laminin receptors: integrins and, most importantly, DG (Fox et al., 1991; Jacobson et al., 2001).

Evolution of *in vitro* models of NMJs

For over one century, different models and approaches have been developed to decipher the mechanisms involved in the formation, maintenance and function of NMJs. Meanwhile, increased incidence and prevalence of inherited neuromuscular diseases require the development of pertinent pathological models for deciphering the pathophysiology underlying these diseases and developing effective treatments (Bhatt, 2016). Although animal models have been crucial for the NMJ field, they have certain limitations, such as ethical issues involving animal welfare, their capacity to fully reproduce some human pathological phenotypes and in their complexity, which hinders the identification of cell-specific mechanisms, including anterograde and retrograde signals. Researchers have tried to overcome these hurdles by developing *in vitro* models to recreate the NMJs. In the following section, we focus on the contribution and the evolution of these *in vitro* models of the NMJ (Fig. 3).

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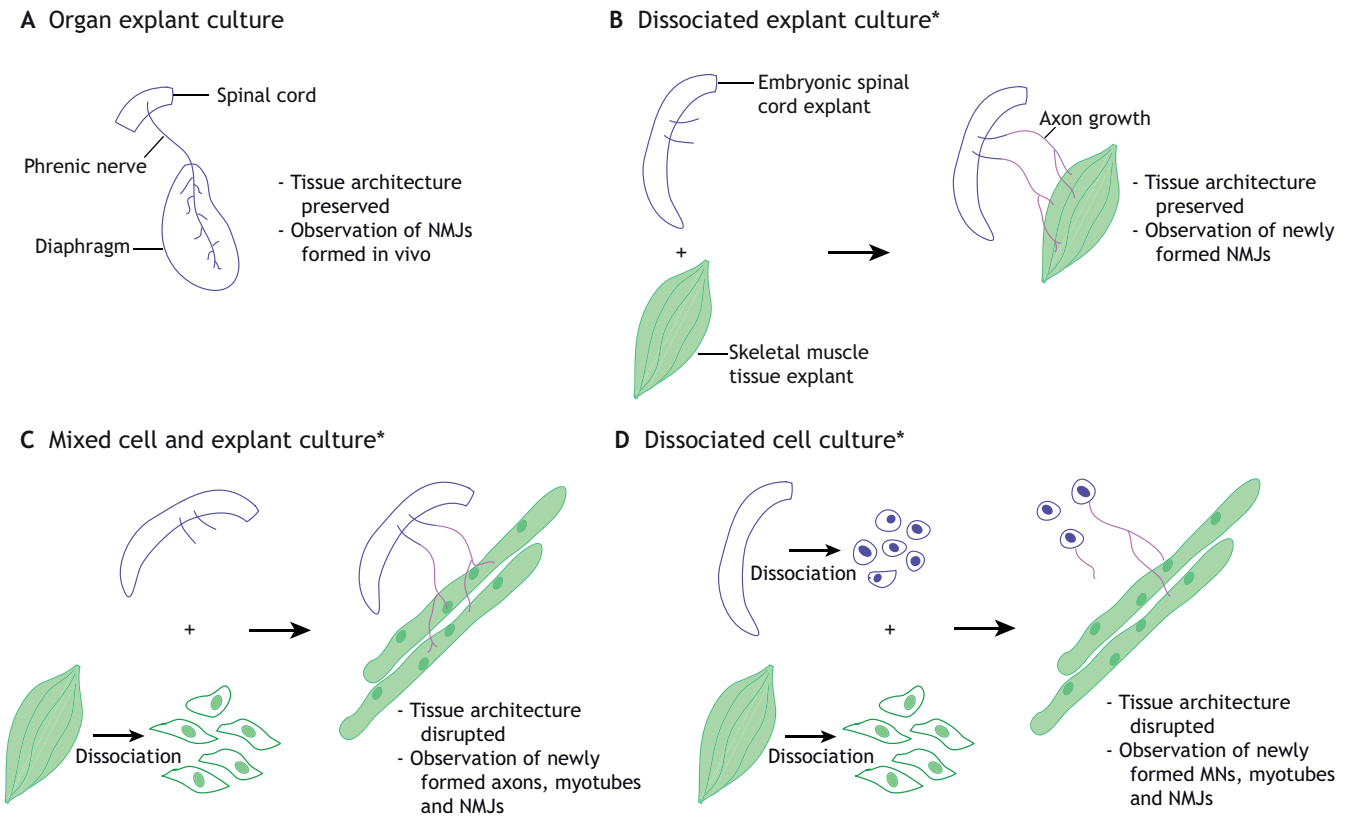

Fig. 3. Different technical approaches to studying the NMJ *in vitro*. (A) Organ explant culture. (B) Dissociated explant culture. (C) Mixed cell and explant culture. (D) Dissociated cell culture. (C,D) Instead of explants or dissociated cells, immortalized cell lines or motoneurons (MNs)/muscle cells generated from induced pluripotent stem cells can be used. *Chimeric model possible (different species of origin for muscle cells and MNs).

The first models of co-cultures: organ explant cultures

Historically, the first neuromuscular junction obtained *in vitro* was reported by R. G. Harrison in 1959 (Harrison, 1959). By cultivating medullary cord and axial mesoderm explants from clotted frog lymph between two glass slides, Harrison observed striated and contracting muscle fibers. As the frog muscle cells do not contract spontaneously, he concluded that the contractions were nerve induced. As the embryos are easy to manipulate, collect and dissect, amphibians have been extensively used as animal models for generating *in vitro* models of neuromuscular junctions (Anderson et al., 1977; Kidokoro et al., 1980; Peng and Nakajima, 1978; Swenarchuk et al., 1990). Among amphibians, two species stand out – *Xenopus laevis* and *Ambystoma mexicanum* – which belong to the anuran and the urodele orders, respectively. *Xenopus laevis* offers a number of advantages, most notably cells that are inexpensive to culture and mature rapidly (Peng et al., 1998; Swenarchuk et al., 1990). Within 3 days of co-culture, myotomal muscle cells are seen to be contracting due to innervation by neurons (Anderson et al., 1977). Cells from *Ambystoma mexicanum*, like *Xenopus* cells, can be cultivated at room temperature and in a basic cell culture medium complemented with 1% horse serum or fetal bovine serum (Anderson et al., 1977). *Ambystoma* cells differ from *Xenopus* cells in terms of the time of maturation. *Ambystoma* cells require a longer period to generate mature myofibers and establish functional synapses (Swenarchuk et al., 1990).

In parallel, a number of other species from chick to rodents have been used to build models for NMJs. These models were justified in part by the lack of tools to study cell biology in frog. Burgen and colleagues used rat phrenic nerve-diaphragm explants to investigate the action of the botulinum toxin (Burgen et al., 1949) (Fig. 3A). In these first studies, models were formed by removing a section of embryonic spinal cord with the phrenic nerve and muscle still attached. These explants were maintained as ‘organ cultures’.

Dissociated explants cultures and dissociated cells cultures

The next breakthrough was the development of physically separated explants at difference with the previous system (Fig. 3B). This strategy allowed the observation of newly formed NMJs (James and Tresman, 1968). In these dissociated explants cultures, most of the NMJ models aimed to visualize and understand the process of neuromuscular synapse formation and also to decipher the mode of action of pharmacological drugs (Evans, 1969). At the same time, the first serial electron microscope studies allowed detailed observations of the ultrastructure of mouse neuromuscular synapses in culture (Bornstein et al., 1968). Crain and colleagues showed that explant cultures of rat spinal cord and skeletal muscle tissues from rat and human formed functional NMJs (Crain et al., 1970).

The development of dissociated neuronal cell culture, where isolated neurons were grown on a single layer along with the availability of inverted microscopes, facilitated electrophysiological recordings *in vitro* (Scott et al., 1969). Using these tools, Fischbach and colleagues demonstrated that co-cultures of cells dissociated from chick skeletal muscle with those from chick spinal cord produced functional synaptic contacts (Fischbach, 1970) (Fig. 3C). These inter-species models, also called chimeric models, confirmed that NMJ formation and function is conserved among a variety of species.

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Chimeric cell cultures

Chicks, frogs and rodents are the most common species used as sources of tissues for studying NMJ formation. Chicks offer easy access and manipulation of the embryo (Frank and Fischbach, 1979; Johnson et al., 1981), and have been used in chimeric models with rat and mouse cells. However, as mammals, the rodents *Mus musculus* (mouse) and *Rattus norvegicus* (rat) provide greater insight into human development or pathology than amphibians or birds (Ionescu et al., 2016). Mouse myoblasts can differentiate into fused and striated myofibers 2-4 days after plating (Ionescu et al., 2016). Functional synapses form 4 days later when they are cultured with murine neuronal explants (i.e. 2 days for contact between axons and myotubes followed by 2 days for synapse maturation) (Vilmont et al., 2016) (Fig. 3D). However, mouse co-cultures are rather inefficient systems, with few synapses forming, in contrast to frog co-cultures (Sanes and Lichtman, 1999).

Chimeric cell cultures models have provided answers to the origin – muscle or nerve – of a number of molecules expressed at the NMJ. For example, species-specific antibodies, one targeting specifically the HSPG from anuran species and the second targeting the HSPG from urodela species, have identified that HSPGs originate from the muscle (Swenarchuk et al., 1990). In the same vein, the development of transgenic animals brought new tools in the field of *in vitro* NMJ modeling. For example, *Hb9::GFP* transgenic mice express GFP in differentiated motoneurons (Wichterle et al., 2002). The use of *Hb9::GFP* mouse cells allow for easy visualization and identification of motoneurons in culture (Ionescu et al., 2016; Umbach et al., 2012).

Limitations and next challenges for dissociated cell models

Embryonic spinal cord explants provide motoneurons *in situ*, within their 3D multicellular environment (Ionescu et al., 2016). Alternatively, dissociated cells from embryonic spinal cord or myotome allow one to enrich specifically for motoneurons or muscle cells (Fischbach, 1972; Nakajima et al., 1980). Clonal and immortalized cell lines are often used to provide a large, convenient and homogenous population of cells ready to culture. However, these cells may harbor abnormal phenotypes that are not completely representative of healthy cells *in vivo*, either because of the immortalization process they have been through or the fact that they are cancer cells. For example, G-8 clonal myogenic cells have a modal chromosome number of 75 instead of 40 (Christian et al., 1977). Nevertheless, muscle cell lines are used largely to study the mechanism of post-synaptic differentiation and more specifically, the mechanisms of AChRs aggregation. For example, C2C12 cells are a subclone originally isolated by Blau (Blau et al., 1983) from a murine cell line generated in 1977 by Yaffe and Saxel (Yaffe and Saxel, 1977). C2C12 cells are useful to study early muscle differentiation, including myoblast fusion as well as AChR aggregation. However, C2C12 cells do not fully differentiate and do not form aggregates of acetylcholinesterase (AChE). A more recently established murine muscle cell line called MLCL forms AChE clusters in addition to the AChR clusters (Cartaud et al., 2004). Finally, collaborations between biologists and physicists lead to the emergence of new culture devices such as microfluidic devices with the objectives of better modeling NMJ pathologies, as well as providing a more suitable system for some mechanistic studies (Machado et al., 2019; Uzel et al., 2016; Zahavi et al., 2015). These developments are discussed below.

Criteria for characterizing *in vitro* NMJs

The establishment of reliable protocols for co-cultures led many groups to consider developing humanized *in vitro* models of NMJs to study human neuromuscular diseases. Whichever *in vitro* model is used, a number of criteria have to be met to ascertain that an NMJ model is representative of those *in vivo* in terms of structure and function, and therefore a valid model. The criteria list that has been used is described in the next few paragraphs and is summarized in Tables 1 and 2.

NMJ morphology

In the first instance, different presynaptic, synaptic and post-synaptic hallmarks can be used as physiological and morphological clues indicative of normal NMJs. Some of them can be detected by immunolabeling of key synaptic proteins (Das et al., 2007; Dorchies et al., 2001; Umbach et al., 2012; Vilmont et al., 2016) (Fig. 2). For skeletal muscle, the first indication of complete differentiation is the morphology of the myofibers. NMJ morphology is dependent on the species as well as the stage of NMJ differentiation and the innervated muscle. Gillingwater and colleagues developed a standardized 'NMJ morph' tool to study NMJ morphology from tissue samples, highlighting these specific parameters in function of the species (Jones et al., 2017; Jones et al., 2016). The best approach to ascertain the presence of a synapse is through electron microscopy (EM). EM allows the visualization of a contact between axon terminals and muscle cells, the presence of synaptic vesicles in the presynaptic element and post-synaptic densities apposed to presynaptic elements (reviewed by Slater, 2017). However, it remains to be shown that this synapse is functional; i.e. muscle contractions induced by nerve activity and the presence of spontaneous/evoked transmission.

Muscle contractions

Striations and spontaneous contractions are also characteristics acquired during differentiation of myoblasts into myotubes in mammals. Mouse, rat and human muscle cells can contract in aneural mono-culture, unlike amphibians (Guo et al., 2014). These contractions are helpful in assessing the differentiation of the cells. Generally, two types of muscle cell contractions can be observed in co-culture models: small twitches independent of innervation (which are short in length and time) and contractions induced by innervation, which are longer and stronger (Ionescu et al., 2016). Innervation-dependent contractions are also characterized by their synchrony from the innervation of several myotubes by axonal branches of the same motoneuron (Vilmont et al., 2016). These two types of twitching movements can easily be distinguished by adding the drugs tetrodotoxin (which inhibits voltage-gated sodium channels) or d-tubocurarine (an antagonist of AChR that prevents ACh binding to its receptor) to the culture medium (Vilmont et al., 2016) (Table S1). The innervation-independent twitches are blocked only by tetrodotoxin, whereas the contractions induced by innervation are abolished by both drugs.

Functional analysis

Electrophysiological demonstration of spontaneous and evoked neurotransmission is the most detailed way to characterize the functionality of a NMJ model. mEPPS can be recorded by a microelectrode in the motoneuron and another in the myotube close to the point of contact. This criterion alone is an unequivocal evidence of a synapse, although it may not be fully mature. A functional neuron can be identified upon electrical stimulation of the motoneuron and evocation of EPPs (Nelson et al., 1993). Alternatively, EPPs can be evoked and synapses revealed by applying glutamate from a microelectrode directly onto glutamatergic motoneurons (Puro et al., 1977).

To assess whether the EPPs or contractions are dependent on neurotransmission and AChRs, different toxins or drugs can be

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Table 1. Non-human *in vitro* models of NMJ – physiological criteria

Reference	Species (muscle cells/ motoneurons/Schwann cells)	Evoked EPP/ contraction: electrical stimulus on presynaptic	Evoked contraction: glutamate iontophoresis	EPP/ mEPP	Muscle fiber contraction	EPP/contraction stopped with d-TC677.69pt or α-BTX (AChR inhibition)	EPP/contraction stop with d-TTX (VGSC inhibition)	EPP/contraction stop with agatoxin or ω-conotoxin (VDCC inhibition)	Calcium imaging
Anderson et al. (1977)	*Xenopus*/*Xenopus*/A	Yes	ND	ND/ND	Yes	Yes	ND	ND	ND
Puro et al. (1977)	Rat/chick/A	ND	Yes	Yes/Yes	Yes	Yes	ND	ND	ND
Peng and Nakajima (1978)	*Xenopus*/*Xenopus*/A	ND	ND	Yes/ND	ND	ND	ND	ND	ND
Nakajima et al. (1980)	Rat/rat/A	ND	ND	ND/Yes	ND	ND	ND	ND	ND
Kidokoro et al. (1980)	*Xenopus*/*Xenopus* /A	ND	ND	ND/Yes	Yes	Yes	ND	ND	ND
Swenarchuk et al. (1990)	*Xenopus*/*Ambystoma*/A or *Ambystoma*/rat/A	ND	ND	ND/Yes	Occasional or Yes	Yes	ND	ND	ND
Nelson et al. (1993)	Mouse/mouse/A	Yes	ND	Yes/ND	Yes	Yes	Yes	ND	ND
Larkin et al. (2006)	Rat/rat/A	Yes	ND	ND/ND	Yes	ND	ND	ND	ND
Das et al. (2007)	Rat/rat/A	ND	ND	Yes/ND	Yes	Not for all myotubes	ND	ND	ND
Umbach et al. (2012)	Mouse/mouse/A	Yes	ND	Yes/Yes	Yes	Yes	ND	ND	ND
Chipman et al. (2014)	Chick/mouse/A	ND	ND	Yes/Yes	Yes	Yes	Yes	Diminution	ND
Zahavi et al. (2015)	Mouse/mouse/A	ND	ND	ND/ND	Yes	ND	Yes	ND	Synchronized Ca^{2+} signals
Ionescu et al. (2016)	Mouse/mouse/A	ND	ND	ND/ND	Yes	ND	Yes	ND	Synchronized Ca^{2+} signals
Vilmont et al. (2016)	Mouse/rat/rat	ND	ND	Yes/Yes	Yes	Yes	Yes	ND	ND
Uzel et al. (2016)	Mouse/mouse (ES cells)/A	Yes (optogenetic)	Yes	ND/ND	Yes	Yes	ND	ND	ND
Machado et al. (2019)	Chick/mouse (ES cells)/A	Yes (optogenetic)	ND	ND/ND	Yes	Yes	Yes	ND	ND

A, absent; α-BTX, α-bungarotoxin; AChR, acetylcholine receptor; d-TC, d-tubocurarine; EPP, end-plate potential; ND, no data; TTX, tetrodotoxin; VDCC, voltage-dependent Ca^{2+} channel; VGSC, voltage-gated Na^{+} channel; mEPP, miniature end plate potential.

used. As mentioned previously, d-tubocurarine is a toxin blocking the AChR functionally, and thus blocking twitching, EPPs and mEPPs mediated by nicotinic AChR (Chipman et al., 2014; Kidokoro et al., 1980). A common test is to record muscle cell twitching, spontaneous EPPs or evoked EPPs, and then add d-tubocurarine in the medium. This addition of d-tubocurarine eliminates twitching and EPPs in the muscle cells, but not the motorneuron action potential (Umbach et al., 2012). This inhibition is reversible and when the d-tubocurarine is removed from the medium, the muscle fibers should re-start twitching or expressing mEPPs and EPPs almost immediately (Anderson et al., 1977; Das et al., 2007; Dorchies et al., 2001; Puro et al., 1977; Umbach et al., 2012; Vilmont et al., 2016).

The first humanized *in vitro* models of the NMJ

As the prevalence of neurodegenerative and muscular disorders is rising, and available treatments for these diseases are scarce, it is a matter of urgency to investigate new treatment strategies. Understanding the pathophysiology underlying diseases, such as amyotrophy lateral sclerosis (ALS) or congenital myasthenic syndrome (CMS), is a crucial step for early diagnosis and effective treatment. Therefore, the remaining challenge was to generate a human *in vitro* NMJ adapted to mimic *in vivo* biology with the aim to decipher pathological mechanisms and perform functional analysis.

In 1970, Peterson and Crain demonstrated that neuromuscular connections could be formed between explants of embryonic spinal cord from rodents and skeletal muscle tissue from adult humans (Crain et al., 1970). Formed with human myofibers, these cultures were maintained up to 7 weeks and electrophysiological analysis indicated that synaptic connections between rodent neurons and human muscle were functional and could be blocked with d-tubocurarine. They also demonstrated an accumulation of acetylcholinesterase (AChE) at sites of synaptic contact (Crain et al., 1970). Extending this first demonstration, Peterson and Crain observed pathological features in long-term co-cultures with human dystrophic muscle. In co-cultures maintained for more than 4 months, unusual focal myofibrillar lesions in a substantial number of innervated mature Duchenne muscular dystrophy (DMD) muscle fibers were described (Peterson et al., 1986). However, the complexity of preparing these co-cultures from human muscle biopsies has restricted their use.

An important breakthrough in the field of NMJ *in vitro* models came with the development of co-cultures between dissociated human primary skeletal muscle cells, derived from satellite cells, with rodent embryonic spinal cords (Table 3). Kobayashi and Askanas were the first to demonstrate that monolayer cultures of adult human muscle cells can be innervated by embryonic rat spinal cord neurons and that the differentiation of human skeletal muscle cells, as well as their electrophysiological properties, are greatly enhanced by innervation (Askanas et al., 1987; Kobayashi et al., 1987). Importantly, dorsal-root ganglia were essential for achieving functional innervation, as demonstrated by the presence of regular contractions and well-organized acetylcholinesterase-positive contacts (Askanas et al., 1987; Kobayashi et al., 1987). Further electrophysiological studies also demonstrated mEPPS and EPPs in these co-cultures were abolished by application of AChR antagonists, such as d-tubocurarine or α-bungarotoxin (α-BTX), which also blocks the contractions of human myotubes in these chimeric co-cultures. These studies also highlight that innervation can occur without the original basal lamina (Kobayashi and Askanas, 1985). Finally, the development of these chimeric models of NMJ provided an important basis for pathological

Table 2. Non-human *in vitro* models of NMJ – molecular criteria and summary of the studies

Reference	Species (muscle cells/ motoneurons/Schwann cells)	Morphology	Mature AChR clusters	Pre-synaptic markers	Synaptic or perisynaptic markers	Post-synaptic markers	Co-culture duration	Conclusion (Table 1 and 2)	Focus of the study
Anderson et al. (1977)	*Xenopus*/*Xenopus* /A	ND	Yes	ND	ND	ND	1 week	Functional amphibians NMJ	Dvlp
Puro et al. (1977)	Rat/chick/A	Plurinucleated striated myotubes	ND	ND	ND	ND	3 to 10 days	Functional chimeric NMJ	Optical synapse formation
Peng and Nakajima (1978)	*Xenopus*/*Xenopus*/A	Mononuclear, striated myotubes	Yes	ND	ND	ND	few days	Functional amphibians NMJ	Dvlp
Nakajima et al. (1980)	Rat/rat/A	Multi-innervated myotubes Vesicles acc. and post-junctional folds	ND	EM: active zones, vesicles	EM: Basal lamina	EM: primary and secondary folds	15 days	Functional mammalian NMJ	Dvlp
Kidokoro et al. (1980)	*Xenopus*/*Xenopus*/A	ND	Yes	ND	ND	ND	8 days	Functional amphibians NMJ	Dvlp
Swenarchuk et al. (1990)	*Xenopus*/*Ambystoma*/A or *Ambystoma*/rat/A	ND	Yes	ND	IF: HSPG	ND	ND	Functional amphibians NMJ	Dvlp
Nelson et al. (1993)	Mouse/Mouse/A	ND	ND	ND	ND	ND	4 weeks	Functional murine NMJ	Synapse elimination
Larkin et al. (2006)	Rat/Rat/A	Multinucleated myotubes	Yes	IF: NF	ND	IF: MHC	16-18 days	Functional rat NMJ	3D NMJ model
Das et al. (2007)	Rat/Rat/A	Multinucleated myotubes	Yes	IF: NF and SYP	ND	IF: fetal myosin	12 days	Functional mammalian NMJ	NMJ model
Umbach et al. (2012)	Mouse/Mouse/A	Enlargment of the terminal bouton	Yes	IF: VaChT, ChT1, Snap25, syntaxin 1a, SYP and SV2	ND	ND	1 week	Functional murine NMJ and characterized presynaptic bouton	NMJ model
Chipman et al. (2014)	Chick/Mouse/A	Mono-innnervated myotubes	Yes	IF: SV2, synaptic vesicle recycling (FM-4-64dye), SYP, NCAM and VDCC	ND	ND	28 days	Functional chimeric NMJ	NMJ model/ disease modeling
Zahavi et al. (2015)	Mouse/Mouse/A	Plurinucleated striated myotubes	Yes	IF: synapsin, synaptic vesicle recycling (FM-4-64 dye), Hb9 and NF-H	ND	ND	A few days	Functional murine NMJ	Dvlp
Ionescu et al. (2016)	Mouse/Mouse/A	Striated myotubes	Yes	IF: SYN1, SYP, Hb9 and NF	ND	ND	3 to 4 weeks	Functional murine NMJ	NMJ model
Vilmont et al. (2016)	Mouse/rat/rat	Pretzel-like AChRs morphology, peripheral nuclei	Yes	IF: TUBB3, basoon, VaChT, ChAT, NF and SYN1	IF: GFAP (Schwann cells)	IF: AChRs, DRPR, VGSC, MuSK, rapsyn, Syne1, mFISH and εAChR	14 days	Functional rodent NMJ	Dvlp
Uzel et al. (2016)	Mouse/mouse (ES cells)/A	Striated myotubes	Yes	MN Hb9-GFP^{+}	ND	IF: α-actinin	9 days	Functional murine NMJ	NMJ model
Machado et al. (2019)	Chick/mouse (ES cells)/A	Striated myotubes	Yes	IF: TUBB3, SV2 and YFP (expressed by MNs)	ND	IF: titin	9 days	Functional chimeric NMJ	Disease modeling

A, absent; acc, accumulation; Dvlp, development; IF, immunofluorescence labeling; ChAT, choline acetyl transferase; ChT1, choline transporter 1; DHPR, dyhydropyridine receptor; EM, electron microscopy; Hb9, homeobox protein 9; HSPG, heparan sulfate proteoglycans; MHC, myosin heavy chain; MN, motoneuron; NCAM, neural cell-adhesion molecule; ND, no data; NF, neurofilament; RyR, ryanodine receptor; SV2, synaptic vesicle 2; SYN1, synapsin 1; SYP, synaptophysin; TUBB3, βIII-tubulin; VaChT, vesicular acetylcholine transporter.

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studies described for myotonic dystrophy (Kobayashi et al., 1990), DMD (Park-Matsumoto et al., 1991), McArdle's myopathy (Martinuzzi et al., 1993), spinal muscular atrophy (SMA), ALS and facioscapulohumeral muscular dystrophy (FSHD) (Braun et al., 1995). Several of these studies revealed some pathological phenotypes. Thus, human skeletal muscle from children affected by a severe form of SMA began to display degeneration only when co-cultured with embryonic rat spinal cords (Braun et al., 1995). Despite these important advances, the only human tissue that could be used in these co-cultures was skeletal muscle cells. Recent advances in stem cell biology have fueled the prospect of generating the main components of the NMJ, offering new perspectives for pathobiological studies and therapeutic development for neuromuscular diseases. Thus, the availability of human pluripotent stem cells (hPSCs), either of embryonic stem cell (ESC) or induced pluripotent stem cell (iPSC) origin, has made the generation of human muscle and nerve cells possible. The first protocols developed from hPSCs concerned the specification of spinal motoneurons. Based on developmental insights generated from animal studies and those gained on mouse ESCs (Wichterle et al., 2002), much progress has been made on the capacity to convert hPSCs into spinal motoneurons. Li and colleagues were among the first to attempt co-cultures between hPSC-derived spinal motoneurons and murine skeletal muscle cells C2C12 (Li et al., 2005) (Table 4). After 3 weeks of culture, contractions were observed only in the presence of hESC-derived motoneurons and immunostaining analysis revealed the presence of clusters of AChR juxtaposed to neurite contacts (Li et al., 2005). Since then, this approach has been extended to disease-specific hiPSC-derived motoneurons such as SMA (Yoshida et al., 2015). In this context, the use of SMA-hiPSC derived motoneurons led to an impaired AChR clustering on C2C12 that precedes motoneuron loss (Yoshida et al., 2015). However, it is important to emphasize the relatively low rate of differentiation of hPSCs into hPSC-derived motoneurons observed in these studies, with only 25-30% of motoneurons generated on average.

To circumvent this limitation, motoneurons enriched by FACS or directly induced from fibroblasts (hi-motoneurons), without passing through an induced pluripotent stem cell stage, have been used (Du et al., 2015; Liu et al., 2016; Singh Roy et al., 2005). When co-cultured with primary rodent skeletal muscle cells, both sources of motoneurons led to the colocalization of synaptic markers such as synapsin with AChR clusters. Electrophysiological analyses also revealed spontaneous end-plate currents detected in murine myotubes when co-cultured with hi-motoneurons generated from human fibroblasts by overexpression of four transcription factors (SOX11, NGN2, ISL1 and LHX3). This electrophysiological activity can be blocked by using either d-tubocurarine, tetrodotoxin or a specific inhibitor of voltage-dependent sodium channels in muscle: ω-connotoxin. When applied to a pathological condition such as ALS, profound dysfunction in the ability of patient-specific hi-motoneurons to control functional muscle maturation is observed (Liu et al., 2016). Although promising, these first descriptions were

Table 3. *In vitro* models of NMJ – human source of skeletal muscle cells

Reference	Source of MN	Co-culture duration	Pre-synaptic markers	Synaptic markers	Post-synaptic markers	Muscle contraction	EPP/ contractions blocked with d-TC or α-BTX	Disease modeling
Crain et al. (1970)	Fetal mouse spinal cord	Up to 7 weeks	ND	HC: AChE	ND	Yes	Yes	ND
Witkowski and Dubowitz (1975)	Embryonic mouse spinal cord	Up to 11 weeks	ND	ND	ND	Yes	Yes	DMD/SMA
Kobayashi and Askanas (1985)	Fetal rat spinal cord	56 days	ND	HC: AChE	IS: ^{125}I-α-BTX	ND	ND	ND
Peterson et al. (1986)	Embryonic mouse spinal cord	Up to 6 months	EM: active zones and vesicles	HC: AChE	EM: primary and secondary folds	Yes	Yes	DMD Myofilament breakdown
Kobayashi et al. (1987)	Fetal rat spinal cord	Up to 4 weeks	ND	HC: AChE	IS: ^{125}I-α-BTX	Yes	ND	ND
Askanas et al. (1987)	Fetal rat spinal cord	Up to 8 weeks	EM: active zones and vesicles	EM: basal lamina	EM: primary and secondary folds	Yes	ND	ND
Kobayashi et al. (1990)	Fetal rat spinal cord	Up to 7 weeks	ND	HC: AChE	IS:^{125}I-α-BTX	Yes	Yes	MD Innervation less mature
Park-Matsumoto et al. (1991)	Fetal rat spinal cord	3 weeks	ND	ND	IF: dystrophin	Yes	ND	DMD Absence of dystrophin
Michikawa et al. (1991)	Rat spinal cord	2 weeks	ND	HC: AChE	IF: α-BTX	Yes	Yes	ND
Braun et al. (1995)	Embryonic rat spinal cord	1 month	ND	ND	EM: primary and secondary folds	ND	ND	SMA (type I, II and III) Degenerating co-cultures
Dorchies et al. (2001)	Rat spinal cord	Up to 1 year	ND	ND	EM: primary and secondary folds IF: desmin, troponin-T, tropomyosin, MHC and ^{125}I-α-BTX	Yes	ND	X-linked MTM No phenotype

α-BTX, α-bungarotoxin; AChE, acetylcholinesterase; d-TC, d-tubocurarine; DMD, Duchenne muscular dystrophy; EM, electron microscopy; HC, histochemistry; IF, immunofluorescence labeling; IS, iodine staining; MD, myotonic dystrophy; MHC, myosin heavy chain; ND, no data; SMA, spinal muscular atrophy; X-linked MTM, X-linked myotubular myopathy.

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Table 4. *In vitro* models of NMJ – human source of motoneurons

Reference	Source of skeletal muscle cells	Co-culture duration	Pre-synaptic markers	Post-synaptic markers	Muscle contraction	EPP/contractions blocked with d-TC/TTX/ω-conotoxin	Disease modeling
Singh Roy et al. (2005)	Rat myoblasts	1 week	IF: SV2, TUBB3 and MAP2	IF: AChRs and desmin	ND	ND	ND
Li et al. (2005)	C2C12	Up to 2 weeks	IF: SYN1 and ChAT	IF: AChRs	ND	ND	ND
Du et al. (2015)	C2C12	10 days	IF: ChAT	IF: AChRs	ND	ND	ND
Yoshida et al. (2015)	C2C12	Up to 60 days	IF: SV2 and NF	IF: AChRs and MHC	ND	ND	SMA type 1 defective AChR clustering
Guo et al. (2010)	Rat skeletal muscle	3 weeks	IF: SYP and TUBB3	IF: AChRs and MHC	Yes	Yes Electrophysiological recordings	ND
Liu et al. (2016)	Mouse myoblasts	1 week	IF: SYN1	IF: AChRs and MHC	ND	ND	ALS (FUS mutations) Decrease AChR clusters and defective AP (amplitude/ frequency)

AChR, acetylcholine receptor; ALS, amyotrophic lateral sclerosis; AP, action potential; ChAT, choline acetyl transferase; d-TC, d-tubocurarine; EM, electron microscopy; IF; immunofluorescence labeling; MAP2, microtubule-associated protein 2; MHC, myosin heavy chain; ND, no data; NF, neurofilament; SMA, spinal muscular atrophy; SV2, synaptic vesicle type 2; SYN1, synapsin 1; TTX, tetrodotoxin; TUBB3, β-III tubulin.

all based on chimeric models and to reach the 'holy grail' of a 'real' human NMJ *in vitro* model, both co-culture components should be of human origin.

Towards entirely humanized NMJ *in vitro* models

Over the past decade, the literature has documented fully humanized *in vitro* models of NMJs based on motoneurons mainly derived from hPSCs cultured with human primary skeletal muscle cells (Afshar Bakooshli et al., 2019; Marteyn et al., 2011; Santhanam et al., 2018; Shimojo et al., 2015; Steinbeck et al., 2016; Vila et al., 2019). Although all these studies diverged in their protocols, they all described the presence of AChR clusters in close proximity to neuritic processes, as well as muscle twitching after 2 or 3 weeks of co-culture. This indicates to some extent the functionality of human primary skeletal muscle cells when co-cultured with human motoneurons (Table 5). To go further ahead in the functional characterization of this system, Steinbeck and colleagues were the first to combine hPSC-based *in vitro* models of NMJs with optogenetics (Steinbeck et al., 2016). The authors used purified hESC-derived motoneurons stably expressing channelrhodopsin 2 (ChR2) coupled to the GFP under the human synapsin promoter. After 6-8 weeks of co-culture with human primary skeletal muscle, muscle twitches were observed and quantified after light stimulation. The addition of vecuronium, an antagonist of the nicotinic AChR, completely blocked the light-induced muscle twitches. Calcium imaging and electrophysiological analyses also confirmed these results. As purification of hPSC-derived motoneurons could lead to the selection of physiologically irrelevant motoneurons, these datasets were reproduced using motoneurons generated with a recent protocol that allows the efficient conversion of hPSC into a homogenous population of spinal motoneurons without using a purification step (Maury et al., 2015). Interestingly, the authors also demonstrated that their 'all human' NMJ cultures could be used in the pathological context of myasthenia gravis (Box 1) (Steinbeck et al., 2016).

Despite these developments, several challenges remain. The first limitation is the source of the human skeletal muscle cells. All the models described are based on the use of human primary skeletal muscle cells, which exhibit substantial donor variation in terms of differentiation (Nikolic et al., 2017). The development of an 'all human' NMJ model in which both neuron and muscle components are derived from the same individual is of great interest for modeling disease of genetic etiology. With the recent development of protocols allowing the conversion of hPSCs into skeletal muscle cells (reviewed by Pourquie et al., 2018), it is possible to produce co-cultures between motoneurons and skeletal muscle cells derived from hiPSCs from the same donor (Demestre et al., 2015; Maffioletti et al., 2018; Mazaleyrat et al., 2020; Osaki et al., 2018; Picchiarelli et al., 2019; Puttonen et al., 2015). Although the maturation level of these co-cultures generated from 'embryonic-like' pluripotent stem cells is still questionable, the detection of AChR clustering, contractions and calcium imaging suggest an acceptable level of functionality of these systems.

Another limitation stems from the fact that most of these studies have used a 2D cell culture system that does not mimic the native tissue structure. Consequently, different attempts have been made recently to evaluate the possibility of developing an 'all human' NMJ *in vitro* model by taking advantage of the advent of 3D cultures and microfluidic lab-on-a-chip technologies (Afshar Bakooshli et al., 2019; Maffioletti et al., 2018; Osaki et al., 2018; Santhanam et al., 2018; Vila et al., 2019). Microfluidics allows precise control over the cell microenvironment, providing an interesting platform for studying the NMJ. Motoneurons and muscle can be in two different chambers connected by axons, which enables spatiotemporal control over the cells. Analysis of the localized actions at the soma, along the axon and at the NMJ can be performed. Santhanam and colleagues were among the first to apply microfluidics to human motoneurons and muscle cells. Two weeks of co-culture were sufficient to observe muscle contractions after electrical stimulation of the motoneurons that can be blocked by treatment with d-tubocurarine (Table S1). However, the percentage of contracting myotubes is lower than the number of NMJs determined immunocytochemically, suggesting that some of the latter may not be mature enough to be functional (Santhanam et al., 2018).

Another approach relies on the use of 3D co-cultures with muscle cells in hydrogels, such as the commercially available basement membrane extract Matrigel followed by differentiating the motoneurons on the surface. 3D cultures tend to be functionally more relevant with respect to contractile muscle. Collagen I is a major component of the native ECM and harbors adequate properties needed for contraction and force generation. However, Collagen I has less stability, degrades faster and appears to be a less effective solution for

Table 5. *In vitro* models of NMJ – human source of skeletal muscle cells and motoneurons

Reference	All from hPSCs	Cell origin	Co-culture duration	2D/3D	Pre-synaptic markers	Synaptic markers	Post-synaptic markers	Muscle contraction	EPP/contractions blocked with drugs	Calcium imaging optogenetics	Disease modeling
Guo et al. (2011)		Human primary myoblasts and hESC-derived MNs	2-3 weeks	2D	IF: SYP and TUBB3	ND	IF: AChRs and MHC	Yes	Yes	ND	ND
Marteyn et al. (2011)		Human primary myoblasts and hESC-derived MNs	2 weeks	2D	IF: TUBB3	ND	IF: AChRs	ND	ND	ND	DM1 (defect in neuritic arborization)
Puttonen et al. (2015)	Yes	hESC mixed differentiation	Up to 6 weeks	2D	IF: SV2, SYP	ND	IF: AChRs, MHC and phalloidin	Yes	Yes	Under stimulation with ACh	ND
Steinbeck et al. (2016)		Human primary myoblasts and hESC-derived MNs	6-8 weeks	2D	IF: SYP	ND	IF: AChRs, desmin and MHC	Yes, after optogenetic stimulation	Yes, after optogenetic stimulation	ChR2	MG (decreased muscle twitches)
Osaki et al. (2018)	Yes	hiPSC-derived muscle cells and hiPSC-derived MNs	2 weeks	3D	IF: ChAT, SMI-32, TUBB3	ND	IF: AChRs, α-actinin	Yes	Yes	ChR2 (CAG promoter, AAV transduction)	ALS (degenerescence, fewer contractions)
Maffioletti et al. (2018)	Yes	hiPSC-derived muscle cells, hiPSC-derived MNs and hiPSC-derived endothelial cells	2 weeks	3D	IF: SMI-32	ND	IF: AChRs, MHC and Titin	ND	ND	ND	Laminopathies Defect in nuclear elongation
Santhanam et al. (2018)		Human primary myoblasts and hiPS-derived MNs	2 weeks	2D microfluidic	IF: NF and SYP	ND	IF: AChRs and MHC	Yes	Yes	ND	ND
Afshar Bakooshli et al. (2019)		Human primary myoblasts and hESC-derived MNs	2 weeks	2D versus 3D	IF: SYP and SMI-32	Laminin β2	IF: AChRs, α-actinin and rapsyn	Yes	ND	GCaMP6/ChR2 (EiFa promoter)	MG (decreased area of muscle response)
Vila et al. (2019)		Human primary myoblasts and ChR2-expressing hiPSC-derived MNs	3 weeks	3D microfluidic	ND	ND	IF: α-actinin	Yes	Yes	ChR2 (EiFa promoter)	MG (difference in contractile properties)
Lin et al. (2019)	Yes	hiPSC-derived muscle cells and hiPSC-derived MNs	Up to 100 days	2D	IF: TUBB3, NF, SV2 and ChAT	ND	IF: AChRs and MHC	No contraction without stimulation	ND	ChR2 (synapsin promoter)	SMA (smaller area of NMJ)
Picchiarelli et al. (2019)	Yes	hiPSC-derived muscle cells and hiPSC-derived MNs	3 weeks	2D	ND	ND	IF: AChRs and α-actinin	ND	ND	ND	ALS (defect in AChR clustering)
Bellmann et al. (2019)	Yes	hiPSC-derived muscle cells and hiPSC-derived MNs	3 weeks	3D microfluidic	IF: TUBB3, ChAT and synapsin EM: vesicles and primary folds	ND	IF: AChRs and α-actinin	No contraction without stimulation	ND	ChR2 (EiFa promoter)	ND
Faustino-Martins et al. (2020)	Yes	hiPSCs and hESCs	Up to 150 days	3D organoids	IF: ChAT, SMI-32, TUBB3, SYP, GFAP, S100β and MBP EM: synaptic cleft and vesicles	IF: laminin sheaths	IF: fast MHC and AChRs EM: sarcomeric units	Spontaneous Blocked by curare	Blocked by TTX	Calcium signal MEA	MG (severe reduction of NMJ)
Kim et al. (2020)		hMPCs and hNSCs	10 days	3D bioprinting	IF: TUBB3, NF and GFAP	ND	IF: MHC AChRs	ND	ND	Calcium signal	Rodent muscle defect injury model
Mazaleyrat et al. (2020)	Yes	Concomitant differentiation of hiPSC-derived muscle cells and hiPSC-derived MNs	30 days up to 7 months	2D	IF: NF and S100β	ND	IF: desmin, Pax3/7, titin, MHC and AChRs EM: sarcomeric units and peripheral nuclei	Spontaneous	Blocked by α-BTX and TTX	Calcium signal	DMD, DM1, FSHD2 and LGMD2A (multiple alterations)

α-BTX, α-bungarotoxin; ACh, acetylcholine; AChR, acetylcholine receptors; ALS, amyotrophic lateral sclerosis; BTX, bungarotoxin; ChAT, choline acetyl transferase; ChR2, channelrhodopsin 2; d-TC, d-tubocurarine; DM1, myotonic dystrophy type I; EM, electron microscopy; GFAP, glial fibrillary acidic protein; IF, immunofluorescence labeling; hMPCs, human mesenchymal precursor cells; hNSCs, human neural stem cells; hPSCs, human pluripotent stem cells; MBP, myelin basic protein; MG, myasthenia gravis; MHC, myosin heavy chain; ND, no data; NF, neurofilament; SV2, synaptic vesicle type 2; SYP, synaptophysin; TTX, tetrodotoxin; TUBB3, β-III tubulin.

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long-term cultures (Leikina et al., 2002). In contrast to Collagen I, Matrigel seems to be better adapted for muscle differentiation (Grefte et al., 2012). Other alternatives to collagen are fibrin, fibrinogen or thrombin (Wu et al., 2005). Such approaches have recently been applied to the development of fully humanized NMJ *in vitro* models in which muscle cell laden hydrogels were formed in specific molds with pillar structures at each extremity sufficient. These then created a continuous uniaxial tension and directed orientation of cells along the axis of tension (Afshar Bakooshli et al., 2019; Maffioletti et al., 2018). A comparative analysis of 2D versus 3D co-cultures reveals a faster and more efficient functional innervation in 3D versus 2D co-cultures when reviewed after 2 weeks in culture. The determination was performed using calcium imaging, optogenetics and electrophysiological recordings (Afshar Bakooshli et al., 2019). A more mature expression profile of AChR isoforms was observed in 3D co-cultures with an increased expression of the adult AChR ε isoform in comparison with 2D cultures. Nonetheless, the small size of muscle fibers, as well as the properties of action potentials recorded in these co-cultures when compared with *in vivo* mammalian models, suggests that the 3D human NMJs co-cultures are not fully mature and that additional cues might be necessary to ensure further maturation of tissues (Afshar Bakooshli et al., 2019). Increasing culture time, adding trophic factors or providing other cell types have been suggested. Recently, complex isogenic multilineage 3D co-cultures have been developed containing skeletal muscle cells, vascular endothelial cells, pericytes and motoneurons, all derived from hiPSCs (Maffioletti et al., 2018). Whereas these complex co-cultures were mainly characterized at the morphological level, these results open new perspectives for the development of humanized organoid-like platforms for studying NMJ development and disease modeling.

Two studies have also described the combination of 3D system and microfluidics (Osaki et al., 2018; Vila et al., 2019). These approaches have the advantage that motoneurons and muscle cells can be compartmentalized while providing spatial guidance cues and signal gradients using 3D hydrogels for better integration. The study uses engineered functional contractile hiPSC-derived skeletal muscle cells co-cultured with hiPSC-derived motoneurons. Interestingly, such approaches are compatible for studying and validating the therapeutic potential of compounds for neuromuscular disorders such as ALS (Osaki et al., 2018).

Finally, 3D bioprinting is an emerging field that could be relevant for the development of *in vitro* NMJ systems. A recent study described the feasibility of using bio-printed 3D hydrogel skeletons to support culture, differentiation and alignment of human primary skeletal muscle cells compatible with the functional integration of immortalized human neural progenitor cells (Kim et al., 2020). Interestingly, these bio-printed constructs with neural cells facilitate rapid innervation and mature into organized muscle tissue, restoring normal muscle function in a rodent model of muscle defect injury (Kim et al., 2020). This development can be pursued in the future for improving the maturity of NMJ systems and highlights an area of considerable interest in the tissue-engineering field.

Future perspectives

For over one century, the field of research around NMJs has grown constantly. Recent advances in stem cell biology have stimulated the development of innovative models of human NMJs. Despite initial successes in developing human motoneuron-muscle cells co-cultures, stem-cell derived NMJs remain immature in comparison with the human and chimeric NMJ models. One challenge is the limited survival of these co-cultures. The first chimeric mouse-human co-cultures could survive for more than 6 months (Peterson et al., 1986), whereas human stem-cell based co-cultures often do not survive beyond 4 weeks. However, a recent protocol of human stem cell co-differentiation enables the co-culture to survive up to 7 months (Mazaleyrat et al., 2020). Another challenge for future studies is to find a different method for generating spinal motoneurons. Among the motoneuron lineages *in vivo*, seven different lower motoneuron subtypes exist, each of them innervating a different muscle group. Most of the NMJ models from iPSCs in the currently published studies used iPS cell-derived general motoneurons. Efforts are under way to characterize the subtypes obtained and to improve the motoneuron differentiation protocols in order to obtain specific motoneuron subtypes (Hester et al., 2011; Lukovic et al., 2017). Similarly, iPS cell-derived muscle fibers are so immature that they cannot be classified as muscle fiber type I or II. Future work to obtain well-characterized motoneurons and muscle fibers subtypes will be particularly important when it comes to modeling neuromuscular pathologies, as all NMJs are not affected in the same way (Nicole et al., 2014). Additionally, tSCs are still a missing partner in current *in vitro* models. Protocols enabling the differentiation of iPS cells into myelinated Schwann cells are available (Liu et al., 2012; Ziegler et al., 2011). To date, there are no protocols for generating tSCs. These cells, together with complex and specialized basal laminas, will likely be required to reach a fully mature *in vitro* human NMJ model. Future research on 3D *in vitro* human NMJ models will be important in this effort. Recently, Faustino Martins and colleagues developed an elegant strategy to derive neuromuscular organoids from hiPSCs. Over a period of 50 to 100 days, these organoids reached a size of 5-6 mm in diameter and displayed an elongated morphology with neural tissue on one side and muscle cells on the other. In this system, electron microscopy confirmed the presence of presynaptic nerve terminals containing synaptic vesicles and active zones: tSCs and post-synaptic muscle fibers with organized sarcomeres and junctional folds. Importantly, these NMJs were functional, as demonstrated by the detection of muscle contraction after 50 days of culture. The functionality could be inhibited with d-tubocurarine, as well as spontaneous calcium oscillations and electrical activities (Faustino Martins et al., 2020). When employed with patient-specific stem cells together with CRISPR/Cas9 genome editing to restore or create mutations identified in patients, these new models will provide more reliable models of human NMJs *in vitro* and of neuromuscular diseases. Finally, to address the needs of new therapeutic approaches for neuromuscular diseases, standardized and miniaturized human *in vitro* NMJ models are a long-awaited drug-screening tool.

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Competing interests

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References

Afshar Bakooshli, M., Lippmann, E. S., Mulcahy, B., Iyer, N., Nguyen, C. T., Tung, K., Stewart, B. A., van den Dorpel, H., Fuehrmann, T., Shoichet, M. et al. (2019). A 3D culture model of innervated human skeletal muscle enables studies of the adult neuromuscular junction. *eLife* **8**, e44530. doi:10.7554/eLife.44530

Anderson, M. J., Cohen, M. W. and Zorychta, E. (1977). Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 731-756. doi:10.1113/jphysiol.1977.sp011879

Askanas, V., Kwan, H., Alvarez, R. B., Engel, W. K., Kobayashi, T., Martinuzzi, A. and Hawkins, E. F. (1987). De novo neuromuscular junction formation on human muscle fibres cultured in monolayer and innervated by foetal rat spinal cord: ultrastructural and ultrastructural–cytochemical studies. *J. Neurocytol.* **16**, 523-537. doi:10.1007/BF01668506

Barik, A., Li, L., Sathyamurthy, A., Xiong, W. C. and Mei, L. (2016). Schwann Cells in Neuromuscular Junction Formation and Maintenance. *J. Neurosci.* **36**, 9770-9781. doi:10.1523/JNEUROSCI.0174–16.2016

Bellmann, J., Goswami, R. Y., Girardo, S., Rein, N., Hosseinzadeh, Z., Hicks, M. R., Busskamp, V., Pyle, A. D., Werner, C. and Sterneckert, J. (2019). A customizable microfluidic platform for medium-throughput modeling of neuromuscular circuits. *Biomaterials* **225**, 119537. doi:10.1016/j.biomaterials.2019.119537

Beeson, D., Higuchi, O., Palace, J., Cossins, J., Spearman, H., Maxwell, S., Newsom-Davis, J., Burke, G., Fawcett, P., Motomura, M. et al. (2006). Dok-7 mutations underlie a neuromuscular junction synaptopathy. *Science* **313**, 1975-1978. doi:10.1126/science.1130837

Bhatt, J. M. (2016). The Epidemiology of Neuromuscular Diseases. *Neurol. Clin.* **34**, 999-1021. doi:10.1016/j.ncl.2016.06.017

Biro, A. A., Holderith, N. B. and Nusser, Z. (2005). Quantal size is independent of the release probability at hippocampal excitatory synapses. *J. Neurosci.* **25**, 223-232. doi:10.1523/JNEUROSCI.3688-04.2005

Blau, H. M., Chiu, C. P. and Webster, C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell* **32**, 1171-1180. doi:10.1016/0092-8674(83)90300-8

Bornstein, M. B., Iwanami, H., Lehrer, G. M. and Breitbart, L. (1968). Observations on the appearance of neuromuscular relationships in cultured mouse tissues. *Z Zellforsch Mikrosk Anat* **92**, 197-206. doi:10.1007/BF00335647

Braun, S., Croizat, B., Lagrange, M. C., Warter, J. M. and Poindron, P. (1995). Constitutive muscular abnormalities in culture in spinal muscular atrophy. *Lancet* **345**, 694-695. doi:10.1016/S0140-6736(95)90869-2

Burden, S. J., Huijbers, M. G. and Remedio, L. (2018). Fundamental Molecules and Mechanisms for Forming and Maintaining Neuromuscular Synapses. *Int. J. Mol. Sci.* **19**, 490-509.

Burgen, A. S., Dickens, F. and Zatman, L. J. (1949). The action of botulinum toxin on the neuro-muscular junction. *J. Physiol.* **109**, 10-24. doi:10.1113/jphysiol.1949.sp004364

Cartaud, A., Strochlic, L., Guerra, M., Blanchard, B., Lambergeon, M., Krejci, E., Cartaud, J. and Legay, C. (2004). MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. *J. Cell Biol.* **165**, 505-515. doi:10.1083/jcb.200307164

Cescon, M., Gregorio, I., Eiber, N., Borgia, D., Fusto, A., Sabatelli, P., Scorzeto, M., Megighian, A., Pegoraro, E., Hashemolhosseini, S. et al. (2018). Collagen VI is required for the structural and functional integrity of the neuromuscular junction. *Acta Neuropathol.* **136**, 483-499. doi:10.1007/s00401-018-1860-9

Changeux, J. P., Galzi, J. L., Devillers-Thiery, A. and Bertrand, D. (1992). The functional architecture of the acetylcholine nicotinic receptor explored by affinity labelling and site-directed mutagenesis. *Q. Rev. Biophys.* **25**, 395-432. doi:10.1017/S0033583500004352

Chipman, P. H., Zhang, Y. and Rafuse, V. F. (2014). A stem-cell based bioassay to critically assess the pathology of dysfunctional neuromuscular junctions. *PLoS ONE* **9**, e91643. doi:10.1371/journal.pone.0091643

Christian, C. N., Nelson, P. G., Peacock, J. and Nirenberg, M. (1977). Synapse formation between two clonal cell lines. *Science* **196**, 995-998. doi:10.1126/science.193191

Court, F. A., Gillingwater, T. H., Melrose, S., Sherman, D. L., Greenshields, K. N., Morton, A. J., Harris, J. B., Willison, H. J. and Ribchester, R. R. (2008). Identity, developmental restriction and reactivity of extralaminar cells capping mammalian neuromuscular junctions. *J. Cell Sci.* **121**, 3901-3911. doi:10.1242/jcs.031047

Couteaux, R. and Pecot-Dechavassine, M. (1970). [Synaptic vesicles and pouches at the level of “active zones” of the neuromuscular junction]. *C R Acad. Hebd Seances Acad. Sci. D* **271**, 2346-2349.

Crain, S. M., Alfei, L. and Peterson, E. R. (1970). Neuromuscular transmission in cultures of adult human and rodent skeletal muscle after innervation in vitro by fetal rodent spinal cord. *J. Neurobiol.* **1**, 471-489. doi:10.1002/neu.480010409

Das, M., Rumsey, J. W., Gregory, C. A., Bhargava, N., Kang, J. F., Molnar, P., Riedel, L., Guo, X. and Hickman, J. J. (2007). Embryonic motoneuron-skeletal muscle co-culture in a defined system. *Neuroscience* **146**, 481-488. doi:10.1016/j.neuroscience.2007.01.068

Demestre, M., Orth, M., Fohr, K. J., Achberger, K., Ludolph, A. C., Liebau, S. and Boeckers, T. M. (2015). Formation and characterisation of neuromuscular junctions between hiPSC derived motoneurons and myotubes. *Stem Cell Res.* **15**, 328-336. doi:10.1016/j.scr.2015.07.005

Dorchies, O. M., Laporte, J., Wagner, S., Hindelang, C., Warter, J. M., Mandel, J. L. and Poindron, P. (2001). Normal innervation and differentiation of X-linked myotubular myopathy muscle cells in a nerve-muscle coculture system. *Neuromuscul. Disord.* **11**, 736-746. doi:10.1016/S0960-8966(01)00221-8

Du, Z. W., Chen, H., Liu, H., Lu, J., Qian, K., Huang, C. L., Zhong, X., Fan, F. and Zhang, S. C. (2015). Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. *Nat. Commun.* **6**, 6626. doi:10.1038/ncomms7626

Eguchi, T., Tezuka, T., Miyoshi, S. and Yamanashi, Y. (2016). Postnatal knockdown of dok-7 gene expression in mice causes structural defects in neuromuscular synapses and myasthenic pathology. *Genes Cells* **21**, 670-676. doi:10.1111/gtc.12370

Engel, A. G., Shen, X. M., Selcen, D. and Sine, S. M. (2015). Congenital myasthenic syndromes: pathogenesis, diagnosis, and treatment. *Lancet Neurol.* **14**, 420-434. doi:10.1016/S1474-4422(14)70201-7

Evans, M. H. (1969). Differences between the effects of saxitoxin (paralytic shellfish poison) and tetrodotoxin on the frog neuromuscular junction. *Br. J. Pharmacol.* **36**, 426-436. doi:10.1111/j.1476-5381.1969.tb07999.x

Fatt, P. and Katz, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320-370. doi:10.1113/jphysiol.1951.sp004675

Faustino-Martins, J. M., Fischer, C., Urzi, A., Vidal, R., Kunz, S., Ruffault, P. L., Kabuss, L., Hube, I., Gazzerro, E., Birchmeier, C. et al. (2020). Self-Organizing 3D Human Trunk Neuromuscular Organoids. *Cell Stem Cell* **26**, 172-186.e176.

Ferns, M. J., Campanelli, J. T., Hoch, W., Scheller, R. H. and Hall, Z. (1993). The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. *Neuron* **11**, 491-502. doi:10.1016/0896-6273(93)90153-I

Fischbach, G. D. (1970). Synaptic potentials recorded in cell cultures of nerve and muscle. *Science* **169**, 1331-1333. doi:10.1126/science.169.3952.1331

Fischbach, G. D. (1972). Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* **28**, 407-429. doi:10.1016/0012-1606(72)90023-1

Fox, J. W., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Mann, K., Timpl, R., Krieg, T., Engel, J. et al. (1991). Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. *EMBO J.* **10**, 3137-3146. doi:10.1002/j.1460-2075.1991.tb04875.x

Fox, M. A., Sanes, J. R., Borza, D. B., Eswarakumar, V. P., Fassler, R., Hudson, B. G., John, S. W., Ninomiya, Y., Pedchenko, V., Pfaff, S. L. et al. (2007). Distinct target-derived signals organize formation, maturation, and maintenance of motor nerve terminals. *Cell* **129**, 179-193. doi:10.1016/j.cell.2007.02.035

Frank, E. and Fischbach, G. D. (1979). Early events in neuromuscular junction formation in vitro: induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed synapses. *J. Cell Biol.* **83**, 143-158. doi:10.1083/jcb.83.1.143

Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R. and Merlie, J. P. (1995). Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* **377**, 232-236. doi:10.1038/377232a0

Gee, S. H., Montanaro, F., Lindenbaum, M. H. and Carbonetto, S. (1994). Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* **77**, 675-686. doi:10.1016/0092-8674(94)90052-3

Granato, M., van Eeden, F. J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J. et al. (1996). Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* **123**, 399-413.

Grefte, S., Vullinghs, S., Kuijpers-Jagtman, A. M., Torensma, R. and Von den Hoff, J. W. (2012). Matrigel, but not collagen I, maintains the differentiation capacity of muscle derived cells in vitro. *Biomed. Mater.* **7**, 055004. doi:10.1088/1748-6041/7/5/055004

Guarino, S. R., Canciani, A. and Forneris, F. (2019). Dissecting the extracellular complexity of neuromuscular junction organizers. *Front. Mol. Biosci.* **6**, 156. doi:10.3389/fmolb.2019.00156

Guo, X., Das, M., Rumsey, J., Gonzalez, M., Stancescu, M. and Hickman, J. (2010). Neuromuscular junction formation between human stem-cell-derived motoneurons and rat skeletal muscle in a defined system. *Tissue Eng, Part C Methods* **16**, 1347-1355. doi:10.1089/ten.TEC.2010.0040

Guo, X., Gonzalez, M., Stancescu, M., Vandenburgh, H. H. and Hickman, J. J. (2011). Neuromuscular junction formation between human stem cell-derived motoneurons and human skeletal muscle in a defined system. *Biomaterials* **32**, 9602-9611. doi:10.1016/j.biomaterials.2011.09.014

Guo, X., Greene, K., Akanda, N., Smith, A., Stancescu, M., Lambert, S., Vandenburgh, H. and Hickman, J. (2014). In vitro Differentiation of Functional Human Skeletal Myotubes in a Defined System. *Biomater Sci.* **2**, 131-138. doi:10.1039/C3BM60166H

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P. et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1-36.

Hamuro, J., Higuchi, O., Okada, K., Ueno, M., Iemura, S., Natsume, T., Spearman, H., Beeson, D. and Yamanashi, Y. (2008). Mutations causing DOK7 congenital myasthenia ablate functional motifs in Dok-7. *J. Biol. Chem.* **283**, 5518-5524. doi:10.1074/jbc.M708607200

Hantai, D., Nicole, S. and Eymard, B. (2013). Congenital myasthenic syndromes: an update. *Curr. Opin. Neurol.* **26**, 561-568. doi:10.1097/WCO.0b013e328364dc0f

Harrison, R. G. (1959). The outgrowth of the nerve fiber as a mode of protoplasmic movement. *J. Exp. Zool.* **142**, 5-73. doi:10.1002/jez.1401420103
Hester, M. E., Murtha, M. J., Song, S., Rao, M., Miranda, C. J., Meyer, K., Tian, J., Boulting, G., Schaffer, D. V., Zhu, M. X. et al. (2011). Rapid and efficient generation of functional motor neurons from human pluripotent stem cells using gene delivered transcription factor codes. *Mol. Ther.* **19**, 1905-1912. doi:10.1038/mt.2011.135
Heuser, J. E., Reese, T. S., Dennis, M. J., Jan, Y., Jan, L. and Evans, L. (1979). Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell Biol.* **81**, 275-300. doi:10.1083/jcb.81.2.275
Higuchi, O., Hamuro, J., Motomura, M. and Yamanashi, Y. (2011). Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. *Ann. Neurol.* **69**, 418-422. doi:10.1002/ana.22312
Hoch, W., McConville, J., Helms, S., Newsom-Davis, J., Melms, A. and Vincent, A. (2001). Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat. Med.* **7**, 365-368. doi:10.1038/85520
Inoue, A., Setoguchi, K., Matsubara, Y., Okada, K., Sato, N., Iwakura, Y., Higuchi, O. and Yamanashi, Y. (2009). Dok-7 activates the muscle receptor kinase MuSK and shapes synapse formation. *Sci. Signal.* **2**, ra7. doi:10.1126/scisignal.2000113
Ionescu, A., Zahavi, E. E., Gradus, T., Ben-Yaakov, K. and Perlson, E. (2016). Compartmental microfluidic system for studying muscle-neuron communication and neuromuscular junction maintenance. *Eur. J. Cell Biol.* **95**, 69-88. doi:10.1016/j.ejcb.2015.11.004
Jacobson, C., Cote, P. D., Rossi, S. G., Rotundo, R. L. and Carbonetto, S. (2001). The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. *J. Cell Biol.* **152**, 435-450. doi:10.1083/jcb.152.3.435
James, D. W. and Tresman, R. L. (1968). De novo formation of neuro-muscular junctions in tissue culture. *Nature* **220**, 384-385. doi:10.1038/220384a0
Johnson, D. D., Bailey, S. and Wenger, B. S. (1981). Trophic activity of sheep sciatic nerve extracts in skeletal muscle cultures from normal and dystrophic chick embryos: failure of dystrophic muscle to respond. *Exp. Neurol.* **73**, 421-429. doi:10.1016/0014-4886(81)90276-4
Jones, R. A., Reich, C. D., Dissanayake, K. N., Kristmundsdottir, F., Findlater, G. S., Ribchester, R. R., Simmen, M. W. and Gillingwater, T. H. (2016). NMJ-morph reveals principal components of synaptic morphology influencing structure-function relationships at the neuromuscular junction. *Open Biol.* **6**.https://doi.org/10.1098/rsob.160240
Jones, R. A., Harrison, C., Eaton, S. L., Llavero Hurtado, M., Graham, L. C., Alkhammash, L., Oladiran, O. A., Gale, A., Lamont, D. J., Simpson, H. et al. (2017). Cellular and Molecular Anatomy of the Human Neuromuscular Junction. *Cell Rep.* **21**, 2348-2356. doi:10.1016/j.celrep.2017.11.008
Kidokoro, Y., Anderson, M. J. and Gruener, R. (1980). Changes in synaptic potential properties during acetylcholine receptor accumulation and neurospecific interactions in Xenopus nerve-muscle cell culture. *Dev. Biol.* **78**, 464-483. doi:10.1016/0012-1606(80)90347-4
Kim, N., Stiegler, A. L., Cameron, T. O., Hallock, P. T., Gomez, A. M., Huang, J. H., Hubbard, S. R., Dustin, M. L. and Burden, S. J. (2008). Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell* **135**, 334-342. doi:10.1016/j.cell.2008.10.002
Kim, J. H., Kim, I., Seol, Y. J., Ko, I. K., Yoo, J. J., Atala, A. and Lee, S. J. (2020). Neural cell integration into 3D bioprinted skeletal muscle constructs accelerates restoration of muscle function. *Nat. Commun.* **11**, 1025. doi:10.1038/s41467-020-14930-9
Ko, C. P. and Robitaille, R. (2015). Perisynaptic Schwann Cells at the Neuromuscular Synapse: Adaptable, Multitasking Glial Cells. *Cold Spring Harb. Perspect Biol.* **7**, a020503.
Kobayashi, T. and Askanas, V. (1985). Acetylcholine receptors and acetylcholinesterase accumulate at the nerve-muscle contacts of de novo grown human monolayer muscle cocultured with fetal rat spinal cord. *Exp. Neurol.* **88**, 327-335. doi:10.1016/0014-4886(85)90195-5
Kobayashi, T., Askanas, V. and Engel, W. K. (1987). Human muscle cultured in monolayer and cocultured with fetal rat spinal cord: importance of dorsal root ganglia for achieving successful functional innervation. *J. Neurosci.* **7**, 3131-3141. doi:10.1523/JNEUROSCI.07-10-03131.1987
Kobayashi, T., Askanas, V., Saito, K., Engel, W. K. and Ishikawa, K. (1990). Abnormalities of aneural and innervated cultured muscle fibers from patients with myotonic atrophy (dystrophy). *Arch. Neurol.* **47**, 893-896. doi:10.1001/archneur.1990.00530080077014
Koenen, M., Peter, C., Villarroel, A., Witzemann, V. and Sakmann, B. (2005). Acetylcholine receptor channel subtype directs the innervation pattern of skeletal muscle. *EMBO Rep.* **6**, 570-576. doi:10.1038/sj.embor.7400429
Larkin, L. M., Van der Meulen, J. H., Dennis, R. G. and Kennedy, J. B. (2006). Functional evaluation of nerve-skeletal muscle constructs engineered in vitro. *In Vitro Cell. Dev. Biol. Anim.* **42**, 75-82. doi:10.1290/0509064.1
LaRochelle, W. J., Witzemann, V., Fiedler, W. and Froehner, S. C. (1990). Developmental expression of the 43K and 58K postsynaptic membrane proteins and nicotinic acetylcholine receptors in Torpedo electrocytes. *J. Neurosci.* **10**, 3460-3467. doi:10.1523/JNEUROSCI.10-10-03460.1990
Leikina, E., Mertts, M. V., Kuznetsova, N. and Leikin, S. (2002). Type I collagen is thermally unstable at body temperature. *Proc. Natl. Acad. Sci. USA* **99**, 1314-1318. doi:10.1073/pnas.032307099
Lennon, V. A., Kryzer, T. J., Griesmann, G. E., O'Suilleabhain, P. E., Windebank, A. J., Woppmann, A., Miljanich, G. P. and Lambert, E. H. (1995). Calcium-channel antibodies in the Lambert-Eaton syndrome and other paraneoplastic syndromes. *N. Engl. J. Med.* **332**, 1467-1474. doi:10.1056/NEJM199506013322203
Li, X. J., Du, Z. W., Zarnowska, E. D., Pankratz, M., Hansen, L. O., Pearce, R. A. and Zhang, S. C. (2005). Specification of motoneurons from human embryonic stem cells. *Nat. Biotechnol.* **23**, 215-221. doi:10.1038/nbt1063
Li, L., Xiong, W. C. and Mei, L. (2018). Neuromuscular Junction Formation, Aging, and Disorders. *Annu. Rev. Physiol.* **80**, 159-188. doi:10.1146/annurev-physiol-022516-034255
Lin, W., Burgess, R. W., Dominguez, B., Pfaff, S. L., Sanes, J. R. and Lee, K. F. (2001). Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* **410**, 1057-1064. doi:10.1038/35074025
Lin, C. Y., Yoshida, M., Li, L. T., Ikenaka, A., Oshima, S., Nakagawa, K., Sakurai, H., Matsui, E., Nakahata, T. and Saito, M. K. (2019). iPSC-derived functional human neuromuscular junctions model the pathophysiology of neuromuscular diseases. *JCI Insight* **4**, e124299. doi:10.1172/jci.insight.124299
Lindstrom, J. M., Seybold, M. E., Lennon, V. A., Whittingham, S. and Duane, D. D. (1976). Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value. *Neurology* **26**, 1054-1059.
Liu, Q., Spusta, S. C., Mi, R., Lassiter, R. N., Stark, M. R., Hoke, A., Rao, M. S. and Zeng, X. (2012). Human neural crest stem cells derived from human ESCs and induced pluripotent stem cells: induction, maintenance, and differentiation into functional schwann cells. *Stem Cells Transl. Med.* **1**, 266-278. doi:10.5966/sctm.2011-0042
Liu, M. L., Zang, T. and Zhang, C. L. (2016). Direct Lineage Reprogramming Reveals Disease-Specific Phenotypes of Motor Neurons from Human ALS Patients. *Cell Rep* **14**, 115-128. doi:10.1016/j.celrep.2015.12.018
Lukovic, D., Diez Lloret, A., Stojkovic, P., Rodriguez-Martinez, D., Perez Arago, M. A., Rodriguez-Jimenez, F. J., Gonzalez-Rodriguez, P., Lopez-Barneo, J., Sykova, E., Jendelova, P. et al. (2017). Highly efficient neural conversion of human pluripotent stem cells in adherent and animal-free conditions. *Stem. Cells Transl. Med.* **6**, 1217-1226. doi:10.1002/sctm.16-0371
Machado, C. B., Pluchon, P., Harley, P., Rigby, M., Gonzalez Sabater, V., Stevenson, D. C., Hynes, S., Lowe, A., Burrone, J., Viasnoff, V. et al. (2019). In vitro modelling of nerve-muscle connectivity in a compartmentalised tissue culture device. *Adv. Biosys.* **3**. doi: 10.1002/adbi.201800307
Maffioletti, S. M., Sarcar, S., Henderson, A. B. H., Mannhardt, I., Pinton, L., Moyle, L. A., Steele-Stallard, H., Cappellari, O., Wells, K. E., Ferrari, G. et al. (2018). Three-dimensional human iPSC-derived artificial skeletal muscles model muscular dystrophies and enable multilineage tissue engineering. *Cell Rep* **23**, 899-908. doi:10.1016/j.celrep.2018.03.091
Marangi, P. A., Forsayeth, J. R., Mittaud, P., Erb-Vogtli, S., Blake, D. J., Moransard, M., Sander, A. and Fuhrer, C. (2001). Acetylcholine receptors are required for agrin-induced clustering of postsynaptic proteins. *EMBO J.* **20**, 7060-7073. doi:10.1093/emboj/20.24.7060
Marchand, S., Devillers-Thiery, A., Pons, S., Changeux, J. P. and Cartaud, J. (2002). Rapsyn escorts the nicotinic acetylcholine receptor along the exocytic pathway via association with lipid rafts. *J. Neurosci.* **22**, 8891-8901. doi:10.1523/JNEUROSCI.22-20-08891.2002
Marteyn, A., Maury, Y., Gauthier, M. M., Lecuyer, C., Vernet, R., Denis, J. A., Pietu, G., Peschanski, M. and Martinat, C. (2011). Mutant human embryonic stem cells reveal neurite and synapse formation defects in type 1 myotonic dystrophy. *Cell Stem Cell* **8**, 434-444. doi:10.1016/j.stem.2011.02.004
Martinuzzi, A., Vergani, L., Carrozzo, R., Fanin, M., Bartoloni, L., Angelini, C., Askanas, V. and Engel, W. K. (1993). Expression of muscle-type phosphorylase in innervated and aneural cultured muscle of patients with myophosphorylase deficiency. *J. Clin. Invest.* **92**, 1774-1780. doi:10.1172/JCI116766
Maselli, R. A., Arredondo, J., Ferns, M. J. and Wollmann, R. L. (2012). Synaptic basal lamina-associated congenital myasthenic syndromes. *Ann. N. Y. Acad. Sci.* **1275**, 36-48. doi:10.1111/j.1749-6632.2012.06807.x
Maury, Y., Come, J., Piskorowski, R. A., Salah-Mohellibi, N., Chevaleyre, V., Peschanski, M., Martinat, C. and Nedelec, S. (2015). Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes. *Nat. Biotechnol.* **33**, 89-96. doi:10.1038/nbt.3049
Mazaleyrat, K., Badja, C., Broucqsault, N., Chevalier, R., Laberthonniere, C., Dion, C., Baldasseroni, L., El-Yazidi, C., Thomas, M., Bachelier, R. et al. (2020). Multilineage differentiation for formation of innervated skeletal muscle fibers from healthy and diseased human pluripotent stem cells. *Cells* **9**. doi:10.3390/cells9061531
Messeant, J., Ezan, J., Delers, P., Glebov, K., Marchiol, C., Lager, F., Renault, G., Tissir, F., Montcouquiol, M., Sans, N. et al. (2017). Wnt proteins contribute to neuromuscular junction formation through distinct signaling pathways. *Development* **144**, 1712-1724. doi:10.1242/dev.146167
Michikawa, M., Kobayashi, T. and Tsukagoshi, H. (1991). Early events of chemical transmission of newly formed neuromuscular junctions in monolayers of

human muscle cells co-cultured with fetal rat spinal cord explants. *Brain Res.* **538**, 79-85. doi:10.1016/0006-8993(91)90379-A

Moransard, M., Borges, L. S., Willmann, R., Marangi, P. A., Brenner, H. R., Ferns, M. J. and Fuhrer, C. (2003). Agrin regulates rapsyn interaction with surface acetylcholine receptors, and this underlies cytoskeletal anchoring and clustering. *J. Biol. Chem.* **278**, 7350-7359. doi:10.1074/jbc.M210865200

Nakajima, Y., Kidokoro, Y. and Klier, F. G. (1980). The development of functional neuromuscular junctions in vitro: an ultrastructural and physiological study. *Dev. Biol.* **77**, 52-72. doi:10.1016/0012-1606(80)90456-X

Nelson, P. G., Fields, R. D., Yu, C. and Liu, Y. (1993). Synapse elimination from the mouse neuromuscular junction in vitro: a non-Hebbian activity-dependent process. *J. Neurobiol.* **24**, 1517-1530. doi:10.1002/neu.480241106

Nicole, S., Chaouch, A., Torbergsen, T., Bauche, S., de Bruyckere, E., Fontenille, M. J., Horn, M. A., van Ghelue, M., Loseth, S., Issop, Y. et al. (2014). Agrin mutations lead to a congenital myasthenic syndrome with distal muscle weakness and atrophy. *Brain* **137**, 2429-2443. doi:10.1093/brain/awu160

Nikolic, N., Gorgens, S. W., Thoresen, G. H., Aas, V., Eckel, J. and Eckardt, K. (2017). Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise - possibilities and limitations. *Acta Physiol. (Oxf)* **220**, 310-331. doi:10.1111/apha.12830

Nishimune, H., Sanes, J. R. and Carlson, S. S. (2004). A synaptic laminin-calcium channel interaction organizes active zones in motor nerve terminals. *Nature* **432**, 580-587. doi:10.1038/nature03112

Ohkawara, B., Cabrera-Serrano, M., Nakata, T., Milone, M., Asai, N., Ito, K., Ito, M., Masuda, A., Ito, Y., Engel, A. G. et al. (2014). LRP4 third beta-propeller domain mutations cause novel congenital myasthenia by compromising agrin-mediated MuSK signaling in a position-specific manner. *Hum. Mol. Genet.* **23**, 1856-1868. doi:10.1093/hmg/ddt578

Okada, K., Inoue, A., Okada, M., Murata, Y., Kakuta, S., Jigami, T., Kubo, S., Shiraishi, H., Eguchi, K., Motomura, M. et al. (2006). The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. *Science* **312**, 1802-1805. doi:10.1126/science.1127142

Ono, F., Mandel, G. and Brehm, P. (2004). Acetylcholine receptors direct rapsyn clusters to the neuromuscular synapse in zebrafish. *J. Neurosci.* **24**, 5475-5481. doi:10.1523/JNEUROSCI.0851-04.2004

Osaki, T., Uzel, S. G. M. and Kamm, R. D. (2018). Microphysiological 3D model of amyotrophic lateral sclerosis (ALS) from human iPS-derived muscle cells and optogenetic motor neurons. *Sci. Adv.* **4**, eaat5847. doi:10.1126/sciadv.aat5847

Park-Matsumoto, Y. C., Kameda, N., Kobayashi, T. and Tsukagoshi, H. (1991). Developmental study of the expression of dystrophin in cultured human muscle aneurally and innervated with fetal rat spinal cord. *Brain Res.* **565**, 280-289. doi:10.1016/0006-8993(91)91660-S

Peng, H. B. and Nakajima, Y. (1978). Membrane particle aggregates in innervated and noninnervated cultures of Xenopus embryonic muscle cells. *Proc. Natl. Acad. Sci. USA* **75**, 500-504. doi:10.1073/pnas.75.1.500

Peng, H. B., Ali, A. A., Daggett, D. F., Rauvala, H., Hassell, J. R. and Smalheiser, N. R. (1998). The relationship between perlecan and dystroglycan and its implication in the formation of the neuromuscular junction. *Cell Adhes Commun* **5**, 475-489. doi:10.3109/15419069809005605

Peterson, E. R., Masurovsky, E. B., Spiro, A. J. and Crain, S. M. (1986). Duchenne dystrophic muscle develops lesions in long-term coculture with mouse spinal cord. *Muscle Nerve* **9**, 787-808. doi:10.1002/mus.880090903

Picchiarelli, G., Demestre, M., Zuko, A., Been, M., Higelin, J., Dieterle, S., Goy, M. A., Mallik, M., Sellier, C., Scekic-Zahirovic, J. et al. (2019). FUS-mediated regulation of acetylcholine receptor transcription at neuromuscular junctions is compromised in amyotrophic lateral sclerosis. *Nat. Neurosci.* **22**, 1793-1805. doi:10.1038/s41593-019-0498-9

Pourquie, O., Al Tanoury, Z. and Chal, J. (2018). The Long Road to Making Muscle In Vitro. *Curr. Top. Dev. Biol.* **129**, 123-142. doi:10.1016/bs.ctdb.2018.03.003

Puro, D. G., De Mello, F. G. and Nirenberg, M. (1977). Synapse turnover: the formation and termination of transient synapses. *Proc. Natl. Acad. Sci. USA* **74**, 4977-4981. doi:10.1073/pnas.74.11.4977

Puttonen, K. A., Ruponen, M., Naumenko, N., Hovatta, O. H., Tavi, P. and Koistinaho, J. (2015). Generation of functional neuromuscular junctions from human pluripotent stem cell lines. *Front. Cell Neurosci.* **9**, 473. doi:10.3389/fncel.2015.00473

Rogers, R. S. and Nishimune, H. (2017). The role of laminins in the organization and function of neuromuscular junctions. *Matrix Biol.* **57-58**, 86-105. doi:10.1016/j.matbio.2016.08.008

Ruegg, M. A. and Bixby, J. L. (1998). Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction. *Trends Neurosci.* **21**, 22-27. doi:10.1016/S0166-2236(97)01154-5

Sanes, J. R. and Lichtman, J. W. (1999). Development of the vertebrate neuromuscular junction. *Annu. Rev. Neurosci.* **22**, 389-442. doi:10.1146/annurev.neuro.22.1.389

Sanes, J. R. and Lichtman, J. W. (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat. Rev. Neurosci.* **2**, 791-805. doi:10.1038/35097557

Santhanam, N., Kumanchik, L., Guo, X., Sommerhage, F., Cai, Y., Jackson, M., Martin, C., Saad, G., McAleer, C. W., Wang, Y. et al. (2018). Stem cell derived phenotypic human neuromuscular junction model for dose response evaluation of therapeutics. *Biomaterials* **166**, 64-78. doi:10.1016/j.biomaterials.2018.02.047

Scott, B. S., Engelbert, V. E. and Fisher, K. C. (1969). Morphological and electrophysiological characteristics of dissociated chick embryonic spinal ganglion cells in culture. *Exp. Neurol.* **23**, 230-248. doi:10.1016/0014-4886(69)90060-0

Shimojo, D., Onodera, K., Doi-Torii, Y., Ishihara, Y., Hattori, C., Miwa, Y., Tanaka, S., Okada, R., Ohyama, M., Shoji, M. et al. (2015). Rapid, efficient, and simple motor neuron differentiation from human pluripotent stem cells. *Mol Brain* **8**, 79. doi:10.1186/s13041-015-0172-4

Sigoillot, S. M., Bourgeois, F., Karmouch, J., Molgo, J., Dobbertin, A., Chevalier, C., Houlgatte, R., Leger, J. and Legay, C. (2016). Neuromuscular junction immaturity and muscle atrophy are hallmarks of the ColQ-deficient mouse, a model of congenital myasthenic syndrome with acetylcholinesterase deficiency. *FASEB J.* **30**, 2382-2399. doi:10.1096/fj.201500162

Silver, R. A., Lubke, J., Sakmann, B. and Feldmeyer, D. (2003). High-probability uniquantal transmission at excitatory synapses in barrel cortex. *Science* **302**, 1981-1984. doi:10.1126/science.1087160

Roy N., S., Nakano, T., Xuing, L., Kang, J., Nedergaard, M. and Goldman, S. A. (2005). Enhancer-specified GFP-based FACS purification of human spinal motor neurons from embryonic stem cells. *Exp. Neurol.* **196**, 224-234. doi:10.1016/j.expneurol.2005.06.021

Slater, C. R. (2017). The structure of human neuromuscular junctions: some unanswered molecular questions. *Int. J. Mol. Sci.* **18**. doi: 10.3390/ijms18102183

Sobel, A., Heidmann, T., Hofler, J. and Changeux, J. P. (1978). Distinct protein components from Torpedo marmorata membranes carry the acetylcholine receptor site and the binding site for local anesthetics and histrionicotoxin. *Proc. Natl. Acad. Sci. USA* **75**, 510-514. doi:10.1073/pnas.75.1.510

Steinbeck, J. A., Jaiswal, M. K., Calder, E. L., Kishinevsky, S., Weishaupt, A., Toyka, K. V., Goldstein, P. A. and Studer, L. (2016). Functional connectivity under optogenetic control allows modeling of human neuromuscular disease. *Cell Stem Cell* **18**, 134-143. doi:10.1016/j.stem.2015.10.002

Swenarchuk, L. E., Champaneria, S. and Anderson, M. J. (1990). Induction of a specialized muscle basal lamina at chimaeric synapses in culture. *Development* **110**, 51-61.

Takahashi, M., Kubo, T., Mizoguchi, A., Carlson, C. G., Endo, K. and Ohnishi, K. (2002). Spontaneous muscle action potentials fail to develop without fetal-type acetylcholine receptors. *EMBO Rep.* **3**, 674-681. doi:10.1093/embo-reports/kvf128

Umbach, J. A., Adams, K. L., Gundersen, C. B. and Novitch, B. G. (2012). Functional neuromuscular junctions formed by embryonic stem cell-derived motor neurons. *PLoS ONE* **7**, e36049. doi:10.1371/journal.pone.0036049

Uzel, S. G., Platt, R. J., Subramanian, V., Pearl, T. M., Rowlands, C. J., Chan, V., Boyer, L. A., So, P. T. and Kamm, R. D. (2016). Microfluidic device for the formation of optically excitable, three-dimensional, compartmentalized motor units. *Sci. Adv.* **2**, e1501429. doi:10.1126/sciadv.1501429

Verschuuren, J., Strijbos, E. and Vincent, A. (2016). Neuromuscular junction disorders. *Handb. Clin. Neurol.* **133**, 447-466. doi:10.1016/B978-0-444-63432-0.00024-4

Vila, O. F., Uzel, S. G. M., Ma, S. P., Williams, D., Pak, J., Kamm, R. D. and Vunjak-Novakovic, G. (2019). Quantification of human neuromuscular function through optogenetics. *Theranostics* **9**, 1232-1246. doi:10.7150/thno.25735

Vilmont, V., Cadot, B., Ouanounou, G. and Gomes, E. R. (2016). A system for studying mechanisms of neuromuscular junction development and maintenance. *Development* **143**, 2464-2477. doi:10.1242/dev.130278

Wallace, B. G. (1989). Agrin-induced specializations contain cytoplasmic, membrane, and extracellular matrix-associated components of the postsynaptic apparatus. *J. Neurosci.* **9**, 1294-1302. doi:10.1523/JNEUROSCI.09-04-01294.1989

Wichterle, H., Lieberam, I., Porter, J. A. and Jessell, T. M. (2002). Directed differentiation of embryonic stem cells into motor neurons. *Cell* **110**, 385-397. doi:10.1016/S0092-8674(02)00835-8

Witkowski, J. A. and Dubowitz, V. (1975). Growth of diseased human muscle in combined cultures with normal mouse embryonic spinal cord. *J. Neurol. Sci.* **26**, 203-220. doi:10.1016/0022-510X(75)90033-7

Wu, X., Chen, X. and Huang, B. (2005). [Relationship between fibrinogen content and the protracted inflammation of secretory otitis media]. *Lin Chuang Er Bi Yan Hou Ke Za Zhi* **19**, 449-450.

Wu, H., Lu, Y., Shen, C., Patel, N., Gan, L., Xiong, W. C. and Mei, L. (2012). Distinct roles of muscle and motoneuron LRP4 in neuromuscular junction formation. *Neuron* **75**, 94-107. doi:10.1016/j.neuron.2012.04.033

Yaffe, D. and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**, 725-727. doi:10.1038/270725a0

Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T. M., Birchmeier, C. and Burden, S. J. (2001). Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* **30**, 399-410. doi:10.1016/S0896-6273(01)00287-2

Yoshida, M., Kitaoka, S., Egawa, N., Yamane, M., Ikeda, R., Tsukita, K., Amano, N., Watanabe, A., Morimoto, M., Takahashi, J. et al. (2015). Modeling the early phenotype at the neuromuscular junction of spinal muscular atrophy using patient-derived iPSCs. *Stem Cell Reports* **4**, 561-568. doi:10.1016/j.stemcr.2015.02.010

Yumoto, N., Kim, N. and Burden, S. J. (2012). Lrp4 is a retrograde signal for presynaptic differentiation at neuromuscular synapses. *Nature* **489**, 438-442. doi:10.1038/nature11348

Zahavi, E. E., Ionescu, A., Gluska, S., Gradus, T., Ben-Yaakov, K. and Perlson, E. (2015). A compartmentalized microfluidic neuromuscular co-culture system reveals spatial aspects of GDNF functions. *J. Cell Sci.* **128**, 1241-1252. doi:10.1242/jcs.167544

Zainul, Z., Heikkinen, A., Koivisto, H., Rautalahti, I., Kallio, M., Lin, S., Haronen, H., Norman, O., Ruegg, M. A., Tanila, H. et al. (2018). Collagen XIII is required for neuromuscular synapse regeneration and functional recovery after peripheral nerve injury. *J. Neurosci.* **38**, 4243-4258. doi:10.1523/JNEUROSCI.3119-17.2018

Zhang, B., Luo, S., Wang, Q., Suzuki, T., Xiong, W. C. and Mei, L. (2008). LRP4 serves as a coreceptor of agrin. *Neuron* **60**, 285-297. doi:10.1016/j.neuron.2008.10.006

Ziegler, L., Grigoryan, S., Yang, I. H., Thakor, N. V. and Goldstein, R. S. (2011). Efficient generation of schwann cells from human embryonic stem cell-derived neurospheres. *Stem Cell Rev. Rep.* **7**, 394-403. doi:10.1007/s12015-010-9198-2

Zong, Y., Zhang, B., Gu, S., Lee, K., Zhou, J., Yao, G., Figueiredo, D., Perry, K., Mei, L. and Jin, R. (2012). Structural basis of agrin-LRP4-MuSK signaling. *Genes Dev.* **26**, 247-258. doi:10.1101/gad.180885.111