

Modeling the synapse and neuromuscular junction using organ-on-a-chip technology

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1 Introduction

The nervous system of our body is the control unit of vital functions, spanning from the molecular levels to the muscles that serve as an engine for our daily motions. This sophisticated operation possesses a complex structure when investigated at the cellular level, in a way that millions of neurons, muscle cells, and all the other cellular components combine to execute special functions such as movement, thought, and sensory perception. This marvelous operation known as the neural system can malfunction in the case of an anomaly in its components, which can cause various diseases and symptoms known as neurological disorders. Drawbacks in the current research and clinical challenges have emerged with deeper insight of nervous system with an increased effort on the subject in recent years. Due to notable obstacles faced when using animal studies and other in vivo models, microfluidic systems have been adopted to form on-chip platforms, which provide numerous advantages over conventional systems in neurological disorder studies including the development of high-throughput, massively parallelized platforms for clinical research. In brief, microfluidics pertains to the meticulous regulation and manipulation of small volumes of fluids within channels or devices that operate on a microscale. The term “on-chip studies” pertains to investigations carried out on microfluidic chips, compact and integrated devices employed for diverse scientific objectives, such as the examination of biological phenomena under regulated settings. Briefly, this chapter discusses the fundamentals of synapses and neuromotor junctions, which are the basic components of the nervous system. For each of these neural systems, design principles,

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applications, and future endeavors with possible challenges will be discussed in the following subsections from the perspective of on-chip platforms. This chapter strategizes to give a basic understanding to the researchers, who are delving into the field of neurological disorders and aim to transfer their know-how into on-chip technologies for a better understanding of biological models.

1.1 Synapses: Fundamental components of neural communication

Plasma membranes of nerve cells, collectively referred to as neurons, have an amazing ability to alter electrical potential difference across the membrane in response to external stimuli (Melvin, 2015). The action potential—a self-propagating depolarization wave—begins as a result of this process. Neurons serve as the fundamental units of the nervous system, playing crucial roles in transmitting electrical signals. Possessing a unique ability to generate signals, neurons are interconnected through specialized junctions known as synapses. These synapses not only link neurons together but also facilitate intricate communication among neurons and their target cells, forming elaborate networks. Due to their distinct excitability, sensory (afferent) neurons may quickly adapt to changes in their internal or external environment and alert the central nervous system (CNS) of these changes (Grider et al., 2023). Around trillions of neurons make up human CNS, which is entrusted with the complex duty of processing this enormous sensory input and transmitting commands through efferent neurons to effectively adapt to environmental changes (Fletcher, 2016). The transfer of information between cells depends heavily on the connections between neurons, as well as those between neurons and target tissues, such as muscular tissue. In the majority of instances, synapses depend on an intricate interplay including both chemical and electrical interactions. Cellular communication is facilitated through the release of chemical signaling molecules from a donor cell, which subsequently triggers a series of processes in the receiving cell (Holler et al., 2021). The chemical transmission plays a crucial role in facilitating the complex activities that take place at synapses inside the CNS (Faber and Pereda, 2018). It is well acknowledged that alterations in synaptic function, encompassing the regulation of both chemical and electrical transmission, are responsible for the cognitive phenomena of human learning and memory (Pereda, 2014). These processes entail the meticulous synchronization of electrical signals and the secretion of neurotransmitters, facilitating the transfer and integration of information inside the CNS.

The complex organization and functionality of synapses are essential to the nervous system's continued healthy operation. Presynaptic terminals, a synaptic cleft, and postsynaptic components make up a synapse (Sun et al., 2023). Synaptic vesicles filled with neurotransmitters stay until signals initiate communications at the presynaptic terminal, which is located at the end of the axon of a neuron (Citri and Malenka, 2008). Neurotransmitters are released into the synaptic cleft, a tiny space that separates the presynaptic terminal and the postsynaptic membrane, when an action potential reaches the presynaptic terminal (Fig. 1) (Castillo, 2012). Neurotransmitters interact with particular receptor proteins ensconced in the postsynaptic membrane within this space. Depending on the

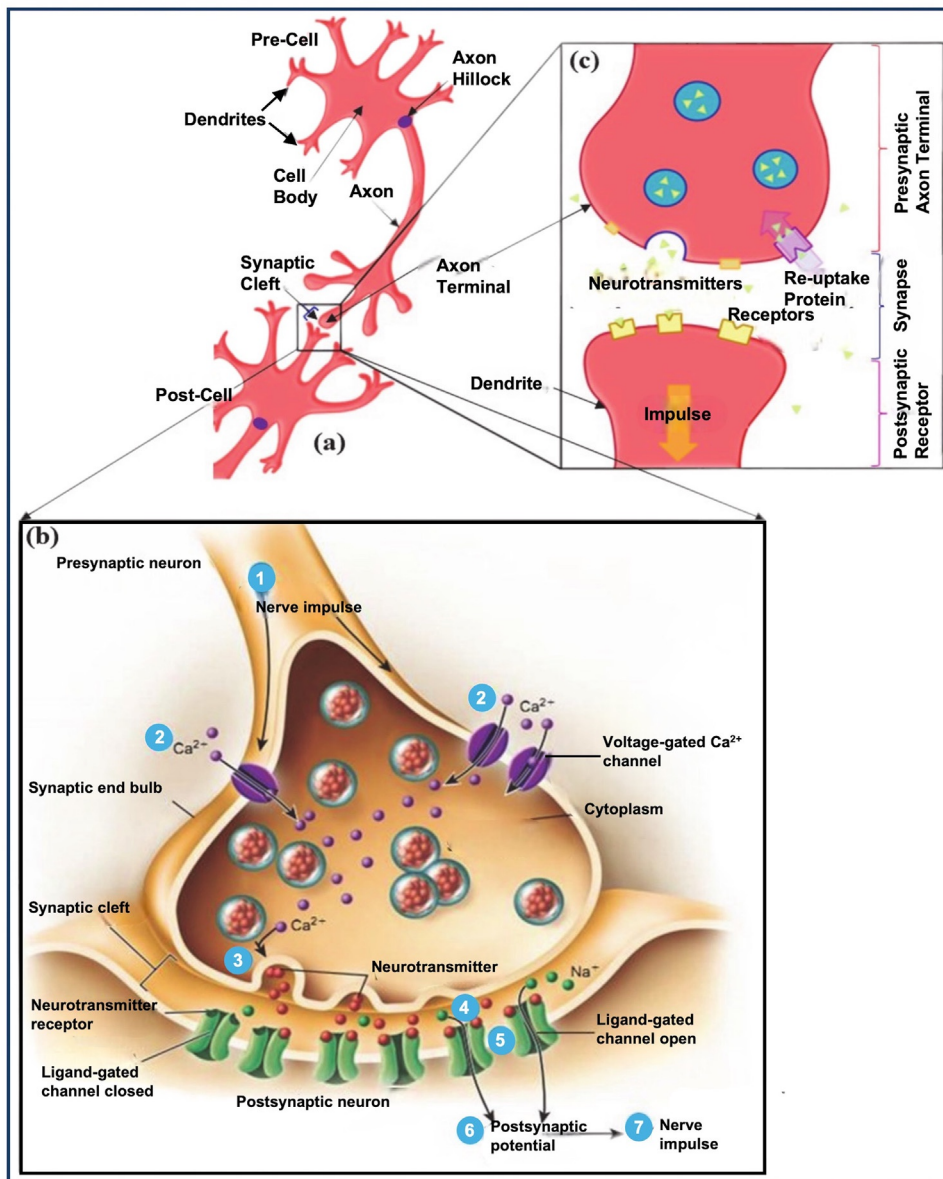


FIG. 1 (A) A schematic representing the communications between neurons within a neural network. (B) The sequential stages involved in the process of biosynaptic transmission. (C) The mechanism by which neurotransmitters are reabsorbed following their release. Reused from Mannan, Z.I., Kim, H., Chua, L., 2021. Implementation of neuro-memristive synapse for long-and short-term bio-synaptic plasticity. *Sensors* 21(2), 644. <https://doi.org/10.3390/S21020644> under the terms and conditions of the Creative Commons Attribution (CC BY) license.

outcome of this interaction, the receiving neuron will either create its own action potential or not, enhancing signal transfer. The process ends with the neurotransmitter system being shut down, ensuring the exact and controlled neuronal transmission required for all brain operations (Croushore and Sweedler, 2013).

One key idea—synaptic plasticity, emphasizes how synapses can adapt and change in response to cerebral activity (Citri and Malenka, 2008). Synaptic plasticity is properly defined as the activity-dependent modification of the strength or efficiency of synaptic transmission at preexisting synapses (Centonze et al., 2007). LTP (long-term potentiation) and LTD (long-term depression) are the two main types of synaptic plasticity. Among the facets of this flexibility is LTP (Bassi et al., 2019). LTP functions as a method for synapses to grow stronger over time, increasing their effectiveness. On the other hand, LTD, a process that sharpens neuronal connections, occurs when there is a need to adjust or weaken an association. This is particularly evident when attempting to overcome habits or adapt to changes in your surroundings (Malenka and Bear, 2004).

Despite their important role in the function of the nervous system, the investigation of synapses is not an easy task. Traditional methodologies, whether carried out in vivo or in controlled laboratory conditions (in vitro), have their own significant hurdles. Because living systems are inherently unpredictable, conducting in vivo investigations might be tough. The complexity of genuine brain networks may not be perfectly replicated by in vitro experiments, despite their control (Azari and Reynolds, 2016). Electrophysiology, which records the electrical activity in neurons, and microscopy, which lets observe brain structures in great detail, are two common standard ways to study synapses (Cavanagh, 2019). Though, these ways have problems because synapses talk to each other so quickly. These old methods make it hard to record and study the fine details of synaptic processes because they happen so quickly. There are also ethical and practical issues playing a significant role especially in studies involving animal participants for studying synapse biology.

To tackle these challenges, organ-on-a-chip platforms and microfluidic devices have been employed in neuroscience studies (Ashammakhi et al., 2023). Microfluidic platforms have been used in neuroscience studies since 1998 (Wang et al., n.d.), providing with it a unique set of qualities that allow scientists to conduct specialized research (Holloway et al., 2021; Amirifar et al., 2022). Achieving this necessitates careful consideration of system design, including the development of well-defined cell cultures in predetermined spatial configurations, the control of axon and dendrite growth, the use of reduced sample sizes, and improved management of the extracellular environment (Gupta et al., 2022). Microfluidic devices provide a controlled and multifaceted settings for generating brain-like complexity; yet, their design and construction hold major challenges. A thorough examination of materials, microchannel geometry, sensor integration (if needed), and the exact regulation of cellular proliferation and testing circumstances are all part of the process that goes into making these customized devices to deal with certain scientific questions properly. Additionally, researchers have formed compartmentalized and perfusion techniques that allow for durations ranging from several days to several

months of successful maintenance of cultures in confined spaces (Habibey et al., 2022). Using surface patterning, gradients, and physical barriers, microfluidics also offers techniques for regulating neuronal growth and separating them from one another for their particular tasks (Habibey et al., 2022). Researchers have been able to properly control and regulate the extracellular space to meet in vivo circumstances thanks to the inclusion of microvalves and precision fluid management (Croushore and Sweedler, 2013). This high level of control proves invaluable when attempting to understand the precise conditions necessary for neurotransmitter release. In addition to cell cultures, microfluidics has created a name for itself in vivo analyses such as microdialysis, which is a technique that involves the sampling of extracellular fluid. Microfluidics technology streamlines the microdialysis procedure and enables real-time sample derivatization, hence augmenting the study of neurotransmitters and other chemical signals (König et al., 2018). The integration of microfluidic devices with conventional detection platforms has enhanced the investigation of the nervous system, allowing researchers to see and evaluate brain activity and neurochemical signaling with more efficiency and accuracy (Rogers et al., 2013). There has been seamless integration between microfluidic devices and several of the same detection platforms such as high-performance liquid chromatography (HPLC) and mass spectrometry used as conventional neurotransmitter analyses (Dai et al., 2023). Consequently, microfluidics is becoming a crucial technology that makes it simpler to examine cellular surroundings and identify crucial signaling molecules that are involved in both healthy and injured brain networks.

1.2 Neuromuscular junctions: Bridging nerves and muscles

Controlling the skeletal muscles requires an advanced interface between the nervous system and the muscle parts that move the bone parts attached to them (Slater, 2017). The neuromuscular junction (NMJ) provides connection between neurons and muscle fibers, and it provides a controlled motor movement by muscle contraction. In this mechanism, a special synapse is formed at the intersection of motor neuron axons and muscle fiber attachment in a way that the signals coming from neural system carried as an axon potential across the nerves. When signal is transmitted to the muscle fiber, calcium voltage-gated channels open at the end of motor axon and cause the release of acetylcholine (ACh) to reach to muscle fibers. After that transfer of signal molecule, ACh binds to the ACh receptor (AChR) on postsynaptic membranes of muscle fibers and causes opening of sodium channels, which results in initiating action potential. Finally, calcium ions are released to the muscle fiber cytoplasm, which fires up the muscle contraction. To reset the process, ACh esterase (AChE) on the postsynaptic membrane is activated, and this inactivates ACh and decreases muscle contraction to reset the signal. Considering all these, an NMJ has three parts: (i) nerve end coming from the nervous system, (ii) the synaptic space that connects nerve end to muscle fiber, and (iii) muscle fiber end plate, which receives the signal for execution (Burden et al., 2018). In addition to these, terminal Schwann cells (TSCs) have a critical role in NMJ formation in which they provide topical

molecules such as neurotransmitters, growth factors, and trophic factors for vital NMJ function. TSCs also play a key role in NMJ repair in case of an injury. Considering all these points, NMJ system is influenced by the extracellular matrix (ECM) that regulates NMJ function and provides an optimal support for the operation (Ko and Robitaille, 2015). Any problem that emerges in this system causes neuromuscular diseases including well-known conditions such as muscular dystrophy, myasthenia gravis, and amyotrophic lateral sclerosis (ALS), which are affecting around 14,000,000 people worldwide (Margeta, 2023). Research and preclinical applications on these diseases require well-developed models, which represent the complete physiology of NMJ as well as the disease models to test the treatment candidates (Leng et al., 2023). Traditionally, animal models are used in NMJ studies to provide effective models on human diseases. However, conducting comprehensive animal studies is costly, and the constraints in the generalizability of these models emphasize the necessity for more progress in the field of neuromuscular junction (NMJ) research. Consequently, there is an increasing momentum toward the development of advanced and efficient models aimed at augmenting the comprehension and management of disorders associated with the neuromuscular junction (NMJ).

2 Synapse- and NMJ-on-a-chip: Design and fabrication

A comprehensive analysis of numerous characteristics is necessary when constructing microfluidic devices for the study of synapses and NMJs. Various elements are essential for ensuring the efficiency and utility of these technologies being applied to the study of the nervous system. For example, distinct compartments or channels need to be considered to achieve precise spatial alignment of neurons or muscle cells with synapses or NMJs within a microfluidic chip (Bartolozzi and Indiveri, 2007). This type of device makes it possible to have controlled interactions and assures that the microenvironment closely mimics the natural environment. Additionally, an efficient flow of culture media and chemical signals is essential for maintaining a conducive microenvironment (Tajeddin and Mustafaoglu, 2021). Therefore, the design of the microfluidic platform should facilitate laminar flow while preventing cross-contamination between different cell types or compartments. The selection of the material to be used in the construction of microfluidic devices should imitate the natural cell environment as closely as possible. These materials must reflect flexibility and mechanical properties of the tissue, along with being biocompatible, to provide necessary mechanical support for cell growth and function (Nielsen et al., 2020). For certain types of studies in neuroscience, cells cultured in a microfluidic device must be exposed to electrical stimulation. Therefore, the materials required for the experimental setup, such as elements that provide seamless integration of electrodes or stimulation mechanisms, need to be included in the design of the chip. Such systems enable researchers to systematically measure biological responses to electrical signals. Furthermore, microfluidic systems should be transparent in order to allow for both observing at the microscopic level and interventions within the chip (Smoak et al., 2019). Developing

microfluidic chips requires a careful approach grounded in biological principles when attempting to integrate the aforementioned elements. Many methods are available for crafting these chips, each with its unique advantages and limitations. Soft lithography, for instance, is one of the most commonly used techniques, utilizing flexible materials such as polymers, for example, polydimethylsiloxane (PDMS), polyurethane, and polyethylene, to generate microfluidic channels and structures (Nguyen et al., 2022; Akceoglu et al., 2021). This process shapes or patterns the polymer, followed by curing it through heat or exposure to ultraviolet radiation. Taylor et al. developed a microfluidic local perfusion (LP) chamber for accessing and modifying pre- and postsynaptic compartments as well as rat synaptic areas in vitro using the soft lithography method. Over 100 parallel rows of synapses can form in this chamber, connecting various neuronal populations. This arrangement allows for precise access to synaptic areas with outstanding spatial and temporal resolution since a perfusion channel connects these parallel rows (Taylor et al., 2010). In a different study, Pigareva et al. introduced a novel two-layer polydimethylsiloxane (PDMS) chip that makes it easier to culture and examine the functional interaction of two interconnected neural networks through soft lithography (Fig. 2A). They used micro-electrode arrays and mouse hippocampus neuron cultures that were cultured in two chambers of a microfluidic chip. Axons grew primarily from the source chamber to the target chamber due to the asymmetric design of the microchannels between the chambers, resulting in the formation of two neural networks with unidirectional synaptic connection (Pigareva et al., 2023).

Micromolding, another commonly used approach, employs mold to create desired structures in a polymer material, such as PDMS (Papautsky and Peterson, 2008). Typically, molds are created using photolithography or other methods and are used to generate channels and structures in polymeric materials (Juang et al., 2022). Micromolding offers advantages including affordable production for the chips (less than 10\$) and can be readily scaled up for high-throughput, extensive production that covers more than hundred copies at once (Tsao, 2016). Gao et al. established an experimental system incorporating ideas from micropatterned surfaces, microfluidic devices, and genetically encoded biosensors to improve the experimental capacities of researching neural circuits in vitro. Micropatterning was performed to place neurons and astrocytes in specific regions and direct connections between the two cell types (Fig. 2B). Microfluidic chambers were positioned atop micropatterned surfaces to enable the administration of various pharmacological agents or viral vectors to the targeted cell types. In this system, astrocytes and neurons of rats interacted through grooves molded into the microfluidic device's floor (Gao et al., 2016).

Micromachining represents another fabrication technology, involving the cutting, drilling, or etching of microfluidic channels and structures into a substrate using various tools and techniques (Scott and Ali, 2021). As an example, concentrated ion beams can be used to shape channels in silicon wafers or drill holes in glass substrates. While this method allows for the creation of extremely small and intricate structures, it can be costly and challenging to control. Duc et al. create a microfluidic platform for simulating

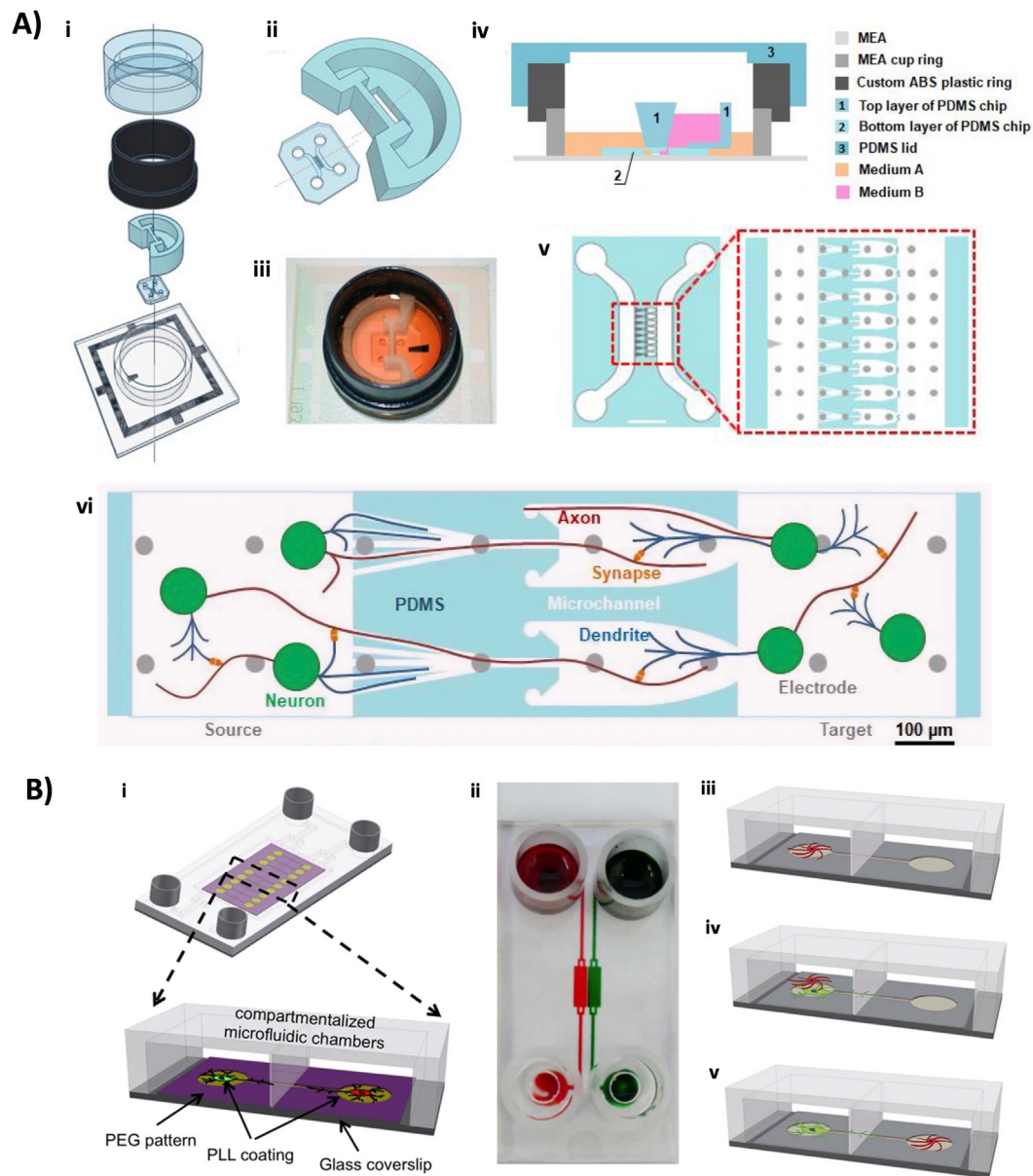


FIG. 2 (A) Polydimethylsiloxane (PDMS) chip for neural cell cultures is shown. (i) Structure and constituent parts of the chip are presented. The microelectrode array, top and bottom layers, plastic ring, and PDMS cover are arranged from bottom to top. (ii) A three-dimensional representation of the PDMS chip's upper and lower layers is displayed. (iii) The microelectrode array (MEA) is shown once the chip is removed from PDMS lid. (iv) The schematic of MEA is represented. (v) A schematic representation of the microfluidic chip (scale bar: 1 mm) and the eight microchannels that connect the two chambers of the microfluidic chip is depicted. (vi) Microfluidic chip-formed scheme of unidirectional synaptic connection between brain networks is represented. (B) (i) The microfluidic device's schematics are provided, showcasing an assembled platform with two sections, (ii) each compartment was dyed with food coloring to reveal the chambers and passageways, (iii) astrocytes alone, (iv) astrocytes implanted into the neuronal chamber, and (v) astrocytes seeded opposite neurons are illustrated. (A) Reproduced from Pigareva, Y., Gladkov, A., Kolpakov, V., et al., 2023. Microfluidic bi-layer platform to study functional interaction between co-cultured neural networks with unidirectional synaptic connectivity. *Micromachines* 14(4), 835. <https://doi.org/10.3390/M14040835> under the terms and conditions of the Creative Commons Attribution (CC BY) license. (B) Reproduced from Gao, Y., Broussard, J., Haque, A., Revzin, A., Lin, T., 2016. Functional imaging of neuron-astrocyte interactions in a compartmentalized microfluidic device. *Microsyst. Nanoeng.* 2(1), 1–9. <https://doi.org/10.1038/micronano.2015.45> under the terms of the Creative Commons CC BY license.

the human neuromuscular junction (hNMJ) using human cells on a chip. Micromachining was used to make structures that would guide muscle growth above the electrodes without stopping the propagation of axons. This made activity tracking more effective. The platform separates motor neurons and muscle fibers into their own areas, which lets scientists treat only certain types of cells to test how well the NMJ works. The study denotes an insight that electrically stimulating motor neurons can cause extracellular muscle action potentials that can be measured. This could be a useful way to learn more about and describe NMJs, especially in the setting of neurodegenerative diseases. For studying hNMJs, this method is better than calcium imaging because it is more sensitive (Duc et al., 2021).

Finally, microfluidic systems can also be manufactured using three-dimensional (3D) printing techniques, which require specialized printers capable of depositing materials with fine resolution, including polymers and metals. These printers are typically guided by computer control, allowing for the precise construction of intricate 3D structures and enabling customization of designs. Kajtez et al. created a novel additive manufacturing technique known as 3D-printed soft lithography to fabricate microfluidic devices. This technique offers a straightforward, yet flexible methodology that enables the production of open-well compartmentalized devices with vertical walls and control over both micro-scale and macroscale features (Kajtez et al., 2020).

3 Applications of synapse and NMJ chips

Many new studies have begun to be carried out in neuroscience research using microfluidic chips and organ-on-a-chip platforms. In this way, many application areas related to brain communication and muscle function have been studied. Robertson et al., for instance, cultivated paired cultures of rat primary hippocampal neuronal and glial cells using microfluidics technology. Despite their ambient isolation, these cultures showed synaptic connections. The presence of synapses and the physical link between the two neuronal populations were confirmed by immunocytochemical labeling for β -III-Tubulin and synaptophysin. To chemically stimulate one culture, the researchers then injected specific fluid quantities as carrier fluid or stimulation agents into the device's inlets. They then used calcium imaging to track the ensuing increase in neuronal activity in the neighboring culture with a calcium-sensitive dye and inverted microscope. Through the use of this cutting-edge technique, the effects of changing neuronal activity can be observed in neural networks that are synaptically coupled yet environmentally separated. This novel method allows insightful modeling and comprehension of the aberrant synaptic transmission and astrocytic behavior linked to diseases of the central nervous system (Robertson et al., 2014).

Majumdar et al. created a microfluidic cell coculture system in a different study that allows different cell types' microenvironments to be customized (Majumdar et al., 2011). A barrier made of microfabricated valves can be slid to alter the distance between the cell culture chambers. This special feature of the platform allowed for the

maintenance of a steady coculture of rat glia and hippocampus neurons for several weeks. Using controlled fluidic exchange between the cell culture chambers, the *in situ* conditioned glia media—which is essential for neuron survival—was continuously supplied. Microfluidic devices are perfect for studying processes such as synapse development since they can sustain healthy neuronal cells for at least 3 weeks following transfection (Majumdar et al., 2011).

Shi et al. created two brand-new microfluidic cell culture setups, a vertically layered setup and a four-chamber setup, for coculturing neurons and glia in the central nervous system (CNS). These devices have pressure-activated valve barriers separating the two types of cells, allowing us to regulate communication between them (Fig. 3A) (Shi et al., 2013). These devices' distinctive architecture made it possible to coculture glia and neurons in close quarters (around 50–100 μm), transfect diverse populations of neurons, and dynamically observe neuronal interactions, such as the formation of synapses (Shi et al., 2013).

Taylor et al. showed that by developing a high-density neuronal culture, axons could be selectively probed from cell bodies (Taylor et al., 2005). Axon development through interconnects into a fluidically segregated axonal compartment was polarized by the platform and its subsequent modifications. The apparatus can be modified to collect analytes at the junctions between two cell cultures to analyze neuron-neuron, glia-neuron, or even glia-glia interactions. It has been used to separate two populations of neurons and to give vital nutrients to their connections (Taylor et al., 2005).

To recreate the retinal neural synapse and grow retinal precursor cells, Su et al. developed a microfluidic chip with numerous arrays of microchannels (Su et al., 2015). Microchannels physically link the cultivated retinal cells together (Fig. 3B). Glycinergic factors were administered to show how they activated the cells' ability to transmit electric signals through the microchannels. Below 100 μM of the inhibitor glycine, the rate of retinal synaptic regeneration (RSR) dramatically dropped and then gradually increased to a level that was more stable. RSR was also improved by potassium chloride chemical stimulation (Su et al., 2015).

To find out how much the microfluidic environment constraint can influence the proliferation of cells and mold neural network (NN) activity, Dupuit et al. tracked the short-term (less than 5 ms delay) and mid-term (11 days) evolution of neuron activation over the length of the culture period at specific points in organized (microfluidic) and random (control) networks by microelectrode arrays (Fig. 3C). In particular, they have looked at the spike and burst rates and correlations between the extracted spike trains in the early stages of growth. The spiking rate, burst, and correlations were greater than the electrophysiological characteristics of ordinary cultures because they were reinforced over time in terms of number and amplitude. They were able to see strong neurite communications through this study, which would have been weaker and more delayed in random networks. As predicted from the microfluidic channels, the confinement of cells seems to enhance brain interactions and cell proliferation throughout the network in addition to increasing detection efficiency (Dupuit et al., 2023).

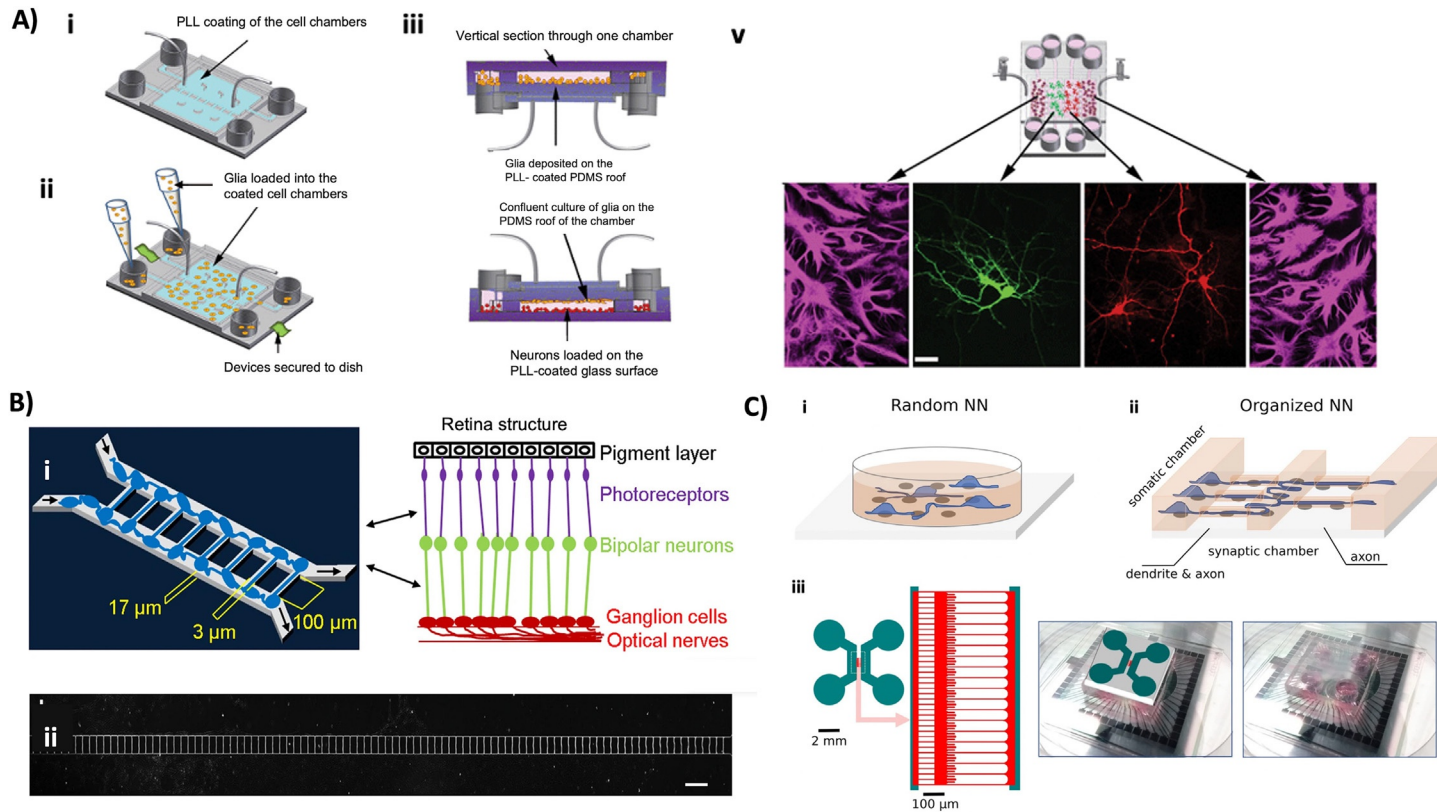


FIG. 3 (A) Diagram showing the flow of cell loading in microfluidic devices with vertical layers. (i) A 1 mg/mL^{-1} PLL coating was applied for 12h to the cell culture chambers (depicted in blue) of the microfluidic devices, (ii) glia, represented as orange spheres, were introduced into the cell chambers, (iii) the devices holding the cells were incubated for 3–5 min, turned over, and left for a further 2 h. (iv) The devices were cultured in glia medium for 4–5 days, (v) the coculture of neurons and glia in the four-chamber microfluidic apparatus is depicted. Neurons, differentially transfected with GFP (green) and mCherry (red) on day 3 of culture, were maintained in coculture with glia and fixed on day 13. Scale bar = 25 μm . (B) (i) The two chambers that make up the retinal synapse regeneration (RSR) chip are joined by 100 microchannels. (ii) The RSR chip's microchannels are shown. Scale bar = 200 μm . (C) The neural network (NN) is described in (i and ii), where the expected network structures in the random (left) and microfluidic (right) data are displayed. (iii) The arrangement and optical micrograph of the microfluidic NNs put together using the arrays of microelectrodes is represented. (A) Reproduced from Shi, M., Majumdar, D., Gao, Y., et al., 2013. *Glia co-culture with neurons in microfluidic platforms promotes the formation and stabilization of synaptic contacts*. *Lab Chip* 13(15), 3008–3021. <https://doi.org/10.1039/C3LC50249J> under a Creative Commons License Attribution 3.0 Unported (CC BY 3.0). (B) Reproduced from Su, P.J., Liu, Z., Zhang, K., et al., 2015. *Retinal synapse regeneration via microfluidic guiding channels*. *Sci Rep.* 5(1), 1–7. <https://doi.org/10.1038/srep13591> under a Creative Commons License Attribution 4.0 Internal (CC BY 4.0). (C) Reproduced from Dupuit, V., Briançon-Marjollet, A., Delacour, C., 2023. *Portrait of intense communications within microfluidic neural networks*. *Sci Rep.* 13(1), 1–13. <https://doi.org/10.1038/s41598-023-39477-9> under a Creative Commons License Attribution 4.0 Internal (CC BY 4.0).

On-chip models of NMJ gained attention with the increasing need of clinical testing. In 2016, Uzel et al. demonstrated a platform to employ in vitro NMJ models more accurate from the perspective of representing neuromuscular diseases (Uzel et al., 2016). By developing an in vitro platform with myoblast-derived muscle strips and motor neurons differentiated from mouse embryonic stem cells, this platform achieves a hydrogel base 3D NMJ model that can represent spinal cord and limb separation in a miniaturized environment. In addition, this unique platform allows measurement of force generation as a quantitative output and optogenetic control of the motor units of NMJ model. Later on, the same research group utilized human induced pluripotent stem cells to have a physiologically relevant 3D model of human NMJ, which could also provide optogenetic control. To achieve this point, a PDMS mold and collagen-coated chambers were used to generate a biomimicking platform for NMJ generation from stem cells. As a result, a microfluidic platform was generated to have a disease-mimicking tool for neurologic disorder drug screening studies (Osaki et al., 2018). As another example, Kamm et al. developed an on-chip model for drug screening in NMJ with patient-derived induced pluripotent stem cells to generate both motor neurons and muscle fibers. Allowing these to form an NMJ in PDMS microfluidic chip, researchers denoted a platform to evaluate different neuromuscular diseases including spinal muscular atrophy (SMA) (Osaki et al., 2020). Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disease, which directly affects motor neurons and causes developed paralysis for patients. Animal models on ALS reached a state to provide important insight for the disease through years; yet, for working directly on the human NMJ, which differs from other species, gained significance with an urgent need of therapeutic development (de Jongh et al., 2021).

Modeling neurological circuits also possess critical information to understand disease conditions. Bellmann et al. developed a microfluidic platform that could harbor a coculture of motor neurons and myotubes derived from human induced pluripotent stem cells (iPSCs) (Bellmann et al., 2019). This work provides a suitable environment for long term (3 weeks), and parallelable (100 unit per microfluidic chip) experiment volume by using PEMA integration in the PDMS microchambers, as well as providing the first proof-of-concept experiment to show usage of rabies tracing assay, which uses a pathogenic rabies virus that can travel through neurons to trace the neuronal circuit. In Bauer et al.'s 2022 study, a similar strategy was used to show the formation and validation of NMJ. Here, iPSC-derived motor neurons (MNs) and myotubes are cocultured in a PDMS chip (Bauer et al., 2022). To visualize the NMJ formation and functioning, engineered rabies virus types used either expressing green or red fluorescent proteins used to prove the natural infection pathway of rabies virus also presented in the model. Another study aiming to transfer NMJ disease modeling studies on microfluidic systems developed a 3D model, which can fit inside a single well of 24-well plate (Yamamoto et al., 2021). Here, iPSC-derived MNs and skeletal muscle fibers (SMFs) are connected to form a functional NMJ in a way that from a separate compartment, MN axons are elongated to reach the SMJs kept in a separate compartment to form a NMJ and reach to a micropost to be bound and used for measuring contractile force generated by the whole system. Using this

design, such model could provide controllable response type from different inducers including glutamate, tetrodotoxin, and vecuronium bromide, which could also be broadened to test various drug response mechanisms. Microfluidic NMJ models have potential to model different neurological disease models. Myasthenia gravis is a neurological disorder, which is observed in case of autoimmune targeting of the body against neuromuscular junction receptors. In a recent study, human derived stem cells are used to differentiate MNs and SMFs and form NMJ system in a compartmentalized experiment scheme. By allowing different cell types to be induced differently and could be stimulated directly or indirectly in a PDMS chip, scientists successfully tested pathogenic functioning of myasthenia gravis (Smith et al., 2021). In addition, the sophisticated microprocessor created by Park et al. allows exact control of axon development conditions, surpassing conventional approaches (Park et al., 2014). Axon growth is guided and isolated by this device, enabling localized biomolecular therapy and length quantification. CSPG's impacts on axon growth in diverse locations illustrate biomolecules' spatial effects. The microchip, like the Campenot chamber but with better fluidic sealing, can be mass produced for high-throughput screening. This invention is useful for investigating axon growth and regeneration in neuroscience and drug screening. Gatti et al. studied how amniotic fluid stem cell-derived extracellular vesicles (AFSC-EVs) protect against muscle loss caused by dexamethasone (Dexa) and how it affects neuromuscular junctions (NMJs) (Gatti et al., 2023). The study uses a microfluidic coculture device to look at how muscles and nerves talk to each other during atrophy. Neurodegeneration due to muscle atrophy is confirmed by Dexa exposure, but the situation gets better with AFSC-EVs treatment, in part because they control reactive oxygen species (ROS). The work is mostly descriptive, but it shows that AFSC-EVs can help change the way muscle atrophy happens in the NMJ. The microfluidic NMJ device that was shown can be used to screen small molecules and do more in-depth studies of how things work. Ionescu et al. present a simplified way that uses a microfluidic chamber to deal with the complexity of NMJ (Ionescu et al., 2016). By growing mouse spinal cord explants and myotubes together, scientists can see how NMJs form, stay together, and break down. The procedure allows precisely control of microenvironments, change them, and tell the difference between local and retrograde signaling. Active NMJs form in 1–2 days, giving researchers a chance to test how well interventions affect NMJ activity. There are different ways to measure NMJ activity, which gives researchers options based on the questions they want to answer and the tools they have access to.

4 Challenges and future directions

Despite the advantages of synapse and NMJ studies performed with microfluidic chip technologies, some challenges must still be considered. The first one is synaptic complexity. Synaptic networks, due to their intricate structure, contain many types of synapses and signaling molecules. Replicating this complexity in microfluidic chips poses a major challenge. Achieving a comprehensive representation of synaptic diversity remains another

significant challenge (Habibey et al., 2022). An additional possible struggle is to integrate different cell types, such as neurons, glia, and muscle cells are all present at synapses and neuromuscular junctions. The issue of finding cell culture media that supports different cell types could be a potential challenge in this regard. The constant challenge of getting these cells to communicate and work properly in chip-based systems is to make them to interact flawlessly (Halldorsson et al., 2015). The replication of in vivo circumstances is a significant obstacle. The in vivo environment, including not only chemical signals along with delicate mechanical and physical cues that neurons and muscles encounter in their natural surroundings, must be properly simulated by these chips (Taylor and Jeon, 2010). Another matter is the functionality and longevity of neuronal and muscular components cultivated inside microfluidic chips. A significant number of research and applications rely on these components, maintaining their functions over long periods of time, which refers from days to weeks depending on the microfluidic systems (Natarajan et al., 2019; Puttonen et al., 2015). Ensuring long-term viability and robustness of these cell cultures is vital for delicate neuronal networks (de Jongh et al., 2021). NMJ models developed in the literature may show drawbacks from many sides, including the generation of correct test environments for mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs). In the future, adaptation of those cell lines with engineering compatible variants would hold great potential to tackle such challenges on NMJ models (Kaufman et al., 2020).

In addition, with the use of sophisticated microfluidic technology, the neuroscience community is actively working to make advancements in understanding brain functionality and disorders. These systems, which are made of materials that are both physiologically and biomechanically compatible, provide high spatiotemporal resolution through wireless or optical controls, multimodal functionalities, switchable neural functions in freely moving animals, and multimodal functionality (Yu et al., 2019). For in vivo pharmacology, chemogenetics, optogenetics, and newly emerging discipline of optopharmacology, the revolutionary microfluidic interfaces hold enormous potential since they enable exact molecular control within particular cells with great temporal and spatial accuracy (Hagmann et al., 2008). Clinical theranostics for disorders such as brain tumors, neurological injuries, and neurodegenerative diseases show potential for localized pharmacological infusions for targeted therapy without damaging neighboring tissues. In vitro tools, including as improved artificial intelligence (AI) analytic techniques and human-derived cells, have the potential to change our understanding of brain diseases and the creation of new treatments by bridging the gap between preclinical and clinical research (Tsai et al., 2023). On-chip nervous system function high-throughput screening can reveal underlying mechanisms and advance therapeutic strategies. In the future, our research would examine how motor neurons and vascular networks interact, perhaps in conjunction with brain organoids. Future organ-on-a-chip devices would include on-chip light microscopes for advanced optogenetics and connectomics, which study the mapping and the connection scheme of neural system (Adamantidis et al., 2007). Moreover, an advanced LOC

technology has the capacity to deliver precise optogenetic monitoring and alteration of neuronal events (Renault et al., 2015). Moreover, microfluidic technologies provide a platform for conducting cutting-edge research on metabolism, drug toxicity, pharmacokinetics, and chemical signaling pathways at the single-cell level for neuroscience studies. Especially, single-cell genetic modification and 3D neural network signal detection are two other recently explored research fields. These advancements have prospects for enhancing current understanding of the intricate composition of the brain and generating groundbreaking therapies within the realm of neuroscience.

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