Neuromuscular junction-on-a-chip: ALS disease modeling and read-out development in microfluidic devices

Rianne de Jongh1 | Xandor M. Spijkers1,2 | Svetlana Pasteuning-Vuhman1 | Paul Vulto2 | R. Jeroen Pasterkamp1

1Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, Utrecht, The Netherlands
2Mimetas B.V., Organ-on-a-chip Company, Leiden, The Netherlands

Correspondence
R. Jeroen Pasterkamp, Department of Translational Neuroscience, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.

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Abstract
Amyotrophic lateral sclerosis (ALS) is a fatal and progressive neurodegenerative disease affecting upper and lower motor neurons with no cure available. Clinical and animal studies reveal that the neuromuscular junction (NMJ), a synaptic connection between motor neurons and skeletal muscle fibers, is highly vulnerable in ALS and suggest that NMJ defects may occur at the early stages of the disease. However, mechanistic insight into how NMJ dysfunction relates to the onset and progression of ALS is incomplete, which hampers therapy development. This is, in part, caused by a lack of robust in vitro models. The ability to combine microfluidic and induced pluripotent stem cell (iPSC) technologies has opened up new avenues for studying molecular and cellular ALS phenotypes in vitro. Microfluidic devices offer several advantages over traditional culture approaches when modeling the NMJ, such as the spatial separation of different cell types and increased control over the cellular microenvironment. Moreover, they are compatible with 3D cell culture, which enhances NMJ functionality and maturity. Here, we review how microfluidic technology is currently being employed to develop more reliable in vitro NMJ models. To validate and phenotype such models, various morphological and functional read-outs have been developed. We describe and discuss the relevance of these read-outs and specifically illustrate how these read-outs have enhanced our understanding of NMJ pathology in ALS. Finally, we share our view on potential future directions and challenges.

KEYWORDS
ALS, iPSC, microfluidics, motor neurons, neuromuscular junction, organ-on-a-chip

Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; ALS, amyotrophic lateral sclerosis; C9ORF72, chromosome 9 open reading frame 72; Ca\textsuperscript{2+}, calcium; ChAT, choline acetyltransferase; COC, cyclic olefin copolymer; EC, endothelial cell; ECM, extracellular matrix; fALS, familial amyotrophic lateral sclerosis; FUS, fused in sarcoma; GDNF, glial cell line-derived neurotrophic factor; hESCs, human embryonic stem cells; iPSCs, induced pluripotent stem cells; NMJ, neuromuscular junction; PDMS, polydimethylsiloxane; sALS, sporadic amyotrophic lateral sclerosis; SOD1, superoxide dismutase 1; TARDBP, TAR DNA-binding protein 43; TSC, terminal Schwann cell; vAChT, vesicular acetylcholine transporter.

Rianne de Jongh, Xandor M. Spijkers and Svetlana Pasteuning-Vuhman, these authors contributed equally to this work.

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Amyotrophic lateral sclerosis (ALS) is one of the most devastating neurodegenerative diseases with a lifetime risk of 1:400. It affects upper and lower motor neurons, which leads to motor weakness, bulbar dysfunction, and premature death, mostly because of respiratory failure, on average 3–5 years after symptom onset (Nijssen et al., 2018; Osking et al., 2019). Classically, 5%–10% of cases are defined as familial ALS (fALS) because of demonstration of direct inheritance (Renton et al., 2014), whereas the remainder of cases lack a clear hereditary or environmental cause and are being referred to as sporadic ALS (sALS). However, it is also becoming clear that the simple distinction between sALS and fALS is no longer tenable (Al-Chalabi et al., 2016). Over the past years, genetic studies have linked mutations in more than 30 genes to fALS, as well as to some sALS cases. The majority of fALS cases can be linked to mutations in one of the following four genes: Chromosome 9 open reading frame 72 (C9ORF72; approx. 40%) (DeJesus-Hernandez et al., 2011; Renton et al., 2011), Superoxide dismutase 1 (SOD1; approx. 20%) (Rosen et al., 1993), Fused in sarcoma (FUS; 1%–5%) (Kwiatkowski et al., 2009; Vance et al., 2009), and TAR DNA binding protein 43 (TARDBP; 1%–5%) (Neumann et al., 2006). These mutations have linked defects in various biological processes to the pathogenic mechanisms underlying ALS, ranging from mitochondrial dysfunction and altered RNA biology to disturbed axonal transport (Berk & Pasterkamp, 2019; van Es et al., 2017; Swinnen et al., 2020; Vandoorne et al., 2018).

Drugs currently used to treat ALS, such as riluzole and edaravone, prolong survival with a few months or slow down functional decline, and no curative treatment is available for ALS (Jaiswal, 2019). Therefore, there is an urgent need for the development of new, effective therapies for treating this disease. This not only relies on obtaining further insight into the mechanisms underlying motor neuron degeneration in ALS, but also on relevant in vitro models that enable testing of new therapeutic agents. The neuromuscular junction (NMJ), the synapse between a motor neuron and muscle fiber, plays a key role in the pathogenesis of ALS. NMJ defects are detected pre-symptomatically in experimental ALS and lead to axon retraction and motor neuron degeneration (Cappello & Francolini, 2017). Therefore, the NMJ is not only an important functional model for dissecting disease mechanisms, but also a relevant target for future therapeutic strategies that target ALS pathogenesis at the early disease stages. In this review, we will highlight recent developments of in vitro NMJ models and various morphological and functional read-outs to phenotype such models. Furthermore, we will discuss the importance of such models and read-outs for enhancing our understanding of NMJ pathology in ALS.

1.1 | Which cell types are required for NMJ modeling?

The design of an in vitro platform for studying NMJ (patho)physiology requires an understanding of the structure and composition of this functional unit, including cells, matrices, and their interactions. The NMJ is a specialized region, a synaptic connection that controls vital body processes such as voluntary movements and breathing (Rudolf et al., 2019). The NMJ is composed of three major elements: the pre-synaptic region containing the nerve terminal, the synaptic cleft, and the post-synaptic endplate. Upon arrival of an action potential at the NMJ, pre-synaptic motor neurons release neurotransmitter acetylcholine (ACh) within the synaptic cleft (Hirsch, 2007; Slater, 2017). ACh binds the ACh receptor (AChR) located in the post-synaptic muscle membrane, which leads to depolarization of the muscle cell and thereby triggers intracellular calcium ($Ca^{2+}$) release to initiate muscle contraction (Hirsch, 2007). The NMJ thus facilitates the transmission of action potentials from a nerve to a skeletal muscle. Below we discuss cell (sub)types and subcellular compartments that should be considered when modeling the NMJ in vitro.

1.1.1 | Different types of spinal motor neurons and motor neuron units exist

Spinal motor neurons and skeletal muscle cells are particularly significant for this review. Motor neurons are very diverse and are classified according to the type of muscle fiber they innervate. Alpha motor neurons innervate extrafusal muscle fibers within skeletal muscle, forming the major force-generating structure. Gamma motor neurons innervate intrafusal muscle fibers within muscle spindles and play an important role in motor control. Less well-defined beta motor neurons innervate both intra- and extrafusal fibers (Kanning et al., 2010). Alpha motor neurons are the most abundant spinal motor neurons and can be classified into three subtypes on the basis of the contractile properties of the motor unit that they form with muscle fibers: fast-twitch fatigable, fast-twitch fatigue-resistant, and slow-twitch fatigue-resistant. Not all motor neuron subtypes are equally affected in ALS. Fast-twitch fatigable motor units degenerate first with a near total loss of NMJ terminals, followed by degeneration of fast-twitch fatigue-resistant motor units, whereas slow-twitch fatigue-resistant motor units remain well-preserved until later disease stages (Pun et al., 2006). This knowledge helps to explain selective neurodegeneration in ALS and may aid therapy development for ALS and other neuromuscular disorders (Kanning et al., 2010).

1.1.2 | Local protein synthesis in spinal motor axons

Spinal motor neurons are located in the spinal cord and extend axons over long distances to form NMJs with muscle fibers. Because of the length of many spinal motor axons and the relatively low speed of axonal protein transport, the distal part of the axon contains its own microenvironment that, in part, functions independent from the soma (Holt & Schuman, 2013). During development, local protein synthesis in distal axons underlies gradient sensing and the...
directional turning of growing axons (Koppers et al., 2019; Leung et al., 2006; Ming et al., 2002; Piper et al., 2006; Yao et al., 2006). Moreover, it plays important roles in nervous system maintenance and repair (Jung et al., 2012; Perry et al., 2012; Verma et al., 2005; Yoon et al., 2012; Zheng et al., 2001). In addition to axons, local protein synthesis can occur at synapses, contributing to synapse development, function, and plasticity (Hafner et al., 2019; Holt et al., 2019). Given this role of local protein synthesis, it is not surprising that accumulating evidence indicates that axons can undergo degeneration by mechanisms independent of the cell body (Kanning et al., 2010). For example, compelling evidence indicates that axonal and NMJ degeneration precedes clinical symptoms in motor neuron diseases and occurs before loss of cell bodies (Conforti et al., 2007; Fischer et al., 2004; Wishart et al., 2006). Furthermore, local axonal mechanisms are affected in neurodegenerative diseases (Baleriola & Hengst, 2015; Fallini et al., 2016; López-Erasquin et al., 2018). Based on these observations, it is tempting to speculate that countering axonal loss or interfering with local axonal disease mechanisms provides a potential therapeutic target to protect axons before motor neuron death and clinical symptoms occur (Krakora et al., 2012).

1.1.3 | Muscle cells are affected in ALS

Muscle cells are an essential component of the NMJ and it is becoming increasingly clear that muscle cells are affected in ALS. For example, oxidative stress and mitochondrial defects are not only observed in motor neurons, but also in skeletal muscle cells of sALS patients (reviewed by Loeffler et al., 2016; Pansarasa et al., 2014). Moreover, skeletal muscle AChRs from ALS patients display distinct electrophysiological properties and differ from patients with other types of denervation (Palma et al., 2011). Furthermore, a recent study using a FUS-ALS mouse model showed that mutant FUS is intrinsically toxic to motor neurons and skeletal muscle cells (Picchiarelli et al., 2019). Thus, it is important to consider this specific contribution of muscle cells to ALS pathogenesis when modeling the NMJ.

1.1.4 | The role of terminal Schwann cells and kranocytes at the NMJ

In addition to an axon terminal and a muscle fiber, the NMJ has a third critical cellular component: the terminal Schwann cell (TSC). By secreting several trophic molecules, TSCs promote AChR clustering and stabilize muscle-nerve connections (Feng & Ko, 2008; Peng et al., 2003). After NMJ formation, TSCs are involved in synapse elimination at NMJs (Bishop et al., 2004; Darabid et al., 2013; Smith et al., 2013; Tapia et al., 2012) and control both synaptic efficacy and structural plasticity, possibly through decoding synaptic transmission and through interaction with neurotrophic factors (Ko & Robitaille, 2015; Robitaille, 1998; Todd et al., 2010). Additionally, TSCs play important roles in NMJ remodeling after injury. TSCs are highly plastic and able to change their properties according to the state of muscle innervation. They can attract and interact with immune cells such as macrophages, which are important for the phagocytosis of cellular debris (Arbour et al., 2015; Ko & Robitaille, 2015; Scheib & Höke, 2016). Age-related changes in TSCs and macrophages may contribute to impaired regenerative capacity of the NMJ (Painter, 2017; Scheib & Höke, 2016) and to the pathogenesis of late-onset motor neuron diseases such as ALS (Pandya & Patani, 2020). TSCs are known to be affected in ALS. For example, in a SOD1 ALS mouse model the ability of TSCs to decode synaptic transmission was altered and intracellular Ca$^{2+}$ responses were enhanced upon motor nerve stimulation, probably as a result of hyper-muscarinic activity (Arbour et al., 2015). These altered TSC properties gradually worsened toward symptom onset, suggesting that TSC dysfunction affects synaptic transmission during ALS progression.

Fibroblast-like cells, named kranocytes, have been identified as cells that cap the NMJ on top of TSCs (Court et al., 2008). The exact role of kranocytes remains to be established. However, it is known that kranocytes respond within 24 hr to muscle denervation and nerve injury by proliferating and spreading throughout the NMJ (Court et al., 2008). This suggests a role in nerve repair and regeneration.

1.1.5 | Extracellular matrix molecules at the NMJ

NMJs are embedded in a specialized extracellular matrix-containing collagen, laminins, agrin and other glycoproteins, which is reviewed more extensively by Barros et al. (2011). NMJs require these molecules for their normal development and function. For example, agrin regulates post-synaptic NMJ development (Wu et al., 2010), laminin induces clustering of Ca$^{2+}$ channels and development of NMJ pre-synaptic active zones (Fox et al., 2007), and Collagen IV is important for NMJ nerve terminal maturation and maintenance (Fox et al., 2007).

The complex cellular and extracellular matrix composition of the NMJ summarized in this section needs to be taken into account when generating in vitro models meant to recapitulate NMJ (patho)physiology.

1.2 | Evidence for early NMJ pathology in ALS

Are NMJ models needed to understand the pathogenesis of ALS? As outlined below in more detail, accumulating evidence indeed suggests that the NMJ is highly vulnerable in ALS and that NMJ defects occur prior to the onset of overt disease symptoms (Fischer et al., 2004; Machamer et al., 2014, 2018; Moloney et al., 2014; Murray et al., 2010).

1.2.1 | Support from clinical studies

Clinical studies show that axonal denervation (Bjornskov et al., 1984; Hanyu et al., 1982), synapse loss (Killian et al., 1994;
Sasaki & Maruyama, 1994), mitochondrial abnormalities (Borthwick et al., 1999; Wiedemann et al., 2002), and increased Ca$^{2+}$ levels (Siklos et al., 1996) contribute to ALS disease onset and progression. In addition, measurements of muscle action potentials and muscle fiber conduction velocity can effectively predict ALS development at a pre-symptomatic stage (Blijham et al., 2007). Finally, ALS patients show changes in axon excitability, which has shown to be more pronounced in distal parts of the axon (Nakata et al., 2006), and muscle biopsies obtained from a sALS patient displayed muscle denervation, whereas the motor neurons in the spinal cord appeared intact (Pollari et al., 2014). Together, these clinical data identify axonal and NMJ defects as early events in the pathogenesis of ALS.

1.2.2 Support from studies in animal models

Further evidence supporting early NMJ involvement and distal axonopathy in ALS derives from ALS animal models, including zebrafish, C. elegans, Drosophila and mice (Fischer et al., 2004; Machamer et al., 2014, 2018; Moloney et al., 2014; Murray et al., 2010). For example, over-expression of mutant FUS results in impaired NMJ function in Drosophila larvae (Machamer et al., 2014, 2018). Likewise, mutant FUS transgenic mice display pre-synaptic defects in motor neurons and over-expression of mutant FUS in motor neurons causes NMJ denervation preceding motor neuron loss (Sharma et al., 2016). These results together with pathological alterations in motor axons at the NMJ, such as mitochondrial failure, impaired axonal transport, an increase in intracellular Ca$^{2+}$, and enhanced reactive oxygen species levels (Fischer-Hayes et al., 2013; Pollari et al., 2014), support an early involvement of the NMJ. Decreased expression of choline acetyltransferase (ChAT) and the vesicular ACh transporter (vAChT) has also been found in ALS mouse models. ChAT is responsible for the biosynthesis of ACh and vAChT is involved in the packaging of ACh into synaptic vesicles before release (Campanari et al., 2016). Other evidence for ALS being a distal axonopathy is that loss of acetylcholinergic function, regulated by ChAT and vAChT at motor nerve endings, occurs before motor neuron loss (Casas et al. 2016). In addition to the work listed above, multiple other animal studies have identified NMJ loss as a key and early feature of ALS pathogenesis (e.g., mouse models for SOD1 (Clark et al., 2016; Tallon et al., 2016; Pun et al., 2006), TDP43 (Chand et al., 2018; Ditsworth et al., 2017), FUS (López-Erauskin et al., 2018; Scekic-Zahirovic et al., 2017; So et al., 2018), and UBQLN2 (Le et al., 2016)). Interestingly, these studies indicate that NMJ denervation is a gradual and highly dynamic process in which motor neurons may grow new branches and connections until eventually, the axon and motor neuron degenerates, as has been shown in SOD1 mice (Martineau et al., 2018). Stabilization of NMJs during this innervation/denervation cycle is an intriguing therapeutic approach.

In conclusion, degeneration of motor axons and the NMJ occurs at the early stages of ALS, and many key cellular elements of the NMJ are affected. These changes are likely to contribute to disease progression and motor neuron degeneration, emphasizing the need for the development of representative NMJ in vitro models.

2 TOWARD ADVANCED NMJ IN VITRO MODELING

Animal models provide valuable insights into NMJ physiology and disease, but it has been challenging to directly study human NMJ pathology. As human NMJs are morphologically different from NMJs in a range of other species, including rodents (Boehm et al., 2020), there is a need for more physiologically relevant in vitro NMJ models. As discussed below, over the past years the field has developed many different NMJ in vitro models ranging from organotypic and co-culture systems to human stem cell-derived models. An overview of important advances with respect to NMJ modeling is presented in Figure 1.

2.1 Classical in vitro NMJ models

The earliest NMJ models were generated by culturing embryonic spinal cord-innervated skeletal muscle from chickens, rodents, and Xenopus (Bornstein & Breitbart, 1964; Crain & Peterson, 1964; Szepesnovol, 1946). Next steps involved the generation of functional NMJ connections between spatially separated explants of somatic muscle and of the lumbar spinal cord (Crain & Peterson, 1964; Grainger et al., 1968; Shimada et al., 1969), and of the differentiation of cells from gastrulating Drosophila embryos into functionally active neurons and myocytes in a dish (Seecco et al., 1972). The advent of the hESC technologies revolutionized NMJ modeling as it allowed the development of motor neurons (Lee et al., 2007; Li et al., 2005; Wichterle et al., 2002) and skeletal muscle cells (Barberi et al., 2007; Darabi et al., 2008) in large quantities and of high quality. Since the early studies listed above, many (non-)ESC-derived NMJ co-culturing studies have been performed using Xenopus (Peng et al., 2003), rodent (Das et al., 2010; Morimoto et al., 2013), and human cells (Guo et al., 2011; Marteyn et al., 2011). Moreover, cross-species co-culturing studies have been conducted (Vilmont et al., 2016) (Figure 1). While ESCs opened new avenues for NMJ modeling, their application is confined by ethical and technical issues. With the development of alternative stem cell technologies (such as iPSCs) these issues have been overcome.

2.2 Patient-derived and iPSC-generated NMJ models

The discovery of induced pluripotent stem cells (iPSCs) in 2006 by Takahashi and Yamanaka was a breakthrough in stem cell biology. The successful reprogramming of human somatic cells into iPSCs was achieved through induced expression of four embryonic transcription factors: Oct4, Sox2, Klf4, and c-Myc (Takahashi & Yamanaka, 2006).
Despite suffering from specific limitations (see Pasteuning-Vuhman et al., 2020), iPSC technology is a powerful tool for the generation of patient-derived cellular models. Thus far, most iPSC-based ALS studies have focused on spinal motor neurons alone (Sances et al., 2016). However, establishing patient-specific NMJ models will advance our understanding of this disease. The formation of functional NMJs in vitro using iPSC approaches was successfully achieved by co-culturing human iPSC-motor neurons with rodent skeletal muscle cells (Umbach et al., 2012; Yoshida et al., 2015) or with primary human skeletal muscle cells (Santhanam et al., 2018; Steinbeck et al., 2016; Afshar Bakooshli et al. 2019). Recent advances in human iPSC differentiation protocols have facilitated the generation of functional NMJs between human iPSC-motor neurons and human iPSC-skeletal muscle cells (Demestre et al., 2015; Lin et al., 2019; Maffioletti et al., 2018; Osaki et al., 2018, 2020; Puttonen et al., 2015)(Figure 1). One of the first studies which described a stem-cell-derived NMJ model introduced a co-maturation method in which neural precursor cells/neurons and myogenic precursors/myotubes grow together and co-develop (Puttonen et al., 2015). This study highlights the necessity for long-term co-maturation of both pre- and post-synaptic cellular components in order to achieve full NMJ maturation. Importantly, iPSC technology allows for the mixing of independently generated iPSC-derived NMJ cellular components from the same genetic background or from mixed backgrounds. This will help the identification of key cellular components contributing to NMJ deterioration in ALS.

3 | The use of microfluidic devices to improve NMJ modeling

The formation of mature NMJs in vitro using the cell (sub)types discussed in the previous sections requires long-term culturing and precise control over the different components of the NMJ. To enable such long-term cultures, researchers have established microfluidic devices to model the NMJ. In such devices, motor neurons and skeletal muscle cells are cultured in separated, interconnected chambers. Microfluidic devices enable cells to get nutrients and oxygen via fluid circulation and allow exposure to spatial cues or signaling gradients needed for differentiation, growth, viability, and proliferation (Natarajan et al., 2019). In addition, spatial segregation allows studies on the effects of drugs on different parts of the NMJ...
(pre- and post-synaptic). Here, we first provide a general introduction into microfluidic technology followed by a discussion on the implementation of this technology in NMJ modeling.

3.1 | Microfluidic technology

The field of microfluidics emerged at the beginning of the 1990s (Harrison et al., 1993; Manz et al., 1990, 1991) and since then has attracted a lot of attention as a powerful technology merging the areas of biosciences and engineering. Around the turn of the century, microfluidic techniques started to be employed for tissue culture, making use of the ability to perfuse cultures and to co-culture tissues in a compartmentalized manner (Hung et al., 2005; Leclerc et al., 2003; Powers et al., 2002; Viravaidya et al., 2004). For CNS applications, microengineering techniques were being used to generate multi-electrode arrays (MEAs) for neuronal activity measurement and stimulation (Gross, 1979; Jimbo & Kawana, 1992; Thiébaud et al., 1997), as well as clustering (Heida et al., 2001). The first materials applied in microfluidic applications were silicon and glass. These manufacturing techniques required elaborate cleanroom facilities and in-depth expertise of microfabrication processing. A particular complex step involved the bonding of multiple substrates, needed for closing of the microfluidic channels. A silicon rubber material, named polydimethylsiloxane (PDMS), circumvented this obstacle (Duffy et al., 1998) and made the construction of microfluidic chips accessible more generally. An additional advantage of the use of elastomers, such as PDMS, is that its flexibility could be deployed for the construction of active valves and pumps, as well as for inducing mechanical strain. A particular advantage of silicone rubbers in the context of cell culture is their oxygen permeability. The use of this prototyping material came with significant drawbacks, however, the most significant being that it is prone to absorption of hydrophobic compounds, preventing its application in drug exposure studies. Other common microfabrication techniques that were utilized for creating cell culture devices include hot embossing and injection molding of polymers, such as poly(methyl methacrylate), polysul- fone, polycarbonate, and cyclic olefin copolymer (COC), followed by solvent bonding (Berthier et al., 2012).

Perfusion flow in microfluidic cell culture devices provides cells with oxygen, nutrients, and growth factors. In addition it is used to induce shear stress. Dynamic cell culture conditions allow precise control over spatial parameters and limit transient changes, such as building up gradients of oxygen or pH. Microfluidic devices typically involve some form of compartmentalization, either by incorporating porous membranes (Ingber, 2018), use of pillars (Osaki et al., 2018; Uzel et al., 2016) or integration of phaseguides (Trietsch et al., 2013; Vulto et al., 2011). This enables researchers to study cell-cell interactions as well as multi-tissue interactions (Marx et al., 2016; Miller et al., 2020). Lately, the integration of 3D scaffolds such as synthetic or natural extracellular matrices has become popular. All these techniques enable full control over the microenvironment and allow cell-cell interaction and cell-matrix interaction, as well as outgrowth and migration of cells. Furthermore, the number of readouts applied in microfluidic devices has been rapidly increasing (Junaid et al., 2017): from integration of magnetic, electrical, mechanical, or optical actuators to compatibility with standard assays and readouts.

3.2 | Constructing NMJs in microfluidic devices

In the field of neuroscience, Campenot chambers were the first compartmentalized culture systems (Campenot, 1977), consisting of a Teflon divider attached to a collagen-coated glass coverslip. Scratches in the coating guide neurites toward another compartment, enabling separation of neuronal cell bodies and axons. Later, a PDMS chip was created to grow and isolate axons as a model of CNS axon degeneration and injury (Taylor et al., 2005). Neurons are cultured in the soma compartment of the device, whereas axons grow through microchannels or microgrooves into the axon chamber. These microchannels prevent cell bodies and dendrites from entering the axon chamber. A more recent study used two-chamber devices to generate a NMJ-on-a-chip (Figure 2a). In this study, rat superior cervical ganglion neurons were seeded into one compartment, and ventricular myocytes into the other. Evoked responses in myocytes after electrical stimulation of the neurons suggested the formation of functional NMJs (Takeuchi et al., 2011). The chip described above (Taylor et al., 2005) was commercialized by Xona Microfluidics and used to design a culture model that in part mimics the anatomical and cellular interactions of the NMJ (Southam et al., 2013). In this study, glial cells were co-cultured with embryonic rat spinal cord motor neurons in the soma chamber to recapitulate the cellular environment of the spinal cord. Motor neuron axons extended into a distal chamber to connect to skeletal muscle cells, which was confirmed by the induction of AChR clustering on postsynaptic muscle cells (Figure 2b).

Microfluidic devices have also been used to demonstrate that trophic factors, such as glial cell line-derived neurotrophic factor (GDNF), act differently when added to the soma as compared to the axon compartment (Zahavi et al., 2015). GDNF facilitates motor neuron outgrowth and muscle innervation when applied to the NMJ. However, following application to the soma compartment GDNF affects cell survival. Using a custom-made microfluidic chip, consisting of two main compartments connected by parallel microchannels, ventral horn spinal cord explants from HBB-GFP mouse embryos were cultured in one compartment and myoblasts derived from primary satellite cells of the adult mouse gastrocnemius muscle in the other compartment. The formation of functional NMJs was confirmed by morphological analysis of AChR clusters and NMJ activity (Zahavi et al., 2015).

Not only rodent but also human cells have been used to assemble NMJ models in microfluidic devices. Human skeletal myoblasts and iPSC-derived motor neurons were spatially separated and cultured in a microfluidic chip leading to the generation of human NMJs (Santhanam et al., 2018). This system is composed of two open top cell culture chambers linked by microchannels allowing axonal...
outgrowth toward the muscle chamber. Formation and function of the NMJ was confirmed by assessment of robust motor neuron-induced muscle contraction. The open top design of the culture chambers allowed for external electrical stimulation of the motor neurons with muscle force measurements as an output. The system was validated for drug and toxicity testing by assessing dose–response curves for the NMJ blocking drugs Bungarotoxin, BOTOX® and curare (Santhanam et al., 2018).
Application of the approaches described above for drug development will require improved reproducibility between individual cultures. Custom-made microfluidic devices suffer from a certain level of variability. This limits their comparative power, especially when high-throughput experiments are desired (Ionescu et al., 2016). These issues can be overcome by commercial devices, such as the above described XonaChip or a similar device from Millipore AXIS™. Xona Microfluidics has transitioned from a PDMS prototype to a chip in thermoplastic COC, providing a reproducible manufacturing technology. Neurons in this new device are more evenly attached and distributed within the somatic compartment as compared to PDMS devices (Paranjape et al., 2019).

### 3.3 The transition toward 3D microfluidic-based NMJ models

Thus far, most NMJ models rely on 2D cultures. However, culturing cells in 2D as compared to their physiological 3D environment affects many cellular functions (Breslin & O’Driscoll, 2013; Edmondson et al., 2014). Tissue explants can be used as a source for 3D approaches (Prpar Mihevc et al., 2017), but this approach is limited to the use of animal material. Human tissue-based 3D approaches include the use of ECM-embedded cells, NMJ organoids and 3D bioprinting. Advantages of ECM-embedded cells over 2D monolayers have been reported for both neuronal and skeletal muscle cultures (Santoso & McCain, 2020). ECM-embedded 3D muscle shows enhanced maturity (Smith et al., 2012) and contractility (Afshar Bakooshli et al., 2019; Shima et al., 2018). Contractility is especially important, as this is only sparsely observed in 2D cultures. 3D neuronal cultures display enhanced survival and distinct gene expression patterns as compared to 2D neuronal cultures (Brännvall et al., 2007; Mertens et al., 2015).

Microfluidic chips can be used to generate 3D cultures. One of the first 3D microfluidic NMJ models utilized ECM-embedded motor neurospheres and ECM-embedded skeletal muscle cells cultured in separate compartments (Uzel et al., 2016). Muscle cells were seeded in a compartment that contained pillar structures, which served as anchors for the formation of myofibers and which facilitated the measurement of contraction (force of pillar displacement) in real-time. Neurospheres were added to an opposing channel with a gel region in between the neuronal and muscle compartments. In time, axons protruded the gel region toward the muscle cells forming NMJs (Figure 2c).

Drug development requires microfluidic devices that support high-throughput screening. Recent studies have reported 3D platforms that allow simultaneous testing of multiple NMJ cultures. In one study, NMJs were generated as described above (Uzel et al., 2016), but the device consisted of six individual tissue culture chips (Osaki et al., 2018). A microfluidic device that holds great potential for high-throughput studies in 3D is the OrganoPlate, a device developed by Mimetas B.V. An OrganoPlate consists of 40–96 microfluidic chips, where each chip contains two or three microfluidic lanes (Trietsch et al., 2013), separated only by pressure barriers termed phaseguides, allowing free cell-ECM interactions (Vulto et al., 2011). The device employs gravity-driven ECM interactions important for the formation and the stability of molecular gradients. Currently, no NMJ models using the OrganoPlate have been reported, but the OrganoPlate 3-lane has previously been used for the generation of stratified culture models, including co-cultures (van Duinen et al., 2019; Wevers et al., 2018).

The studies discussed above (Osaki et al., 2018; Uzel et al., 2016) still lack some of the essential cell types present at the NMJ, and the methods used to generate the neurospheres in these studies do not allow full control of the cell types developing within the neurospheres. A recent study used more defined spheroid-like structures utilizing a device containing open chambers connected by microgrooves and 6–9 chips per plate (Machado et al., 2019). In this device, the middle chamber contained ECM-embedded skeletal muscle cells, forming fibers wrapped around anchor points, similar to Uzel et al. (2016) and Osaki et al. (2018). Muscle contraction could be quantified by visual inspection of muscle displacement. The outer two chambers contained 3D aggregates consisting of sorted motor neurons and astrocytes, where motor neurons extended their axons through the microgrooves to the muscle cells, in a similar fashion as in the Xona device (Figure 2d).

Future inclusion of 3D structures derived from stem cell sources, such as organoids, may further increase the physiological relevance of NMJ models but could also increase variability and thereby affect reproducibility (Carcamo-Orive et al., 2017). If needed, homogenous cell populations can be obtained through cell sorting. Following sorting, of for example motor neurons and astrocytes, cells could be combined into more controllable 3D structures. Protocols to generate or obtain pure populations of other cells, for example, TSCs, still need to be developed. This also holds true for protocols that allow the combination of several different cell types into a single 3D structure, for example, related to the composition of media and growth factors.

In conclusion, various platforms have recently been used to develop 3D NMJ models. While there is a consensus among scientists that 3D cultures reflect human physiology more closely than 2D cultures, further characterization and development of 3D platforms for NMJ models is needed, for example, inclusion of additional cell types or improved compatibility with high-throughput screening. The development of physiologically relevant human NMJ models in a high-throughput platform will be essential for advancing drug development for ALS.

### 4 ALS-on-a-chip: dissection of disease mechanisms and therapy development

Accumulating evidence suggests that neuromuscular disorders such as ALS are distal axonopathies, where dysfunction at the NMJ is already evident before any clinical symptoms (Moloney et al., 2014; Murray et al., 2010) (see section 1.2). To acquire more knowledge
of NMJ function and its contribution to ALS pathology, an increasing number of studies utilizes NMJ-on-a-chip approaches (Machado et al., 2019; Maimon et al., 2018; Osaki et al., 2018). Various functional and morphological readouts exist to measure NMJ function and pathology in vitro, and their details are discussed in the subsequent sections (see Figure 3 and Table 1). First in general but followed by a discussion of their use in ALS research.

4.1 Assays for studying NMJ morphology and function in vitro

Various morphological read-outs are used to study the NMJ in vitro (Table 1). Time-lapse microscopy is used to visualize neurite outgrowth, neuron-muscle contacts, and myofiber differentiation. For a more detailed analysis of the NMJ, immunocytochemistry is used and includes α-bungarotoxin detection to visualize the presence of clustered AChRs, a sign of motor axon innervation (Wu et al., 2010). Muscle denervation is one of the hallmarks of ALS (Fischer et al., 2004) and changes in muscle innervation can be assessed by determining the co-localization of a pre-synaptic motor neuron marker and the aforementioned AChR clusters. Furthermore, a recent study describes how AChR clustering can be used to assess the maturity of NMJs in vitro (Picchiarelli et al., 2019). Finally, mitochondrial density is elevated at the NMJ (Ly & Verstreken, 2006) and NMJ formation leads to the accumulation of mitochondria at the NMJ, which might be relevant for motor neuron function (Altman et al., 2019). A detailed protocol for tracking and analyzing axonal transport of fluorescently labeled organelles in motor neurons has been published, including a simplified microfluidic chip manual (Altman et al., 2020). This method will be valuable for studying the effect of impaired mitochondrial transport on NMJ function.

A second approach for studying the NMJ relies on the use of functional assays. A functional NMJ facilitates communication between the motor neuron and skeletal muscle, and cellular responses can be measured in the muscle upon motor neuron stimulation. Assessment of NMJ function is crucial, as morphological read-outs may not reflect how well a NMJ actually functions. Various approaches to assess NMJ function have been described (Table 1). As detailed in section 3, these include micropillars around which skeletal muscle can assemble. Bundles exert force on these pillars when contracting, which can be measured (Uzel et al., 2016). Methods to quantify muscle displacement without the use of micropillars also exist (Machado et al., 2019; Santhanam et al., 2018). The inclusion of a MEA is often used to assess neuronal activity, but it can also measure electrical activity generated by contracting myotubes (Rabieh et al., 2016). Another approach to detect muscle activity is recording of the Ca²⁺ fluctuations that underlie muscle contraction.

In order to study neuron-muscle communication at the NMJ, motor neurons can be genetically modified to express light-sensitive cation channels, typically channelrhodopsin-2, a technique known as optogenetics. Optogenetics allows precise control of neuronal activity by stimulation with light (Boyden et al., 2005). Light stimulation induces receptor activation, causing specific motor neuron firing. This, in turn, leads to muscle contraction, thereby creating a highly controllable model of the NMJ.

Thus, several methods have been developed to study NMJ morphology and function. With the inclusion of additional cell types into current NMJ models and further technological advances in the area of microfluidics the number readouts for assessing the NMJ in vitro is likely to increase in the future.

4.2 How can axon degeneration in ALS be studied using microfluidic platforms?

Axon degeneration occurs preceding neuronal cell body loss in ALS (see section 1.2). The mechanistic details of this early pathogenic event are poorly understood but underlying several axonal defects have been reported in ALS models (Figure 3a). These include defects in axonal transport. Axonal transport ensures the dynamic supply of proteins, lipids, and organelles (such as mitochondria) to the NMJ from the soma. Moreover, it supports the clearing of recycled and misfolded proteins from the axon thereby preventing the formation of toxic aggregates (Millecamps & Julien, 2013). Since impaired axonal transport is a common axonal defect, analysis of this process

![Figure 3](image-url)
(a) Protein aggregation
- Defective transport of mitochondria
- Axon degeneration
- Changes in axonal transcriptome
- TSC dysfunction
- Chronic muscle injury
- Morphological NMJ changes

(b) Soma/glia compartment
- Protein aggregation (IHC)
- Control of neuronal activity (optogenetics)
- Glial toxicity (inclusion of control/ALS glial cells)

Microgrooves/gel region
- Impaired axonal transport (mitotracker)
- Changes in neuronal activity (calcium imaging/MEA)

Legend:
- Motor neuron
- Microglia
- Astrocyte
- Terminal Schwann Cell
- Macrophage
- Skeletal muscle fibre
- Skin fibroblast
- iPSCs
- Mitochondria
- Protein aggregates
- Secreted cues
| NMJ Characterisation | Readout | Assay | Microfluidic chip characteristic |
|----------------------|---------|-------|----------------------------------|
|                      |         |       | Transparent | Compartmentalization | Micropillars | MEA |
| Neurite outgrowth    | Time-lapse microscopy | ✓ | ✓ | ✓ |
|                      | Immunocytochemistry | ✓ | ✓ | ✓ |
| Neuron-muscle contact| Time-lapse microscopy | ✓ | ✓ | ✓ |
|                      | Immunocytochemistry | ✓ | ✓ | ✓ |
| Myofiber differentiation| Time-lapse microscopy | ✓ | ✓ | ✓ |
|                      | Immunocytochemistry | ✓ | ✓ | ✓ |
| Succinylcholine Clustering | Immunocytochemistry | ✓ | ✓ | ✓ |
| Mitochondrial density| Immunocytochemistry | ✓ | ✓ | ✓ |
| Neuronal activity    | MEA\(^a\) | ✓ | ✓ | ✓ |
|                      | Calcium imaging | ✓ | ✓ | ✓ |
|                      | Optogenetics(control of)\(^b\) | ✓ | ✓ | ✓ |
| Muscle activity      | MEA\(^a\) | ✓ | ✓ | ✓ |
|                      | Calcium imaging | ✓ | ✓ | ✓ |
|                      | Pillar displacement\(^c\) | ✓ | ✓ | ✓ |
|                      | Particle image velocimetry\(^d\) | ✓ | ✓ | ✓ |
|                      | Image subtraction video recordings\(^e\) | ✓ | ✓ | ✓ |
| Axonal transport     | (Mito)tracke + time-lapse microscopy\(^f\) | ✓ | ✓ | ✓ |
| Axonal mRNA expression| Axon-seq\(^g\) | ✓ | ✓ | ✓ |
|                      | FISH\(^h\) | ✓ | ✓ | ✓ |
| Mitochondrial functioning | Calcium imaging | ✓ | ✓ | ✓ |
|                      | Mitochondrial dyes [TMRM, JC-1] | ✓ | ✓ | ✓ |

Note: ✓ Required. 
✓ Preferred.

AChR, acetylcholine receptor; FISH, fluorescent in situ hybridization; JC-1, 1,1′,3,3′-tetraethyl-5,5′,6,6′-tetrachloroimida carbocyanine iodide; MEA, microelectrode array; NMJ, neuromuscular junction; TMRM, tetramethylrhodamine, methyl ester.

\(^a\) Rabieh et al., 2016; \(^b\) Uzel et al., 2016; Osaki et al., 2018; \(^c\) Uzel et al., 2016; \(^d\) Machado et al., 2019; \(^e\) Santhanam et al., 2018; \(^f\) Lu et al., 2012; Altman et al., 2020; \(^g\) Nijssen et al., 2018; \(^h\) Lee et al., 2015.
will provide insight into the contribution of NMJ denervation to axonal degeneration.

Axonal transport can be visualized by following fluorescently labeled organelles or proteins using fluorescence time-lapse microscopy. One of the organelles suffering from impaired anterograde transport are mitochondria, leading to reduced mitochondrial density at the motor nerve terminal, and accumulation of mitochondria in the cell body (reviewed by De Vos and Hafezparast 2017). Mitotracker, a fluorescent dye that specifically labels mitochondria, can be used in combination with live cell fluorescent imaging to, for example, determine if NMJ denervation affects mitochondrial transport in the axons or vice versa. This method can be used in vitro as well as in vivo. However, since the use of this technology in vivo is challenging and because many in vivo models do not completely recapitulate the human phenotype, in vitro models are a valuable resource. As axons grow randomly toward muscle cells in regular co-cultures these models often do not allow for the identification of an isolated axon and its growth direction. Within compartmentalized microfluidic chambers, the connecting microchannels define the direction of transport (Figure 3b). Axonal transport studies benefit from transparent microgrooves and gradients of signaling molecules to guide directional outgrowth of the axons from the proximal to the distal compartment (Lu et al., 2012).

Not only the transport but also the functional properties of mitochondria are affected in neurodegenerative diseases. Mitochondria are involved in energy metabolism and Ca$^{2+}$ homeostasis. Motor neurons rely on mitochondria for Ca$^{2+}$ buffering (reviewed by Grosskreutz et al., 2010) and increased intracellular Ca$^{2+}$ concentrations are a hallmark of axon degeneration. Normally, an influx of Ca$^{2+}$ as part of an action potential is harmless. However, it has been shown that in many neurodegenerative disorders, including ALS, recovery from the Ca$^{2+}$ influx is impaired (Fischer-Hayes et al., 2013). This is often caused by defects in mitochondrial Ca$^{2+}$ buffering which can be studied by combining Ca$^{2+}$ indicators with microscopy to measure intracellular Ca$^{2+}$ levels.

The distal axon is an important subcellular compartment (see section 1.1.2). It contains local translation machinery, which is important for signaling responses to internal and external triggers. Cargo synthesized in the soma would take too long to reach distal parts of the axon for acute responses, as motor axons can be up to one meter in length. However, distal parts of the axon do communicate with the cell soma using retrograde axonal transport while anterograde transport supplies molecules and organelles to distal axons (reviewed by Sahoo et al., 2018). To specifically study the axonal transcriptome, isolation of axonal RNA without somatic contaminants is a key requirement. Multiple studies have focused on obtaining pure axonal RNA for sequencing using cell culture inserts (Minis et al., 2014; Rotem et al., 2017). In these experiments, the axonal fraction is highly enriched in axonal RNA, but some dendrites were able to cross into the axonal compartment (Rotem et al., 2017). The use of a Xona Microfluidic device circumvented this problem as it allowed isolation of axonal RNA without contamination of non-axonal material (Briese et al., 2015; Taylor et al., 2005). More recently, a very elegant axon-sequencing (axon-seq) approach was reported (Figure 3b). This technique combines microfluidics, spatial single-cell RNA sequencing, and bioinformatics analysis, including a control step to remove samples containing cellular cross-contamination (Nijssen et al., 2018). This work revealed that the transcriptome of axons is enriched for mRNAs with roles in mitochondrial and ribosomal functions and that axonal and somal transcriptomes differ. Interestingly, SOD1$^{G93A}$ motor axons showed deregulated expression of key transcripts involved in neuronal function, axon maintenance, and growth (Nijssen et al., 2018). Axon-seq is a very promising technique for elucidating disease mechanisms in ALS, especially when combined with iPSC-generated cultures. It would also enable studies into differences between the proximal and distal parts of the axon by using three-compartment microfluidic devices. To further assess transcripts identified by axon-seq or other axon-specific techniques, multiplex fluorescent in situ hybridization (e.g., RNAscope) can be used. This method can simultaneously visualize thousands of mRNAs at the same time in intact cells and preserves the spatial information of each transcript (Lee et al., 2015). This approach would also allow comparison of proximal and distal axonal compartments.

4.3 Investigating the role of non-cell autonomous processes in ALS

Cell types other than motor neurons contribute to the pathogenesis of ALS (see section 2.1, Figure 3a). Microfluidic devices are an excellent platform for investigating how these cell types interact and influence motor neurons. For example, co-cultures of primary myocytes from pre-symptomatic SOD1$^{G93A}$ mice (in the distal compartment) with spinal cord explants from mouse embryos (in the proximal compartment) have demonstrated that axons are less likely to cross the microgrooves toward SOD1$^{G93A}$ muscle cells as compared to SOD1$^{wt}$ cells (Maimon et al., 2018). After introducing SOD1$^{G93A}$ myocytes, axons retracted, degenerated, or remained static. Similar axonal phenotypes were observed when motor neurons were cocultured with muscle cells carrying ALS-associated C9orf72 and TARDBP mutations. This suggests that ALS muscle cells secrete destabilizing factors. Further experiments revealed a therapeutic effect of microRNA126-5p over-expression in ALS myocytes that was mediated via a down-regulation of the axon repellent Semaphorin 3A. MicroRNA126-5p over-expression rescued NMJ disruption and axon degeneration. These data support the idea that targeting cells other than motor neurons may modulate ALS pathogenesis (Maimon et al., 2018).

A contribution of glial cells to ALS pathogenesis has also been established. The expression of mutant SOD1 in motor neurons determines disease onset and early progression, but not worsening at later stages. Deterioration at the later stages can be modulated by the expression of mutant SOD1 in microglial cells and astrocytes (Boillée et al., 2006; Ilieva et al., 2009; Yamanaka et al., 2008). Furthermore, areas of reactive astrogliosis and microgliosis surround degenerating...
motor neurons in ALS patients and animal models (Figure 3a) (Saberi et al., 2015), and glial cells like oligodendrocytes and oligodendrocyte precursors are affected in ALS (Ferraiuolo et al., 2016; Kang et al., 2013; Philips et al., 2013). Microfluidic devices offer a unique opportunity to mix cells from patients and controls in a highly controlled fashion to dissect (non-) cell autonomous disease mechanisms. Protocols have been developed to include glial cells in the soma compartment of Xona devices (Southam et al., 2013) and mixed populations of motor neurons and astrocytes have been applied in microfluidic platforms (Machado et al., 2019). In the latter study, mouse ESC-derived motor neurons were co-cultured with ESC-derived ALS-mutated astrocytes. At day 1, no differences in motor axons extending into the myofiber compartment were found in co-cultures with control and mutated astrocytes, but by day 7 SODI\textsuperscript{G93A} astrocytes caused a significant decrease in motor axon innervation. Furthermore, reduced muscle contraction was measured in cultures containing SODI\textsuperscript{G93A} astrocytes. Both findings resemble key features of early peripheral pathology of ALS in vivo and underscore the need for including glial cells in microfluidic NMJ models. These include astrocytes but also TSCs, oligodendrocytes, and Schwann cells. Moreover, it has been demonstrated that inflammatory processes occur within the skeletal muscle and near NMJs in a SODI\textsuperscript{G93A} rat model (Van Dyke et al. 2016). Therefore, the inclusion of immune cells such as microglia or macrophages should also be a future goal.

### 4.4 Application of ALS-on-a-chip approaches in drug discovery

Drug development for the treatment of ALS is challenging because of patient heterogeneity and other factors. In combination with iPSC technology for generating patient-derived cells, microfluidic devices are a promising system for improving drug development, as they enable high throughput screening in combination with quantitative analysis of functional parameters. Microfluidics-based drug screening could for example assist in compound screening, clinical trial design, prediction of disease progression and contribute to the development of precision medicine approaches for individual patients (for review see Pasteuning-Vuhman et al., 2020). Several studies have started to use NMJ models for compound testing. One microfluidic-based NMJ model was employed to test the therapeutic effects of the Receptor Interacting Serine/Threonine Kinase 1 (RIPK1) inhibitor Necrostatin, an ALS drug candidate, on myofiber denervation. This work found a Necrostatin-induced reversal of myofiber denervation and decreased muscle contraction in co-cultures of motor neurons and SODI\textsuperscript{G93A} astrocytes (Machado et al., 2019). Another study that demonstrated the use of NMJ models for compound screening tested pridopidine, a promising compound for the treatment of Huntington’s disease, in SODI\textsuperscript{G93A} mutant-related pathology (Ionescu et al., 2019). Application of pridopidine improved contraction of SODI\textsuperscript{G93A} mutant muscles, as assessed by live cell imaging. Together with an increased number of NMJs, demonstrated by AChR clustering, this showed that pridopidine restores neuromuscular activity. Furthermore, analysis of retrograde axonal transport, visualized by addition of fluorescently labeled BDNF particles to the distal compartment and live cell imaging, showed that pridopidine enhanced axonal transport, whereas Riluzole, a drug currently used to treat ALS, was ineffective. Furthermore, it was shown that pridopidine mediates its neuroprotective effects via activation of the sigma-1 receptor. Taken together, this study illustrates how microfluidic chips, with a wide variety of possible morphological and functional readouts, can effectively contribute to drug validation studies.

An innovative addition to microfluidic NMJ cultures in light of drug screening is the inclusion of (iPSC-derived) endothelial cells (ECs). A common issue in therapy development for the nervous system is drug delivery to the brain and spinal cord (Bartanusz et al., 2011). Therapeutic compounds are typically administered in the blood, meaning that these compounds need to be able to cross endothelial barriers in order to reach the tissue involved. The blood–brain barrier (BBB) and the blood–spinal cord barrier hamper such crossing, as the endothelial barriers prevent the entry of various molecules into the brain (Wevers & de Vries 2016; Bartanusz et al., 2011). In a recent study, iPSC-derived ECs were seeded against a collagen gel layer forming an EC monolayer and then co-cultured with iPSC-derived ALS neurospheres (Osaki et al., 2018). This 3D ALS-on-a-chip model with EC barrier was used to test two drugs that recently entered clinical trials for ALS: bosutinib (an inhibitor of the ubiquitous non-receptor tyrosine kinase Src/c-Abl pathway) and rapamycin (an inhibitor of mechanistic Target Of Rapamycin (mTOR)). Intriguingly, co-treatment with rapamycin and bosutinib altered the vascular permeability of the drugs, restored muscle strength loss, and protected the ALS motor unit from cell death. These results highlight the importance of an EC barrier for in vitro NMJ modeling, especially for drug screening purposes. The BBB may be relevant for future, highly complex co-culture systems, in which brain and spinal cord components are both present. Such models can be used to identify compounds that restore NMJ defects, by targeting upper motor neurons, which are located in the brain in vivo.

Despite the fact that only a few studies have reported ALS-on-a-chip models, these studies highlight the potential of these platforms for dissecting disease mechanisms and compound testing. The possibility of including relevant cell types other than motor neurons and skeletal muscle cells, such as astrocytes, microglia, and TSCs, creates an opportunity for further defining the contribution of non-cell autonomous processes to motor neuron degeneration (Figure 3). In combination with iPSCs from patients, a microfluidic device is a powerful tool for studying NMJ defects on a patient-specific basis. We, therefore, believe that NMJ/ALS-on-a-chip platforms will rapidly evolve in the upcoming years, thereby furthering our understanding of NMJ defects in ALS and aiding the development of patient-specific therapies.

### 5 | FUTURE DIRECTIONS

Microfluidic technology is used increasingly for the generation of in vitro NMJ models. While these microfluidic models were initially
hampered by the lack of human material, the introduction of iPSC technology has overcome this problem. It is becoming clear that NMJ pathology is jointly influenced by both motor neurons, skeletal muscle, and other cell types. To further advance the physiological properties of current NMJ disease models, glial cells can be added, as they play important roles in NMJ maintenance and disease. Increasing culture complexity may also concern the modeling of different subtypes of motor neurons and inclusion of ECs. We expect that the addition of vasculature to NMJ models will improve candidate drug selection for various neuromuscular diseases. Furthermore, incorporation of vasculature may allow the development of body-on-a-chip approaches. Various organs may be co-cultured in a microfluidic platform, interlinked by vasculature. Multi-organ models will allow the study of pharmacokinetic processes related to absorption, distribution, excretion, and metabolism of novel therapeutics, and would even allow researchers to study off-target effects (Kimura et al., 2018). However, directly targeting the muscle may also be beneficial for drug delivery purposes. It is becoming increasingly clear that skeletal muscle plays an important role in ALS pathogenesis (see section 1.1.3) and that it is highly accessible for drug administration. Muscle delivery of drugs may be beneficial as substances can be applied in close proximity to the NMJ. Furthermore, molecules are known to be retrogradely transported from muscle to the CNS following intra-muscular injections to exert their therapeutic effects in the CNS. Microfluidic platforms are useful for optimizing these approaches, as they allow specific access to the muscle compartment.

Another future improvement for NMJ modeling is the generation and application of NMJ organoids (Faustino Martins et al., 2020). Miniaturized NMJ organoids can be generated from human iPSCs. Under appropriate growth factor conditions, human iPSCs differentiate into axial stem cells, the building blocks of the posterior body that are able to self-organize and generate NMJ organoids containing functional NMJs supported by TSCs. Incorporation of these models into a microfluidic platform can enhance their differentiation and maturity through the introduction of perfusion flow and vascularization (reviewed by Grebenyuk & Ranga, 2019). However, organoid-based NMJ models in microfluidic devices need further validation before they can be widely implemented.

Aging is an important factor in ALS. iPSC-derived neurons have lost most of the epigenetic memory associated with aging and the environmental risks that may contribute to ALS pathogenesis. Various strategies to induce cellular aging and stress in iPSC cultures are currently being investigated (Ziff & Patani, 2019). Such strategies include the addition of compounds to artificially induce aging, but also novel reprogramming strategies. Many reports suggest that bypassing the iPSC stage by direct conversion of somatic cells into, for example, neurons prevents loss of epigenetic markers and disease-related phenotypes, (Mertens et al., 2015). However, it remains challenging to obtain efficient and pure cell numbers when using direct conversion methods (Mertens et al., 2015).

6 | CONCLUDING REMARKS
The combination of microfluidic and iPSC technologies opens new avenues for studying NMJ pathology. Current read-outs focus on NMJ morphology and physiology, and on motor axon-specific processes. Despite the generation of microfluidic-based 3D NMJ models, future studies are needed to enhance the physiological relevance of these models, for example, through the inclusion of additional cell types. Because microfluidic systems provide highly controlled environments for studying disease pathology, these platforms hold great potential as screening platforms for the identification and testing of novel therapeutic compounds. It is therefore expected that the field will start implementing procedures aimed at automation. This will streamline drug development studies, subsequently enhancing candidate drug selection for a wide variety of neuromuscular disorders.

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ORCID
R. Jeroen Pasterkamp https://orcid.org/0000-0003-1631-6440

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