High-throughput platforms for the screening of new therapeutic targets for neurodegenerative diseases

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ABSTRACT

Despite the recent progress in the understanding of neurodegenerative disorders, a lack of solid fundamental knowledge on the etiology of many of the major neurodegenerative diseases has made it difficult to obtain effective therapies to treat these conditions. Scientists have been looking to carry out more-human-relevant studies, with strong statistical power, to overcome the limitations of preclinical animal models that have contributed to the failure of numerous therapeutics in clinical trials. Here, we identify currently existing platforms to mimic central nervous system tissues, healthy and diseased, mainly focusing on cell-based platforms and discussing their strengths and limitations in the context of the high-throughput screening of new therapeutic targets and drugs.

INTRODUCTION

Neurodegenerative diseases (see Glossary) are incurable and highly debilitating conditions that can lead to impaired cognitive and sensorial functions and/or problems in movement (ataxia). These include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and multiple sclerosis (MS), among others. Some central nervous system (CNS) neurodegenerative diseases are known to have a genetic/familiar component, whereas others are highly multifactorial [1].

Neurodegenerative diseases were initially investigated using anatomical studies followed by biochemical analysis. Currently, a common practice is the study of familial genes that could be involved in these diseases or identification of major pathways involved in the mechanisms of the disease, recurring to mutation integration (knock-in and knockout) in animal models. This enabled the massive progress seen recently in the knowledge of these diseases. Nevertheless, these conditions remain untreatable, and several important mechanisms of neurodegeneration are still to

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be fully understood. In 2010, the annual costs of mental and neurological pathologies in Europe were €798 billion. Owing to the nature of these diseases, 40% of these costs were related to loss of productivity of the affected people [2]. Costs are probably higher today because these pathologies usually have a long-term impact and the population is aging. Taking all this into consideration, it is clear that there is the need to increase our understanding of CNS neurodegenerative disorders to address them effectively.

Over time, scientists have been looking forward to making more-human-relevant studies mainly because of the difficulty in translating the obtained results in preclinical animal models to humans, the costs associated with animal models, as well as all the ethical issues raised with the use of the latter. As such, there is a growing need for biologically and clinically relevant *in vitro* models to be used to increase the knowledge of CNS function, define disease biomarkers, study and test new therapeutic targets and drug candidates before the start of clinical trials, as well as enable the discovery of new interesting applications for known therapeutic molecules.

In vitro screening systems need to recreate an environment that is as representative as possible to the one found *in vivo* in the target tissue or system unit of interest. Although simple *in vitro* systems are appealing, there is a need to find equilibrium between model simplicity and physiological relevancy for data understanding and interpretation to be possible and relevant. Ideally a platform must allow the full control of system complexity and existing variables. Additionally, it should enable the researcher to start with a simple platform that, in time, with the increasing understanding of the system, can become more and more complex, giving rise not only to a higher understanding of the system but also to more-relevant results and knowledge. Moreover, with the increasing need to test as many variables as possible at once, high-throughput screening (HTS) assays have been evolving. Here, we identify the existing platforms to mimic the healthy and diseased CNS, mainly focusing on cell-based platforms, discussing their strengths and limitations in the context of the HTS of new therapeutic targets and drugs for the treatment of neurodegenerative diseases.

Considerations for HTS platforms

Typically, in the context of biomedicine, HTS is applied in the design of chemical structures to be used in drug discovery and further testing in a therapeutic setting or to test compounds and/or conditions to address biological questions in fundamental research [3]. The need for HTS platforms is increasing among industry and academic researchers, because they can solve the problem of lengthy, resource-intensive procedures that can, sometimes, make the screening of the required large number of conditions impossible. One of the main causes of failure during clinical trials is the lack of efficacy of the existing *in vitro* systems to test drug toxicity, which ends up being revealed only at late stages of the research [4].

HTS assays are usually divided into two categories: biochemical assays, studies of protein–protein interactions, proteases, enzymatic activity, among others; and cell-based assays. Biochemical assays usually assume a starting knowledge of a molecular target; by contrast cell-based assays often aim to identify modulators of a pathway of interest.

There are several published studies that focus on the considerable potential of HTS-based biochemical assays. Pedro *et al.* [5] developed a method in which the activity of the leucine-rich repeat kinase 2 (LRRK2) was measured, because increased LRRK2 activity was proved to be related

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to PD. The group used the AlphaScreen® assay which relies on the emission of fluorescence if there is a biomolecular interaction between the donor and the acceptor (the kinase and a moesin which is its putative physiological substrate). This HTS platform has been proposed to test the capacity of small molecules in inhibiting the activity of LRRK2. Crowe *et al.* [6] carried out another interesting study, screening almost 30,000 compounds to assess their influence on tau protein assembly. Formation of toxic tau oligomers in the brain is one of the main observed pathologic events of AD. With this in mind, using an HTS assay based on complementary thioflavin T fluorescence and fluorescence polarisation methods, the authors were able to observe the effects of inhibitors of tau oligomerisation and found that aminothienopyridazines (ATPZs) caused inhibition of fibril assembly as well as fibrillisation of tau. Because the normal ability of tau to stabilise microtubules was not affected, ATPZs were shown to be promising drugs to treat AD. In addition, Wang *et al.* [7] developed a study focused on the process of hydrolysis of acetylcholine by acetylcholinesterase, which is a central mechanism in the control of neural response. In AD the levels of acetylcholine are low, making it an interesting therapeutic target. The authors measured acetylcholinesterase activity and screened acetylcholinesterase inhibitors thus proving the feasibility of the assay that has the potential to be, in the future, used as an HTS platform for screening inhibitors of acetylcholinesterase. Further evidence supporting the relevance of biochemical-based assays in the context of neurodegenerative diseases was conducted by Shu and co-workers [8] who devised an HTS assay based on a novel reporter substrate of autophagin-1 composed of a natural substrate (LC3B) fused to an assayable enzyme (PLA2) that becomes active upon cleavage by this cysteine protease. This platform can serve for identification and characterisation of autophagin-1 inhibitors – inferring the role of autophagy, because its regulation could be the cause of many diseases, including neurodegenerative ones. More recently, Kashem *et al.* [9] studied the inhibition of sphingosine-1-phosphate (S1P), a sphingolipid whose degradation by S1P phosphatases and sphingosine-1-phosphate lyase (SPL) is involved in AD and MS pathologies. Briefly, the group created a scintillation proximity assay able to screen possible inhibitors in 384-well microplates in a rapid way. By testing a library of 10^6 compounds the team identified several classes of SPL inhibitors amenable to be used as a new class of immunosuppressive drugs.

With cell-based assays, entire pathways can be subjected to questions creating multiple potential points of interest, in contrast to the study of single predetermined steps as in the biochemical assays. Furthermore, cell-based assays can provide information that a biochemical assay cannot, such as the nature of the pharmacological activity of the screened compound at a specific receptor or intracellular target. Furthermore, there are also some targets that cannot be properly reconstituted in a biochemical assay 10, 11, 12, 13, 14, such as complex interactions between receptors or cellular factors that are not easily reproducible outside the cell. Consequently, cell-based platforms are particularly promising because they can be powerful tools in the study of cell growth and differentiation, to investigate the influence of small molecules and cell growth conditions on cell function and physiology, and also to understand signalling pathways in mammalian cells. These have also proven to be very useful in some CNS studies 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25. In this context, Thid and co-workers [15] designed a new neural cell culture substrate based on the phospholipid bilayer as a biomimetic platform for cell behaviour studies, which was shown to support cellular adhesion and growth. Kang *et al.* [19] have established a microelectrode array to measure neuronal network activity in a platform that could constitute an interesting drug screening platform. In fact, HTS has also been used to ease and standardise manual methods for the measurement of neurites [24]. There are many important factors that need to be considered when planning a cell-based platform. These include choosing the biological system (primary cells, cell line or explants),

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choosing the assay approach (functional, phenotypic or reporter gene) [26] and another crucial factor is the assay readout.

Primary cells of human origin are possibly the most physiologically relevant model system and, although primary cells can sometimes be difficult to obtain, several primary cell types from humans and other species are already commercially available [27]. Nevertheless, transformed cell lines of human origin are currently the most commonly used systems. Many of these lines preserve a highly differentiated phenotype being good platforms to screen complex physiological responses. Additionally, cell lines can be engineered to express a specific target [27], which can increase their interest as models for certain studies. Diverse transformed and primary mammalian cells have been exploited to study diseases, including diabetes, cancer and neurodegenerative diseases 3, 28, 29. More recently, the development of induced pluripotent stem cell (iPSC) technology provided a new powerful tool for drug screening, allowing the use of cells with the same genetic background of the patients without certain cell type constraints associated with the use of the patient primary cells, as in the case of CNS cells [30].

Issues such as cell viability, doubling time (when applicable) and recovery from freeze–thaw cycles must all be considered [31]. Additionally, cell seeding density and passage number can significantly affect the output and, consequently, the size of the cell bank needed [27]. Differences in cell growth across the plate that can occur with long incubation periods also need to be taken into account, being easily prevented by careful regulation of the humidity and temperature of the incubator and by an even distribution of the plates in the incubator.

When using a cell-based assay focused on a signalling pathway there is flexibility on the choice of the readout. Particularly, if an antibody is available, possible readouts can be any step in which a protein is modified (e.g. phosphorylated), translocated or its abundance changed [32]. When talking about HTS applications, the most applied detection method seems to be fluorescence-based assays. This is mainly because of high sensitivity, diverse selection of fluorophores and a variety of readouts based on different environment-sensitive fluorescence properties [33] that, altogether, allow miniaturisation, flexibility in assay design, ease of manipulation and even the possibility to monitor multiple events simultaneously [33]. Fluorescence readouts are also appealing because fluorescence microscopy techniques have significantly evolved in the past decades with increasing sensitivity, image definition and automatisation features.

Cell-based 2D platforms

Glass coverslips and tissue-culture polystyrene (TCPS) are the most frequently used substrates to culture cells. These can be adequate and convenient substrates for 2D cell cultures and have been widely used for toxicity assays, for example. Malik and co-workers [34] recently reported a 2D model to assess neurotoxicity profiles of several drugs. The authors have used pluripotent stem cells to develop a highly reproducible HTS system and identified cardiac glycosides as potential drugs to target glioblastomas, as a result of their toxicity for human neural stem cells. Although several cell culture models have been successfully established using glass or TCPS substrates, it is well known that these offer cells a very different mechanical environment compared with the one experienced in physiological conditions *in vivo* 35, 36, 37, 38. With this in mind, the biomaterial engineering field has been contributing to the development of physiologically relevant *in vitro* assays by the use of 2D materials that can better mimic *in vivo* mechanical properties. These substrates include polymeric

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substrates [39] or hydrogels 40, 41, 42, 43, 44. In fact we have recently shown that substrate mechanical properties can influence neuronal morphology and intracellular signalling pathways [39]. In this section, relevant 2D platforms within the context of CNS disorders are discussed.

Disease models

Neurons can exist in the body over a lifetime and neurodegenerative diseases are progressive conditions that affect neurons in time. Consequently, to mimic the physiological conditions *in vitro*, long-term culture systems are necessary. Lesuisse and Martin developed a long-term system using cortical neurons to study mechanisms of neuronal maturation, aging and death and concluded the appearance of an age-related biochemical phenotype in the culture [45]. Interestingly, long-term neuronal cultures were later used to study AD progression by Bertrand *et al.* [46], in this case using cultures of hippocampal neurons. More recently, Todd and co-workers established a method for long-term high-density culturing of hippocampal neurons [47]. The authors claim that their organoid system can serve as a model for AD and in other neurodegeneration studies, because these cultures can provide distinct advantages when testing the effects of long-term therapeutic strategies.

Envisaging AD therapies, Seyb *et al.* [16] developed a cell-based HTS to screen molecules for their ability to inhibit calpain, because this has been correlated with beta-amyloid (Aβ) cytotoxicity. Here the authors explored differentiated Sh-SY5Y cells (a commercially available cell line established by subsequent cloning of the neuroblastoma cell line SK-N-SH, which was established from a metastatic bone tumour). Although this appears to be an interesting screening platform, no direct inhibitors of calpain were found in this study.

Recently, several studies with stem cells have been directed towards the identification of novel therapeutic approaches to understand and ultimately cure neurodegenerative diseases 48, 49, 50. Using cell models of mouse and rat neural stem cells treated with hydrogen peroxide to induce oxidative stress, Wang *et al.* achieved interesting insights regarding the action mechanism of Tecfidera™ (dimethyl fumarate) [51]. This drug, which has been approved in Europe and is already available on the market, shows an ability to reduce relapse rates in MS patients. The team saw an increase in the survival of stem cells through regulation of *Nrf2* and the extracellular-signal-regulated kinase (ERK)1/2/mitogen-activated protein kinase (MAPK) signalling pathway. Zhang *et al.* [52] used a HD-specific iPSC line to develop an *in vitro* HD cell model. Being a cell model based on human cells makes it potentially more clinically relevant and, consequently, useful for the screening of therapeutic drugs.

Salvador and co-workers developed an *in vitro* model of traumatic brain injury (TBI) [53] based on cultures of murine brain microvascular endothelial cells from the cerebral cortex conducted on collagen-coated flexible-bottomed culture plates, in the presence or absence of astrocytic factors. By using these substrates which can be easily subjected to stretch, simulating the primary mechanical disruption typically occurring in TBI, together with oxygen and glucose deprivation, the authors achieved a closer to reality replication of the events occurring in TBI.

Co-cultures have also been widely studied and used as cell models to understand neurodegeneration. Several co-culture models have been proposed to address myelination in the CNS context 54, 55, 56. For instance, Howe and co-workers published the effects of the Fc portion of human IgM (Fcμ) in CNS cells. To achieve this, the group used a mixed culture of oligodendrocytes, microglia and astrocytes

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and found that, under treatment of Fcμ, the microglia changed to a phenotypic activated state and started to release cytokines such as interleukin (IL)-1β. The authors hypothesised that molecules produced in response to IL-1β kill the leukocytes that trigger inflammation in diseases such as MS. Thus, Fcμ could be an interesting target to explore against demyelination in MS [57].

Microfluidic platforms

Molecular gradients are important regulatory components of tissue processes. Numerous molecular gradients can be found in the majority of tissues, and to mimic such milieu authors have elegantly used microfluidics technology in 2D environments 20, 21, 58, 59, 60. Although microfluidics chambers were initially used in the context of neurosciences to force the physical isolation of axons from each other and from their respective cell bodies, these systems are now useful for several applications on CNS (co-)culture studies 61, 62, 63 because they introduced an easy and feasible way of studying neurobiology, as a result of the precise control of the microenvironment of the cells; for a review, see [64]. Molecular and cellular interactions are both easily achieved with this methodology.

Microfluidic-based neuronal circuits

There are several good examples of *in vitro* neuronal cell cultures grown and manipulated in microfluidic devices and their value as a tool to study fundamental physiological changes that occur in the CNS. In 2009, Park *et al.* produced a multicompartment co-culture microfluidic platform to study mammalian CNS axon–glia interactions for the screening of growth factors and potential myelin repair drug candidates [20]. In the same year, the same researchers [21] showed that a multicompartment microfluidic neuron culture platform could be used for biochemical analysis of the proteic axonal fraction allowing the simultaneous use of multiple experimental conditions in parallel on a single platform (Fig. 1a). Park *et al.* also [63] developed a multicompartment microfluidic platform where oligodendrocyte progenitor cells (OPCs) were directly seeded on top of isolated axons. OPCs were shown to differentiate successfully into myelin-producing oligodendrocytes. This platform was used to study the localised effects of chondroitin sulfate proteolgycans (CSPG), coming to the conclusion that CSPG at concentrations lower than 250 ng/ml did not cause the retraction of pre-established axons.

Hosie *et al.* explored microfluidic platforms to study glutamate excitotoxicity, which is a pathogenic condition in several neurodegenerative diseases. The authors isolated distal axons from cell bodies in a microfluidic device and concluded that the most susceptible part of the neuron to excitotoxicity is the distal axon [65]. More recently, Roberston *et al.* [66] developed a high-throughput platform to monitor neuronal synaptic communication for drug screening purposes. The authors have combined a microfluidic system with a calcium-imaging array to study primary hippocampal cultured networks. This system can overcome some limitations of microelectrode arrays such as the lack of interface between some electrodes and cells as a result of random distribution of cells, assuring the quality of the signal and data collected.

Kang and co-workers [19] used an agarose hydrogel to build a multi-well neuronal microcircuit microelectrode array platform, which enables the reduction of data collection times. This tool could prove to be powerful for neurobiological assays assuring the statistical power needed for data analysis in a short period of time and it would be interesting to combine it with a microfluidic system.

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Microfluidic-based disease models

Microfluidic platforms have also been widely explored as disease models. Cho *et al.* [67] developed a microfluidic chemotaxis platform to mimic the AD brain, namely creating gradients of soluble Aβ to study microglial behaviour and unravel the reason behind microglial accumulation near Aβ plaques. This microfluidic platform, which contains a central Aβ reservoir and Aβ gradients in the cellular compartments, could be used for other migration studies in the framework of other neurodegenerative diseases such as the study of OPC migration in demyelinating diseases such as MS. Lee and Park [68] also developed an AD model. The formation of Aβ aggregates is known to be influenced by several external factors of the surrounding microenvironment. High concentrations of metal ions such as Fe^{2+} are present within and peripheral to the senile plaques, and in AD patient neutrophils. The authors address the issue of Aβ deposition by immobilising monomers on a polydimethylsiloxane microgrooved surface, and then comparing the clearance effects of several chelators on Fe^{3+}-induced formation of Aβ aggregates.

Kunze *et al.* [69] developed a microfluidic cell culture platform to improve the understanding of astrocyte–neuron interactions in amyotrophic lateral sclerosis (ALS) pathology by co-culturing neurons with super oxide dismutase (SOD)-mutant astrocytes: astrocytes genetically modified to overexpress a mutated form of SOD1, a mutation known to occur in ALS. This microfluidic device prevents the direct contact between cultured cells enabling the study of metabolic communication between the cultured cell types (Fig. 1b).

Microfluidic-based BBB models

The blood–brain barrier (BBB) is known to be a highly selective barrier between the brain vasculature and brain tissue, composed of endothelial cells (ECs) of the cerebral microvasculature, which are interconnected by tight junctions. Although the BBB is absolutely essential for the normal function of the brain, preventing the entry of pathogens and unwanted substances, it also constitutes the main impediment for drug entry into the brain when drugs are intravenously administered. Therefore, a large number of systems have been proposed and used to investigate the cellular and molecular mechanisms underlying BBB establishment, to test new drugs, evaluate conditions and mechanisms that cause the barrier disruption, among others [70]. *In vitro* models of the BBB have been traditionally achieved using cell culture trans-wells but microfluidic systems have enabled the establishment of dynamic and more physiologically relevant *in vitro* BBB models. The use of microfluidic platforms has enabled the application of shear stress to the cultured ECs via fluid flow, which was shown to increase the barrier properties of the BBB in *in vitro* models 71, 72, 73 – better mimicking the physiological conditions.

Prabhakarpandian *et al.* [74] have recently developed an innovative microfluidic BBB model that comprises a microcirculation two-compartment chamber (Fig. 1c). In this study, ECs were cultured in the presence of astrocyte-conditioned medium. The authors focused on the permeability of the BBB *in vitro* model and have not really addressed trans-endothelial electrical resistance (TEER) measurements. BBB integrity alteration can be routinely assessed *in vitro* by determining cell monolayer permeability to different solutes and measuring the TEER.

The presence of astrocytes *in vitro* was shown to increase TEER values significantly in BBB models 75, 76, 77. Xue and co-workers [78] have gone further in the pursuit of a clinically relevant culture model

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and established a platform comprising a tri-culture of astrocytes, neurons and ECs achieving TEER values significantly higher than those obtained in other models. Another study using a tri-culture of astrocytes, pericytes and endothelial cells also showed increased TEER values [75]. Although these studies suggest that the presence of more cell types that are present in the *in vivo* scenario is extremely important to obtain increased TEER they also suggest that the presence of the different cell types might not be as relevant for permeability issues 75, 79. As such, it is important to consider both factors when considering a model to test new drugs for CNS applications.

Cell-based 3D platforms

Whereas 2D approaches allow a well-controlled analysis of the impact of individual components on cells, our tissues have additional layers of complexity that arise from the three-dimensionality that strongly conditions cell function. To achieve a more native-like environment many researchers have started to work in 3D environments. By comparing cell culture systems with 2D and 3D architectures several authors observed significant differences in cell behaviour that suggest that neural cells cultured in 3D behave more closely to those *in vivo*. Therefore, the more physiologically relevant microenvironment of a 3D culture could enhance the quality and biological relevance of the obtained data. In the following subsections 3D models of increasing complexity will be approached; namely spheroid cultures, tissue slices and biomaterial-based platforms.

Spheroid culture models

In vitro multicellular spheroids have begun to bridge the complexity gap between monolayer cell culture and *in vivo* tissue structure and have become valuable *in vitro* models for developmental and drug resistance studies. These can also be very useful when studying a neurodevelopmental disease. To promote cell assembly while preventing spheroid aggregation, culture systems developed for cell spheroid formation such as hanging drops and microwells could be used to obtain uniform neurospheres [80]. There is currently a commercially available 3D spheroid culture platform named Perfecta3D® hanging drop plates (3D Biomatrix), which is a high-throughput 3D cell culture device that aims to simplify spheroid formation, culture and subsequent testing of the achieved 3D constructs. However, static suspension cultures also present limitations, such as the ones observed in the case of cultures with large-diameter aggregates, in which limited supply of oxygen, nutrients and/or growth factors to the centre of the aggregates could lead to the development of necrotic cores, and alter culture dynamics. In this case, dynamic culture systems are more appropriate [81].

Lancaster *et al.* settled a human pluripotent stem cell derived cerebral 3D organoid culture system in which various cerebral zones were represented. The authors maintained neuroectoderm from embryoid bodies in 3D Matrigel® droplets. The structure was then moved to a bioreactor, rapidly evolved, recreating different brain tissue areas [82]. Moreover, Tieng *et al.* generated neurospheres that differentiated in dopaminergic neurons and studied the influence of gamma secretase inhibitors (compound E and DAPT) on neuronal differentiation and maturation and concluded that these molecules could control maturation and induction of neural precursors. This model is of distinct interest for PD and demyelinating diseases [83].

Seidel and co-workers [84] developed a 3D model of tauopathy that recapitulates pathological processes known to occur in AD. This culture model is based on a spheroid culture of a human cell line and promises to provide an efficient system for the screening of new therapeutic drugs. This work

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further shows the relevance of 3D models and it is of added value when compared with 2D systems because the authors found increased neuronal differentiation and interesting levels of degeneration being achieved *in vitro*. Also aiming to recreate the AD environment *in vitro*, Park *et al.* [85] developed a 3D neurospheroid culture-based microfluidic system as an *in vitro* brain model (Fig. 2a). The cultured cells were then treated with Aβ to simulate the AD environment. The combination of spheroid cultures with microfluidic technology enabled the recreation of the 3D architecture and the interstitial fluid flow simultaneously, which could be of added value because the authors have shown that Aβ toxicity in dynamic culture conditions is significantly higher than the one seen in static conditions. This *in vitro* brain model constitutes an interesting model for the *in vitro* study of neurodegenerative disorders in the brain.

Tissue-explant-based models

The use of tissue explants is also a strategy followed by several authors to achieve 3D cultures where tissue architecture, cellular and molecular content must be preserved. Because these closely recreate the *in vivo* environment, keeping cell-matrix composition and relative content similar to the ones in native tissue can be of added value when the aim is to create a model of a pathology that involves several cell types or even brain regions, and/or where tissue architecture and composition are key factors.

Reinhart *et al.* [86] have used rat brain tissue explants to which Huntingtin clones where delivered through biolistic transfection. Using a library of 74 compounds known to be involved in processes of cell death and inflammation, the authors concluded that IκB kinase complex compounds (WAY-717 and WAY-781), $CXCR_3$ chemokine receptors (compounds 6c and T_{487}), a c-Jun N-terminal kinase inhibitor (SP600125) and an adenosine 2A receptor agonist (CGS21680) and antagonist (KW-6002) showed neuroprotective features in the disease explant model. This platform was developed to study HD but it can be useful to establish other disease models for other pathologies that have a known genetic profile. Zhang *et al.* [87] have also used *ex vivo* tissue explant cultures of brain and spinal cord tissues to establish an MS *in vitro* model (Fig. 2b). The proposed system has been shown to constitute a good platform for the study of myelination and remyelination mechanisms. In this report, the authors used image stacks obtained from confocal microscopy and, through image software, quantified the myelin. An automated myelin quantification method is of added value for the screening of remyelination-promoting drugs. To test potential neuroprotective agents, Ravikumar *et al.* [88] established a protocol where spinal cord slice cultures were treated with lipopolysaccharide (an antigen used to induce neurodegeneration) and co-cultured in trans-wells in the presence of microglia cells seeded on poly(l-lysine)-coated glass coverslips. Polystyrene beads were used to activate the phagocytic capacity of the microglia. As bead concentration increased the axonal disruption also increased. Resveratrol was then tested as a neuroprotective agent and this treatment led to reduced levels of neurodegeneration when compared with controls.

More recently, Hutter-Schmid *et al.* [89] developed a model to study the relationship between damaged brain vessels and neurodegeneration. In the neurovascular unit vascular cells closely interact with astrocytes. Hence, understanding angiogenesis mechanisms in the brain, as well as simulating its reaction to growth factors and drugs, is of extreme importance. In this model, tissue slices were cultured in membrane inserts and shown to maintain the normal structural vessel network of the brain. This is relevant once alterations in the vascular system can contribute to the development of degenerative diseases such as AD [90]. As such, this model can be further used to

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screen new drugs to improve vascular function or also to study responses of the vascular cells to some common neurodegenerative drugs.

TBI incidence is rapidly increasing because of recent military conflicts. Miller and co-workers [91] developed a model of blast-induced TBI, in which mouse brain slices and a shockwave tube were used to mimic shockwave overpressure. Cell death and effects of the injury in neuron cells were assessed. The authors concluded that neuronal death was prominent. Besides, astrocytes and microglia were activated in the model, thus showing the feasibility of the model to mimic blast TBI. To screen neuroprotective compounds to be used in ischemic stroke, Beraki and co-workers [92] created a two-step robust screen methodology. Firstly, the authors used primary cortical neurons and subjected them to oxygen glucose privation for 2 hours. The compounds were then added to the culture and cell death, viability and toxicity were measured. Remaining compounds were then tested in a mouse brain organotypic slice culture and, among them, Carbenoxolone® proved to be the most efficient. This added a great therapeutic value for the treatment of this disease.

Biomaterial-based models

In the past decades, the fields of biomaterials and tissue engineering have been paramount to the developments observed in HTS platforms, helping the recreation of physiologically relevant *in vitro* systems. Nevertheless, adapting 3D assay systems to high-throughput testing brings additional challenges because miniaturisation might not be as straightforward as one would desire, however such platforms are emerging 93, 94.

Lai and Kisaalita [95] have developed a 3D culture system for human neural stem cells. The authors have transformed the regular polystyrene 2D culture wells into 3D culture wells by building a porous polystyrene scaffold into standard 96-well plates. Although this solution has the added value of being cheaper than any of the previously mentioned commercial solutions, polystyrene might not present the ideal mechanical properties for many CNS cell-culture-based studies. Hydrogels have been more frequently explored, mainly because of their biological properties, because these more closely resemble the natural extracellular matrix and, consequently, positively impact cell behaviour. Neurons in a 2D microenvironment have been shown to have high Ca^{2+} dynamics in comparison with neurons cultured in 3D hydrogels 17, 96, 97, 98 showing that the 3D environment is more relevant for electrophysiology studies. Irons *et al.* [17] have shown that neurons and astrocytes cultured in a 3D environment acquire a complex 3D morphology and are able to maintain cellular viability for several weeks. Additionally, network properties (pore size, mechanical properties, etc.) and degradation rate are tunable through changes in the cross-linking degree and gel chemistry [99]. Currently, different hydrogels have been explored for neural cell culture, either from natural mammalian extracellular-matrix-derived sources such as Matrigel® and collagen or synthetic or chemically defined hydrogels. Among the latter, several systems are already commercially available that have also been tested for culture of neural cells (most commonly for glioblastoma cell culture). These include: the HyStem™ (Advanced Biomatrix), a chemically defined hydrogel rich in hyaluronic acid; AlgiMatrix®, an alginate-based 3D culture system (ThermoScientific); the synthetic peptide nanofibre scaffold HydroMatrix™ Peptide Hydrogel (Sigma-Aldrich); and the QGel assay system (QGel). The gel-forming method will vary for each case, but nowadays photopolymerisation and chemical polymerisation also represent good approaches because mild reaction conditions can currently be applied, which can be performed at physiological pH and temperature, allowing even cell encapsulation at little or no cost to cell viability.

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Choi and co-workers [100] have used a Matrigel®-based platform to culture human neural cells to recapitulate AD tauopathy and Aβ pathologies. The results of this study support the idea that 3D models better recreate clinically relevant environments and the hypothesis that Aβ deposition leads to tauopathy.

We have developed a 3D *in vitro* tissue engineered glial scar [101] by culturing primary astrocytes within alginate hydrogels, in the presence of meningeal-fibroblast-conditioned medium. Hydrogel mechanical properties were shown to influence cell behaviour significantly with intermediate stiffness gels promoting astrogliosis. This platform enabled the identification of RhoA as a pivotal mediator of astrocyte reactivity and, as such, identified it as a potential therapeutic target (Fig. 2c). Ibuprofen and chondroitinase ABC were shown to reduce RhoA levels and recover the astrocytic phenotype. These 3D cultures were shown to recapitulate many biological features of glial scars and could be useful in the future for the screening of therapeutic drugs to target astrogliosis.

Bioprinting is another appealing advancement because it can answer some problems of cell distribution throughout the 3D environment, particularly significant in the case of multiple-cell-type cultures, because cells and biomaterials can be deposited layer-by-layer in a controlled fashion 102, 103. Lee [103] used such a strategy to pattern embryonic neurons and astrocytes in a collagen 3D environment.

Polymeric materials have also been used as artificial axons for *in vitro* testing of rapid myelinating systems. These have been used as neuron-free models to study oligodendrocytes and the myelination process (Fig. 2d). Studies with artificial axons go back to 1990 when Bullock and Rome [104] used glass microfibres to study the behaviour of OPC differentiation and myelination. Although this was shown to be a suitable system to study oligodendrocytes, the authors did not find oligodendrocytes consistently wrapped around the glass fibres. More recently, Li *et al.* [105] produced electrospun poly(ε-caprolactone) and gelatin co-polymer fibres as artificial axons, which were shown to support growth and differentiation of OPCs. In this work, the authors claim that cells wrap the fibres but their data are not sufficient to fully assess the existence of myelin sheath all around the polymeric fibres. Lee *et al.* [106] also used electrospinning techniques to produce polystyrene and poly(l-lactic acid) nanofibers coated with poly(l-lysine). The authors produced fibres with different diameters and showed that OPCs react to this physical stimulus, because they were shown only to myelinate fibres with a diameter higher than 0.4 µm. This result is not extraordinarily surprising because *in vivo* CNS axons are known to present diameters that range from 0.3 to 2 µm [107] but it stresses the importance of the physical stimulus for myelination processes. This work was further able to show the existence of concentric wrapping of the polymeric fibres. All together, these studies have shown that axonal signals are not needed for OPC differentiation and myelination to occur. Because these systems allow the myelination process to occur in the absence of neurons, they may be particularly interesting to discriminate the cross-talk between myelinating oligodendrocytes and other CNS cells besides neurons.

Concluding remarks and future perspectives for HTS platforms

Here we reviewed the current state-of-the-art of existing platforms for the study of CNS function in homeostasis and disease. Some already comply with the requirements to serve as an HTS platform; others can easily be extrapolated to such systems. In Table 1, a summary of the added value and

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limitations of illustrative examples is presented, in the framework of the development of such screening platforms.

When discussing HTS in the context of the CNS one must not forget the particularities of this intricate system. Neurons and glia establish complex and orchestrated cellular networks that are difficult to reproduce in simple culture systems. The CNS is one of the most densely cellularised tissues of our body, and the extracellular matrix, which occupies 20% of the CNS space, is an extremely important constituent and adds an extra layer of complexity to the system. Mechanotransduction is also becoming a relevant player in CNS disease. Consequently, the further exploration of extracellular-matrix-derived and biomaterial-based matrices to support 3D neural cultures is expected to improve the advanced models greatly. Nevertheless, future progress will not solely rely on biomaterial science but also on the adaptation of many of the commonly used readout techniques to the 3D culturing setting; namely the challenge of recovering or accessing cells from the matrices for further analysis and the necessity of using microscopic techniques that allow evaluation of multiple focal planes. Miniaturisation and automated handling of 3D cultures can also be defiant. The combination of microfluidics technology with 3D architectures is rapidly evolving 108, 109, 110, 111 and, once 3D culture hurdles are overcome, work in this field will progress and afterwards microfluidics and co-culture systems can be combined.

In fact, a major obstacle for the advancement of the strategies discussed here has been the lack of solid fundamental knowledge on the etiology of many of the major neurodegenerative diseases. Many neurodegenerative disorders are multifactorial, with several players involved in the process. iPSC technology will open new avenues to the development of patient-specific models and personalised medicine. More recently, researchers have also developed platforms to mimic multi-organ interaction that are not present in the conventional tissue culture plate, by using microfluidics with a co-culture system with the aim of studying pharmacokinetics of drugs [59] or cell-based drug metabolism [60]. Although not yet applied to the CNS, 'quasi-all-body' model systems can bring new key information to the field.

Finally, new algorithms for image analysis and large dataset processing will also need to emerge to handle the terabytes of information generated, sometimes even in one experiment. Therefore, a truly multidisciplinary approach will be vital for the evolution of the field. Ultimately, this will translate into new drug targets, biomarkers, biomolecule-based therapies and novel and more-efficient disease treatment or management approaches.

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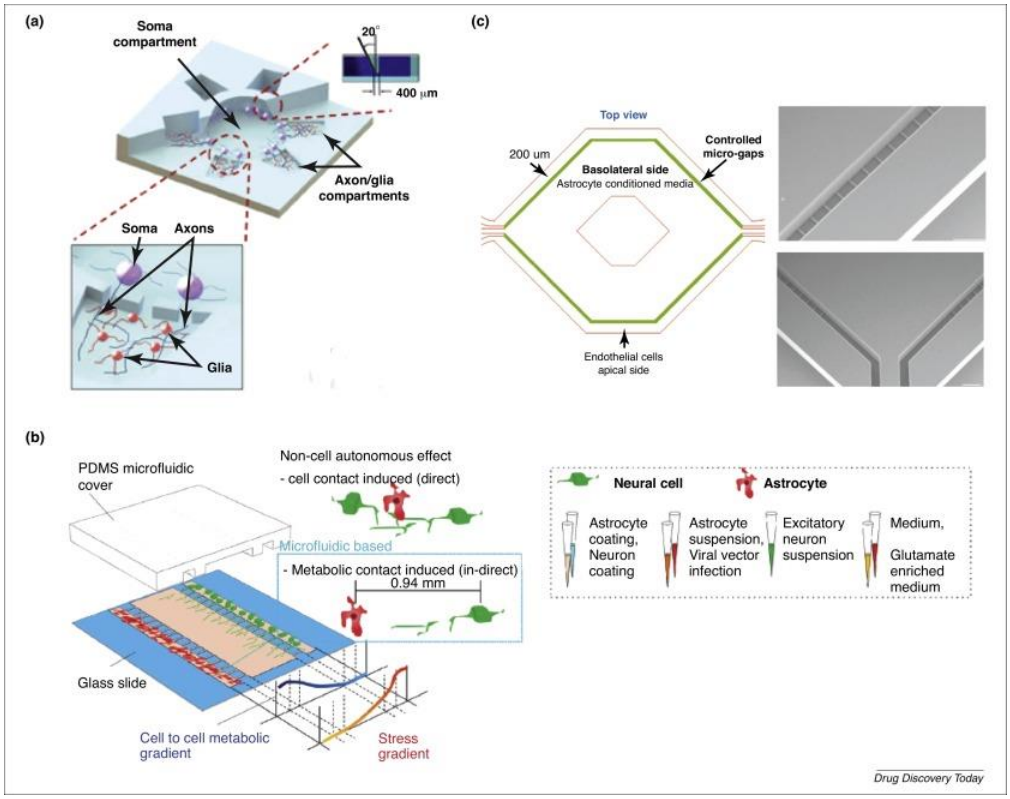

Figure 1. Schematic representations of 2D cell-based platforms. **(a)** Multicompartment neuron–glia co-culture system, which enables multiple localised axon treatments in parallel. Reproduced, with permission, from [21]. **(b)** Microfluidic platform that allows the co-culture of astrocytes and cortical neurons to address non-cell autonomous effects in amyotrophic lateral sclerosis (ALS) disease, induced through the metabolic communication between neurons and astrocytes over a distance of 0.94 mm. Reproduced, with permission, from [69]. **(c)** Microfluidic blood–brain barrier (BBB) model that comprises a microcirculation two-compartment chamber – top view of the basolateral chamber which is supported in the centre by a support structure to prevent the collapse of the top of the microfabricated chamber. Scanning electron microscopy (SEM) images of the 3 µm gaps and apical and basolateral fluidic chamber. Reproduced, with permission, from [66].

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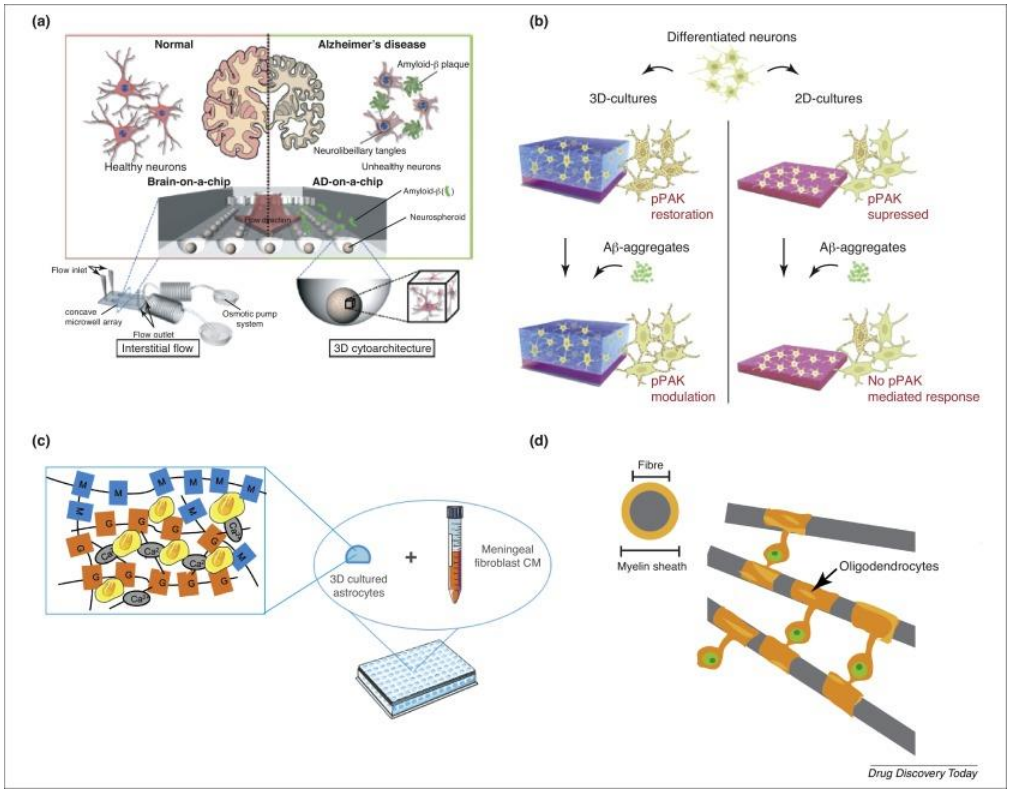

Figure 2. Schematic representations of 3D cell-based platforms. **(a)** 3D brain-on-a-chip with an interstitial level of flow. Reproduced, with permission, from [85]. **(b)** The impact of a 3D microenvironment. Differences in phosphorylated P21-activated kinases (pPAK) expression and responses to beta amyloid (Aβ) oligomers in 2D cultures and 3D self-assembling peptide (SAP) matrix cultures. Reprinted, with permission, from [112]. **(c)** Alginate-based *in vitro* tissue engineered glial scar. **(d)** Artificial axon system for myelination studies. Adapted, with permission, from [108].

Table 1. Illustrative examples of 2D and 3D platforms proposed in the context of CNS *in vitro* cellular models

Study focus	Dimensionality	Type of platform	Added value	Limitations	Refs
Neurotoxicity	2D	Human and rat monocultures	Scale up potential Human neural cells Possibility to achieve multiple neural cell types	Difficulty of access to large quantities of human neural cell types	[34]

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Study focus	Dimensionality	Type of platform	Added value	Limitations	Refs
			differentiated from the NSCs		
Myelination	2D	Microfluidic co-culture	Possibility of testing six different culture conditions in parallel Ability to manipulate local physical and chemical environment	Inability to achieve robust myelination	[21]
Neuronal connectivity	2D	Microfluidic co-culture	Combination of calcium imaging with microfluidics technology	Inability to distinguish synapse formation origin Reduced volumes of aqueous solutions could lead to evaporation and extensive changes in imaging	[66]
BBB	2D	Microfluidic co-culture	Ability to reproduce a triple co-culture system using primary cells. BBB function and resemblance to *in vivo* events	Time-consuming readout	[78]

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Study focus	Dimensionality	Type of platform	Added value	Limitations	Refs
Dopaminergic nervous tissue	3D	Spheroid culture	Spontaneous electrical activity Controlled induction and maturation of neural precursors	No spatially organised structures	[83]
Alzheimer's disease	3D	Microfluidic and spheroid culture	Combination of 3D and microfluidics technology	Time-consuming readout	[85]
Multiple sclerosis	3D	Slice culture	Readout method – myelination, demyelination and re-myelination were reproduced and automatically quantified in a rapid and precise way	No recreation of the inflammatory component of the disease	[52]
Neurotoxicity	3D	Slice culture	Semi-automated readout method Tissue architecture is conserved	LPS-induced degeneration	[88]
Stroke	3D	Slice culture	Readout method Easiness to study a target pathway	Platform potential needs to be further confirmed with other drugs	[92]

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Study focus	Dimensionality	Type of platform	Added value	Limitations	Refs
Astrogliosis	3D	Biomaterials	System simplicity Resemblance of *in vivo* features Ability to tune mechanical properties	Time-consuming readout method	[101]

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; LPS, lipopolysaccharide; NSCs, neural stem cells.

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Glossary

Biolistic transfection :A physical method of transfection in which the target tissue is bombarded with DNA-coated gold particles.

Bioprinting: The process of generating spatially controlled cell patterns or constructs using 3D printing technologies – typically it involves dispensing cells in an injectable biomaterial, like hydrogels.

Electrospinning: A fibre production method that uses electric force to draw charged threads of polymer solutions or melts up to fibre diameters in the nano- to micro-metre range. It is used in the biomaterials field because of close resemblance between electrospun polymer fibres and the extracellular matrix fibrillar components.

High-throughput screening :A process in which large numbers of conditions (chemicals, biological agents, etc.) are tested with high efficiency, to identify biologically active molecules and/or cellular targets as candidates for further validation in additional experiments.

Mechanotransduction: The process by which a cell translates mechanical stimulus into biochemical signals – the transduced signals can vary in properties, being electrical (i.e. the ones involved in the depolarisation of cellular membranes), chemical (i.e. those producing a second messenger) or transcriptional (i.e. in the activation of gene expression), among others.

Microelectrode arrays or multielectrode arrays (MEAs): Devices that contain multiple microelectrodes through which neural signals are obtained and/or delivered, essentially serving as neural interfaces that connect neurons to electronic circuitry – MEAs can be used *in vivo* or *in vitro*, neuronal cultures on MEAs can survive for over a year *in vitro*.

Microfluidics: The behaviour, precise control and manipulation of fluids that are typically geometrically constrained to a submillimeter scale.

Multicellular spheroids: Microscale, spherical cell clusters–spheroids can be monoculture or multiculture.

Neurodegenerative diseases: A disease group characterised by progressive nervous system dysfunction, being associated with atrophy of affected structures of the nervous system.

Tissue engineering: Generally involves the use of materials (scaffolds), cells and bioactive molecules to prepare *de novo* tissues *in vitro* or *in vivo* with the goal of trying to understand tissue function or as part of tissue regenerative or repair strategies.

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Daniela N. Rocha is currently the Deputy R&D Manager at Blueclinical (Porto), and worked at INEB (Biomedical Engineering Institute), before her current post, under the supervision of Ana Paula Pêgo. In 2015, she received her PhD degree in biomedical engineering from the University of Porto and her MBA from Porto Business School. She has eight years of experience in research within the biomaterials for neuroscience field, including polymers and hydrogels. Her expertise includes primary cell isolation, 3D cell culture techniques, mechanotransduction events and related signalling pathways.

Eva D. Carvalho is an MSc bioengineering student from University of Porto (Portugal) and is currently developing a master's thesis in Ana Paula Pêgo's group on platforms to study demyelinating diseases. In 2013, Eva enrolled a research project at IBMC (Portugal) in which the TGF-β signalling pathway in *Drosophila melanogaster* was studied. In 2015, at REMEDI (Ireland), Eva was focused on developing iPSC from *Retinitis pigmentosa* patients. Her main research interests are on neurodegenerative diseases and neuroregeneration.

Ana Paula Pêgo (PhD) is the Coordinator of the nBTT: nanoBiomaterials for Targeted Therapies Group at INEB|i3S. By using nanomedicine strategies her team aims at providing *in situ* and in a targeted manner the required signals to promote nervous tissue regeneration. The research on new biomaterials for application in neurosciences includes the development of new polymers for the design of alternative vectors to viruses for efficient nucleic acid delivery to neuronal cells and preparation of nerve grafts for spinal cord injury treatment. Her team recently developed a tissue-engineered astroglial scar to serve as a platform for the identification of glial scar features or elements that might represent therapeutic targets to treat central nervous system lesions.

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