Using stem cell-derived neurons in drug screening for neurological diseases

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Abstract

Induced pluripotent stem cells (iPSCs) and their derivatives have become an important tool for researching disease mechanisms. It is hoped that they could be used to discover new therapies by providing the most reliable and relevant human *in vitro* disease models for drug discovery. This review will summarise recent efforts to use stem cell-derived neurons for drug screening. We also explain the current hurdles to using these cells for high throughput pharmaceutical screening and developments that may help overcome these hurdles. Finally, we critically discuss whether iPSC-derived neurons will come to fruition as a model that is regularly used to screen for drugs to treat neurological diseases.

1. Introduction

A report by the World Health Organisation concluded that neurological disorders are a substantial burden and contributed to 12% of deaths worldwide in 2005 (WHO, 2006). Since many neurological disorders are age-related, this figure is expected to rise with our aging population. Given the increasing burden and low availability of effective treatments for neurological disorders there is a high demand for new therapies. The lack of effective treatments suggests there is still much to be known about neurological disease. These diseases are difficult to study due to inaccessibility of diseased tissue, forcing researchers to make conclusions from post-mortem tissue or unrelated cell or animal models.

A key challenge in drug discovery for many decades has been the choice of good model cell lines for primary screening. In particular, certain cell types such as neuronal cells are not accessible for *ex vivo* screening approaches. Therefore, many cell models are based on cancer cell lines, which have many genetic changes compared to the cell type of interest and may not be representative of the disease (Skibinski and Finkbeiner, 2011). Furthermore, primary animal cells and immortalised cell lines are far removed from human cells (Horvath et al., 2016). A recent analysis of success rates in drug development calculated that the likelihood of a compound in phase I to gain final approval is as low as 10.4% (Hay et al., 2014). The majority of failures in phase II and III trials are attributed to lack of efficacy, however a large proportion of failures are due to safety concerns. This suggests that our understanding of disease mechanisms, and the relevance of current cellular and animal models, are inadequate. Clearly new drug screening models are needed to facilitate the discovery of new therapeutics in a timely and cost-efficient manner, with lower failure rates. Pluripotent stem cells may offer a solution to this problem.

The two main types of pluripotent stem cells are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are typically derived from the forming inner cell mass of a blastocyst-stage embryo. By isolating the embryo at this stage, researchers are able to isolate cells that retain the capacity to differentiate into any somatic cell type, known as embryonic stem cells ESCs. This raises ethical dilemmas when human embryos are used, and indeed some countries have banned the use of human ESCs (Robertson, 2010). Furthermore, it is challenging to obtain ESCs carrying specific genetic abnormalities without genetic manipulation (Barker and de Beaufort, 2013). Use of mouse ESCs somewhat circumvents the issues associated with human ESCs, however these cells have their own limitations regarding the relevance of animal models to human disease pathogenesis. iPSCs provide a potential solution: by expressing a set of key transcription factors, somatic (fully differentiated) cells can be “reprogrammed” to adopt a pluripotent state. iPSCs may circumvent some of the difficulties associated with cancer cell lines and animal models. Furthermore, iPSCs are an ideal model for investigating diseases of known genetic causes because cells can be generated from patients with known mutations.

The brain is comprised of several different cell types including a wide variety of neuronal subtypes. Neurological diseases can affect particular subtypes of neurons and protocols have been developed to differentiate iPSCs into distinct neuronal subtypes and glial cells. The protocols for differentiation of ESCs and iPSCs into different neuronal and glial subtypes have been recently reviewed extensively elsewhere, and so will not be discussed in detail here. ESCs and iPSCs have been differentiated into cortical (Maroof et al., 2013; Shi et al., 2012), cholinergic (Hu et al., 2016), dopaminergic (Kirkeby et al., 2012; Kirkeby et al., 2017; Kriks et al., 2011; Sanchez-Danes et al., 2012), GABAergic (Nicholas et al., 2013; Yang et al., 2017), hippocampal (Yu et al., 2014), hypothalamic (Merkle et al., 2015), motor (Nizzardo et al., 2010; Wada et al., 2009), serotonergic (Lu et al., 2016; Vadodaria et al., 2016) and purkinje neurons (Wang et al., 2015). Furthermore, neuronal precursor cells (such as neural stem cells (NSCs) and neural progenitor cells) are also often used (Cezar et al., 2007; Han et al., 2009; Kaufmann et al., 2015; Lorenz et al., 2017; Mali et al., 2013; Pei et al., 2015). These are cells that have been directed towards a neuronal fate, and therefore lack pluripotency, but are not committed to a specific neuronal subtype. Advantages of using these cells over terminally differentiated cells are that they continue to proliferate, and the time needed in culture to generate differentiated neurons is reduced. However, they may not recapitulate disease phenotypes of mature neurons. ESCs and iPSCs can also be differentiated into different glial cells: astrocytes (Emdad et al., 2012; Juopperi et al., 2012), oligodendrocytes (Nistor et al., 2005) and microglia (Abud et al., 2017). These glial cells may play a role in the pathogenesis of many neurological diseases.

The advent of these different differentiation protocols has facilitated research on many neurological diseases using iPSCs. The current state of this research has been recently reviewed for a range of diseases (Gibbs et al., 2018; Inak et al., 2017; McKinney, 2017; Poon et al., 2017; Tamburini and Li, 2017) including Parkinson's disease (Cobb et al., 2018; Singh Dolt et al., 2017), Alzheimer's disease (Arber et al., 2017; Robbins and Price, 2017), amytrophic lateral sclerosis and frontotemporal dementia (Guo et al., 2017; Preza et al., 2016), Huntington's disease (Tousley and Kegel-Gleason, 2016), childhood neurological diseases (Barral and Kurian, 2016; Santos and Tiscornia, 2017), and psychiatric disorders (Adegbola et al., 2017; Watmuff et al., 2017). These reviews cover differentiation of specific neuronal subtypes and relevant disease phenotypes observed in these cells, therefore these topics will not be covered here. Many of these reviews highlight the possibility of harnessing iPSCs to discover new therapies for neurological disorders, but most do not detail how successful this strategy has been to date. In this review we summarise recent efforts to use iPSC-derived neurons in drug screening applications and critically assess whether this technology is currently viable for the discovery of therapeutics for neurological diseases.

2. Using iPSC-derived neurons in screening applications

2.1 Testing candidate drugs

iPSC-derived neurons have found widespread use in early stage drug discovery pipelines, from testing a limited set of candidate compounds to mini-screens using a focused set of compounds through to medium-scale screening where automated protocols for handling 384-well microplates is required. Candidate compounds, hormones and growth factors with a known action have been tested in multiple disease models for neuronal diseases. These studies have laid the groundwork for establishing the use of iPSC-derived cells as an appropriate proxy for human disease. Furthermore, these studies facilitate the development of robust assays prior to screening with large compound libraries. For example, candidate drugs for Rett syndrome, an X-linked neurological disorder considered part of the autism spectrum disorder, have been tested on iPSC-derived neurons (Marchetto et al., 2010). Insulin-like growth factor 1 (IGF1), a compound previously identified to rescue Rett phenotype in a mouse model, was tested on iPSC-derived neurons following characterisation of a cellular phenotype. IGF1 treatment increased glutamatergic synapse number, thus rescuing the disease phenotype (de Souza et al., 2017; Tang et al., 2013). A phase 2 trial has recently been conducted using Trofinetide, a small molecule analog of IGF1 (Glaze et al., 2017). Treatment with Trofinetide resulted in an improvement in several

outcome measures which represented clinically meaningful improvement in disease symptoms.

Another successful model utilised Alzheimer's disease iPSC-derived neurons to test the impact of compounds with a known mode of action on β-amyloid secretion (Yagi et al., 2011). A γ-secretase inhibitor and a modulator of amyloid precursor protein cleavage (compound names not published) were tested and found to suppress β-amyloid secretion in a dose-dependent manner. In a similar study using iPSCs from sporadic and familial Alzheimer's disease patients, docosahexaenoic acid treatment was found to alleviate two phenotypes identified in these cells: endoplasmic reticulum stress and oxidative stress (Kondo et al., 2013). These findings are promising because they identify phenotypes that could be used in screens, and suggest modes of action that may have therapeutic potential for Alzheimer's disease.

In schizophrenia, five known antipsychotic drugs were tested for their effect on neuronal connectivity in iPSC-derived neurons from four schizophrenia patients (Brennand et al., 2011). One of these drugs, Loxapine, was successful in increasing neuronal connectivity in neurons from all patients. Loxapine was shown to be successful in relieving agitation associated with schizophrenia in a phase III clinical trial (Lesem et al., 2011) and is now licenced for use in the USA and the EU. This study demonstrates that effects of drugs in iPSC-derived neurons may correlate with clinical efficacy in patients.

An iPSC-derived neuronal model of familial dysautonomia (a neurodegenerative disease that affects sensory and autonomic neurons) found that Kinetin improved the identified splicing defect and also increased the percentage of neurons during differentiation (Lee et al., 2009). Kinetin has been tested in patients in a small clinical trial and increases wild-type IKBKAP mRNA production (Axelrod et al., 2011). A phase II trial of Kinetin is ongoing (ClinicalTrials.gov Identifier: NCT02274051). These studies show that iPSC-derived neuronal disease models can be predictive of a therapeutic effect in patients and lead to clinical trials. Two candidate drugs for the treatment of spinal muscular atrophy (SMA), valproic acid and tobramycin, have been tested in iPSCs from a child with SMA (Ebert et al., 2008). In iPSC-derived neurons stained for SMN (the protein associated with SMA) both of these drugs were found to increase the number of nuclear SMN gems (the spherical structures where most SMN protein resides). A similar assay has been used to screen a much larger number of compounds, as detailed in section 2.3. Unfortunately, valproic acid has not been effective in improving outcomes in either children or adults with SMA in clinical trials (Kissel et al., 2014; Kissel et al., 2011).

Neurological disorders caused by mutations in mitochondrial DNA have also recently been modelled using human iPSC-derived neural progenitors (Lorenz et al., 2017). A library of 130 compounds were tested as a proof of concept study using an imaging-based assay of mitochondrial membrane potential as a cellular readout. Patient lines with MT-ATP6 mutations displayed increased mitochondrial membrane potential and 10 compounds were found to ameliorate this. The study then focused on one compound named Avanafil, a PDE5 inhibitor. Interestingly we have used a similar high-content assay to detect decreased mitochondrial membrane potential in iPSC-derived dopaminergic neurons from patients with Parkinson's disease (Little et al., 2018). This suggests that such an assay could be used to identify potential novel therapies for Parkinson's disease too.

iPSC-derived neurons may also provide an ideal tool for assessing neurotoxicity. Measuring neurotoxicity is important for identifying environmental toxins, testing the safety profile of new drugs and to identify compounds that may be protective against neurotoxins. One study used Parkinson's disease iPSC-derived dopaminergic neurons to test 44 compounds of known neuroprotective activity (Peng et al., 2013a). Interestingly, the cells were exposed to the neurotoxin MPP+ to induce toxicity before the test compounds were added and their effect on cell viability was measured using an MTT assay, a common colorimetric cell viability assay based on the reduction of a dye by cellular enzymes (Kupcsik, 2011). This screen identified 16 hits including known neuroprotective agents such as selegiline (an established treatment for Parkinson's disease), nicotine (which has been trialled for Parkinson's disease) and antioxidants. Anti-inflammatory agents and neurotransmitters were also identified. However, some compounds that are currently in clinical trials for Parkinson's disease did not produce a positive effect, including rasagiline and creatine, although the authors suggest that this could be due to the dosage used. Furthermore, this study assessed whether these compounds protected against 1-methyl-4-phenylpyridinium (MPP+) toxicity. It would be interesting to see whether similar results would be seen in iPSCs derived from PD patients. It is not yet known how accurately this screen will predict compound efficacy in patients. A separate study tested 80 compounds from the National Toxicology Program, 37 of which were known neurotoxins (Ryan et al., 2016). Using commercially available iPSC-derived neurons the authors developed a high-content assay that measured neurite outgrowth and the number of viable cells. This identified 16 compounds that selectively inhibit neurite outgrowth and 6 of these were confirmed by repeat studies. All 6 of the confirmed hits have previously been shown to be neurotoxic, demonstrating that this assay is a reliable assessment of neurotoxicity.

A metabolic screen using mass spectrometry has also been performed to assess the impact of valproate on human ESC-derived neural progenitors, as a model of neurodevelopmental impairment (Cezar et al., 2007). This is reported as the first time small molecules have been identified following secretion from human ESCs and human ESC-derived cells. Several molecules were found to be upregulated following valproate exposure. However, the authors were unable to identify all of the secreted molecules identified in this screen, highlighting a potential downside to this screening approach. However, the field of metabolomics could provide a promising screening platform for use with iPSC-derived neurons through the sensitive detection of molecular changes. This may overcome some of the hurdles associated with high-content screening such as accurate identification of cellular features in complex neuronal cultures whilst still generating large amounts of data from very few cells. These examples show that using iPSCs to test candidate drugs may be a powerful method to identify which candidates may or may not be successful in humans. Often, a handful of known neuronal modulators are used in such studies with the aim of “re-purposing” FDA-approved drugs for use in other conditions. A major advantage is that these drugs are well characterised and thus have already been established to have the right pharmacodynamics and safety profile for treating neurological diseases. Sometimes, a small screen using a collection of FDA-approved drugs is performed to increase the chances of finding hits (see below).

2.2 Small screens

By screening small (<10,000 compounds) or medium-to-large (>10,000 compounds) libraries, it is possible to test an unbiased hypothesis and identify novel structural scaffolds or functions of a chemical entity. Typically, such screening employs 96- or 384-well plates and requires cells to be seeded into these plates at some point during the differentiation process. The main readouts are high-content analysis (immunofluorescence), reporter gene expression, and cell viability (Ketteler and Kriston-Vizi, 2016). Figure 1 shows a workflow using iPSC-derived neurons in high-content screening. In addition, the availability of high-throughput electrophysiology instrumentation has made it possible to screen compounds in such assays as well, even though this has not been employed extensively to date. Other phenotypic profiling methods such as mass spectrometry have potential but are challenging to execute on larger scales. Common screening methods have been reviewed elsewhere (Walters and Namchuk, 2003; Yao et al., 2015). There are several challenges associated with using pluripotent stem cells in screening that we will highlight in section 3. However, iPSC-derived neurons have been used to test small libraries of compounds, which we will

discuss here.

One study screened 750 FDA-approved drugs for their effect on the viability of human ESCs and human iPSC-derived NSCs (Han et al., 2009). Using adenosine triphosphate (ATP) levels as a measure of cell viability in a multi-well plate the authors identified nine drugs that were significantly more toxic to NSCs than ESCs, but no drugs were found to be more toxic to ESCs than NSCs. The authors followed up one compound, amiodarone, which was unexpectedly found to be non-toxic to dopaminergic neurons, despite being toxic to NSCs. In a related study 2000 compounds were screened using NSCs alongside rat cortical neurons and an MTT assay was used to assess the effect of these compounds on cell viability (Malik et al., 2014). The authors found that the human cells were more sensitive to neurotoxins than the primary rat cells. The study identified 43 active compounds and investigated these further. The authors used an elegant way of assessing cell morphology following treatment with these 43 compounds by using human NSCs that ubiquitously express the fluorescent reporter dTomato. Pyrithione zinc and sanguinarine elongated cells, whilst cardiac glycoside increased cell area. This raises the possibility of using reporter ESC lines to improve high-content analysis of cellular properties, although it may be possible to use intracellular dyes to achieve the same effect without having to genetically modify cell lines. A more recent study investigated whether iPSC-derived cells could be used to discover neurotoxins by testing 80 compounds on iPSCs, neural stem cells, neurons and astrocytes (Pei et al., 2015). Cell viability following incubation with these compounds was then assessed in 96–well plate format using an MTT assay. This identified 50 compounds that reduced cell viability, with more compounds affecting neurons than the other cell types investigated. This study shows that these cells can be used to determine whether environmental compounds are toxic to human cells. As an additional refinement, iPSC reporter lines were developed which express luciferase when differentiated into neurons or astrocytes. When these cells were employed in a similar screen, toxic compounds caused a reduction in luminescence, demonstrating a decrease in the number of neurons or astrocytes. Together these studies demonstrate techniques that could be used to screen libraries of drugs with iPSC-derived neurons and demonstrate that relevant data on the impact of test compounds can be collected using these cells.

2.3 High-throughput screens

iPSC-derived cells have been used to test candidate drugs in studies based on phenotypes established in these cells, or to test drugs identified in screens using other cell types. The

next step is to use of iPSC-derived neurons in large scale high-throughput screens. Details of large screens that have utilized stem cell-derived neurons are summarised in Table 1. One large scale screen from a collaboration between pharmaceutical companies Pfizer and ArunA Biomedical used mouse ESC-derived neurons to screen over 2.4 million compounds (McNeish et al., 2010). This screen aimed to identify compounds that enhanced agonist-evoked currents from AMPA glutamate receptors which could be used to treat cognitive impairment. Amongst the 37 confirmed hits were known AMPA potentiators and two novel molecules, one named CE-382349. A smaller study screened 5000 molecules in mouse ESC-derived motor neurons to identify compounds that promote survival following trophic factor withdrawal (Yang et al., 2013). A large number of hits were found that act on targets previously shown to be neuroprotective or involved in amyotrophic lateral sclerosis including inhibiting protein or DNA sythesis, MMP inhibitors, a cannabinoid receptor agonist, and a calpain inhibitor as well as ligands for neurotransmitter receptors and kinase inhibitors. This primary screen was then expanded upon by using cells from a human ESC line and iPSCs from two amyotrophic lateral sclerosis patients. This study discovered that the molecule kenpaullone (a GSK-3 inhibitor) substantially increased survival of iPSC-derived motor neurons following trophic factor withdrawal. Another amyotrophic lateral sclerosis screen used high-content analysis to identify TDP-43 aggregates in iPSC-derived neurons (Burkhardt et al., 2013). From 1757 compounds, 38 hits were identified which reduced the percentage of cells with aggregates. These hits were then tested using cortical neurons from the same iPSCs. From this study four classes of compounds were identified that reduced TDP-43 aggregates in a dose dependent manner in amyotrophic lateral sclerosis patient iPSC-derived cortical neurons including two types of kinase inhibitors and cardiac glycosides. A study related to Parkinson's disease includled the generation of iPSCs from patients with a mutant form of α-synuclein and controls produced via genome editing (Ryan et al., 2013). However, human ESC-derived neural precursor cells were used to perform the screen of 2000 compounds. These cells were transfected with a luciferase reporter plasmid that produced luminescence upon activation of the MEF2 transcription factor. One hit, isoxazole, was then further assessed in the iPSC-derived dopaminergic neurons with mutant α-synuclein. Isoxazole conferred a degree of protection from apoptosis following exposure to mitochondrial toxins.

One elegant high-content screen was performed using mouse ESC-derived motor neurons to screen for neuroprotective agents (Hoing et al., 2012). The ESCs used here were derived from a mouse carrying a bacterial artificial chromosome that contained a green fluorescent protein reporter vector under the control of an Hb9 promoter, which produced green fluorescence in mature motor neurons. After FACS of differentiated mature motor neurons,

these cells were co-cultured with astrocytes. The BV2 cell line was then used as a source of activated microglial cells to induce motor neuron death. The high-content analysis performed here included assessing total neurite length, number of branch points and neurite area. From a screen of 10,000 small molecules, 37 active compounds were identified, 26 of which were confirmed and 12 selected for further testing. These hits were not named but one is an inhibitor of the nitric oxidase synthase L-NIO.

A recent screen measured concentrations of different amyloid β peptides (Aβ38, Aβ40 and Aβ42) in the media of iPSC-derived cortical neurons in a 96 well plate using cells with trisomy of chromosome 21 to increase amyloid β (Brownjohn et al., 2017). This system was used to screen the Prestwick chemical library for modulators of amyloid β. Multiple hits were identified including known γ-secretase inhibitors and members of the avermectin class of macrocyclic lactones that were followed up in further experiments. A similar screen measured alterations in Aβ40 and Aβ42 production by ELISA (Kondo et al., 2017). iPSCs with doxycycline-inducible neurogenin-2 expression were used to improve efficiency of cortical neuron differentiation. This identified 129 initial hit compounds, that were then classified based on fingerprinting of the compound chemical structure to identify diverse compounds that may have a synergistic effect. Six compounds were then chosen as lead compounds: bromocriptine, cilostazol, cromolyn, fluvastatin, probucol and topiramate. All of these lead compounds, except for bromocriptine, have previously been shown to have anti-amyloid β effects *in vivo,* confirming the reliability of this screen whilst highlighting the potential to identify compounds that could be missed by using animal models.

A high-throughput screen used iPSC-derived motor neurons to screen 200,000 compounds in a drug discovery screen for SMA (reviewed in Grskovic et al., 2011). High-content image analysis was used to detect the number of gems of SMN, the protein that is reduced in SMA. This high-content analysis was also able to determine the localization of these gems. The results of this screen were not published and it is not known whether any hits were identified or have been taken forward for clinical trials. A high-throughput and high-content screen has also been performed by researchers at the pharmaceutical company Novartis using iPSC-derived neuronal progenitors from a patient with Fragile X syndrome (Kaufmann et al., 2015). In this screen 50,000 compounds were tested to identify inducers of Fmr1 expression, measured by staining cells for fragile X mental retardation protein and performing image analysis. Two different algorithms were used to perform single-cell multiparametric analysis, resulting in 2099 compounds being identified that induced a small increase in signal. Of those identified compounds, 790 were chosen for further dose response experiments. The only compound with a previously reported mode of action was a histone deacetylase inhibitor. The authors state that image and data analysis was critical for the sensitive

detection of weaker hits, and indeed this kind of analysis may be integral to using iPSC-derived neurons in screening applications.

2.2.4 Commercially available cells

A key challenge in using iPSC-derived cells for screening is the variability between batches of differentiated cells and between different lines as well as the time it takes to generate neurons from iPSCs. One approach to overcome this limitation is the use of commercially available iPSC-derived cells that can be bought in large quantities with quality controlled purity and reproducibility.

Commercially available iPSC-derived neuronal cells may reduce some of the time and effort needed to generate sufficient cells with which to perform a screen. iCell neurons, commercially available iPSC-derived cortical neurons (Cellular Dynamics), were used in a screen to investigate the effect of toxic β-amyloid Aβ1-42 aggregates on neuronal survival and neurite length in a screen performed through a collaboration between Harbin Medical University, GlaxoSmithKline and Cellular Dynamics (Xu et al., 2013). The design and implementation of this screen was based on results generated from iPSC-derived forebrain neurons. This system was used to screen several hundred compounds and 19 hits were identified including a cyclin-dependent kinase 2 inhibitor. A separate screen used commercial human neural precursors (from Clonetics) to screen 640 molecules from two small commercially available libraries and 25,000 proprietary compounds (Richards et al., 2006). Data was collected on neurite number, length and complexity to assess the induction of neurite outgrowth. Immunolabelling was fully automated in this study and compound addition was semi-automated, thus increasing throughput. However, the authors noted that reconfirmation rates were lower for this screen than for a conventional high-throughput screen. The 14 hits identified were then further investigated for their mechanism of action in human and rat neurosphere cultures. One mechanism of action investigated related to changes in intracellular calcium levels. The compounds 2-chloro-ATP, 1,3-dipropyl-8-p-sulfophenylxanthine and bethanechol increased intracellular calcium levels. Interestingly, many of the hits identified from the screen in human cells did not have the same effect in primary rat cortical neurons, highlighting differences between human and non-human cell models. A more recent screen measured 11 parameters from images of iCell neurons including seven parameters of neurite morphology. A total of 5212 compounds were tested and 50 confirmed compounds identified that promote neurite outgrowth (Sherman and Bang, 2018). These hits were classified into reported targets including kinase inhibitors, steroid hormone receptor modulators and neurotransmitter modulators. In this screen, nuclear

staining provided a useful first step in data analysis; by measuring parameters of nuclear staining, compounds were identified that exhibited significant cytotoxicity and were thus removed from further analysis.

3. Technical limitations and potential future advances

The previous sections demonstrate that pluripotent stem cell-derived neurons have been used in large scale drug screens. However, only a small number of such screens have so far been published. In this section we will discuss the challenges facing the adoption of these cells for screening. We will also discuss recent advances in technology that may improve the uptake of these cells for screening applications and improve the accuracy of drug screening.

The generation and differentiation of iPSCs is a relatively novel technology and there are difficulties associated with establishing these as cell models for drug screening. Many of these hurdles concern the culturing of iPSCs and their differentiated progeny. Growing and culturing iPSCs can be very time consuming and expensive. It is difficult to maintain the cells in a pluripotent state and small changes in growth conditions can lead to unwanted spontaneous differentiation or a reduction in proliferation. Automated cell culture using robotics will be essential for scaling up production to generate the number of cells needed for use in the pharmaceutical industry (Konagaya et al., 2015; Thomas et al., 2008). However this is far from trivial due to the manner in which iPSCs are grown. Development of better growth substrates and media should improve the reliability of iPSC culture and reduce spontaneous differentiation. Recent advances, including media that can maintain growth for longer than one day (for example Essential 8 Flex, ThermoFisher), and more specific attachment matrices to reduce spontaneous differentiation and improve survival, are making culture conditions more suited to automated systems (Rodin et al., 2014; Tsai et al., 2015). For truly automated high-throughput work, differentiation protocols also need to be applicable to automation. Automation can potentially increase the reliability and reproducibility of a procedure through standardisation (Daniszewski et al., 2018). Arguably automation should be employed wherever possible as best practice when using iPSCs in screens in order to enhance reproducibility (Engle et al., 2018). Applying automation to significant steps in a screening system, such as cell culture, compound addition and staining, could limit variability in screens but the complexity of these procedures may hinder automation efforts. However, many aspects of iPSC culture have been shown to be amenable to automation including reprogramming, maintenance and differentiation (Archibald et al., 2016; Efthymiou et al., 2014; Konagaya et al., 2015; Paull et al., 2015) with equivalent or improved reliability over manual techniques. Furthermore, an economics tool

applied to a study of producing iPSC-derived neurons for screening has shown that automated processing can significantly reduce labour costs associated with stem cell processes (Jenkins et al., 2016).

The next step will be to further miniaturise assays reviewed here in order to increase the throughput of screens whilst further decreasing costs. A recent study demonstrated a novel technique to wash 1536-well plates using centrifugation (Knight et al., 2017). This method can be used in assays involving immunofluorescence as well as in applications where plates need to be coated before using. This may be particularly useful when using iPSC-derived neurons in high-throughput imaging based assays and could be used for plate coating, media changes and the multiple washing steps involved in immunofluorescence without disturbing the delicate cell layer. It is also possible to avoid the need to coat plates before seeding neurons by the addition of extracellular matrix to the culture medium when seeding cells (Dai et al., 2016). Plating cells with medium containing an extracellular matrix protein is sufficient for the adherence of iPSC-derived NSCs in 1536-well plates. This one-step seeding method simplifies the cell seeding procedure, thus making the process more amenable to automation and reducing the time required to seed cells.

It is important to consider how iPSCs are generated when using them to model disease. The generation and growth of iPSCs is usually clonal, meaning that all cells in an individual iPSC line are derived from a single cell (Hong et al., 2013). This raises the question of whether this cell is representative of the patient it was taken from. The cell in question may have a spontaneous DNA mutation due to mosaicism or have undergone genetic changes during reprogramming. Equivalently, is a particular control cell line representative of the healthy population? Could a “healthy” volunteer subject have an undiagnosed genetic disorder? For these reasons it is necessary to use multiple patient iPSC clones, and iPSC lines from multiple control donors to be sure that observed differences between cell lines are really due to the mutation being studied. However, using multiple different lines for high-throughput screening greatly increases the cost, duration and complexity of the screen. A compromise is to perform preliminary characterisation studies on multiple lines and then identify one line in which to perform high throughput screening.

Genetic engineering has been used to correct gene mutations in patient lines to create a control line that is otherwise genetically identical, known as an isogenic control. This helps ensure that any differences seen are due to the specific genetic mutation in the patient. The advent of CRISPR/Cas9 genome editing technology has improved the speed, reliability and efficiency of genome editing and has many applications for drug screening (Agrotis and Ketteler, 2015). Importantly it has now been used to create isogenic controls in many iPSC

studies. Mutations can also be introduced into control cells to further increase confidence in observed phenotypes. These genetic engineering techniques will enable researchers to confidently identify robust phenotypes in patient lines with more confidence, and select the most suitable line to use in screening.

Protocols for the differentiation of iPSCs into neurons raise additional difficulties separate from those discussed above. Differentiation of neurons is particularly time consuming and often involves multiple passaging steps, which hinders full automation. Furthermore, these protocols require expensive recombinant proteins such as cytokines. Over time, small molecules have been discovered that can replace some of the recombinant proteins traditionally used in differentiation protocols and are far less expensive to produce (Reinhardt et al., 2013). Current differentiation protocols can sometimes be unreliable with many sources of variability. Often, differentiated cell populations are heterogeneous, containing different types of neurons alongside other cells such as glia. For reliable screening data this heterogeneity must be controlled for, to ensure inter-well and inter-plate consistency. Neuronal cultures can be sorted by fluorescence-activated cell sorting (FACS) or by magnetic-activated cell sorting to select only neuronal progenitors and thus help remove non-neuronal contaminants (Burkhardt et al., 2013; Doi et al., 2014; Kim et al., 2012; Schondorf et al., 2014). Indeed, a thorough set of protocols have recently been published detailing a method for the generation of expandable neuronal progenitor cells using FACS purification based on cell surface markers (Cheng et al., 2017). This publication also details a protocol for the differentiation of neuronal progenitor cells into cortical neurons through the incorporation of a doxycycline-inducible Neurogenin-2 cassette into the cells. This protocol is not only faster than traditional differentiation methods but it also produces a more homogenous population of neurons. It is also possible to cryopreserve banks of Neurogenin-2 neuronal progenitor cells from different patients to then differentiate and screen at a later date. Another option is to control for heterogeneity at the analysis stage, using sophisticated high-content screening analysis. If an imaging-based screen is performed, neurons can be identified by image analysis software via the presence of neuron-specific immunofluorescence, or even by cell morphology. This enables the analysis to be restricted to the neuronal cells (or comparison between cell types) without the need for complicated cell culture procedures.

Another consideration regarding neuronal differentiation is the maturity of iPSC-derived neurons, which is of particular importance for the study of diseases of aging such as Parkinson’s disease and Alzheimer’s disease. Neurons produced from current differentiation protocols are more akin to embryonic or neonatal neurons than adult neurons (Cornacchia and Studer, 2015; Patterson et al., 2012). Indeed, comparing the gene expression profile of

iPSC-derived dopaminergic neurons with that of human post-mortem midbrain dopaminergic neurons revealed large differences in global gene expression between the two groups of cells (Papapetropoulos et al., 2006; Xia et al., 2016). Many of the gene expression differences were associated with cell maturity. A similar study of iPSC-derived cortical neurons compared single cell transcriptomics data with that of human adult and fetal cortical neurons (Handel et al., 2016). Again, these iPSC-derived cortical neurons exhibited high levels of expression of genes associated with fetal neurons.

Current differentiation protocols usually take 50 to 100 days before the cells are used for downstream experiments, which greatly increases the length and expense of studies and screens. Research is ongoing to decrease the time needed to generate neurons (Qi et al., 2017), however these protocols may need additional interventions to induce the effects of aging such as exposure to toxic stressors. Indeed, many studies have used toxins to reveal differences between control and diseased iPSC-derived neurons. Toxins such as H_2O_2, 3-methyladenine, Calcimycin, rotenone, MG-132, 6-hydroxydopamine, and MPP+ have been used to induce oxidative or nitrosative stress, mitochondrial dysfunction or ER stress (Consortium, 2012; Nguyen et al., 2011; Peng et al., 2013b; Schondorf et al., 2014). Aging has been induced in iPSC-derived dopaminergic neurons by short term expression of progerin. This protein is a truncated form of lamin A produced from mutations in the LMNA gene and is associated with progeria, a disease of premature aging (Miller et al., 2013). Treatment with progerin revealed phenotypes that had not previously been seen, including a reduction in dendrite length, which was more pronounced in Parkinson's neurons.

As with all cell models there is a major difference between the complexity of iPSCs and animal models. Efforts have been made to increase the complexity of cell models by using 3D cultures, fluid flow systems or co-cultures with other cell types (reviewed in Horvath et al., 2016). Co-culture with other cell types differentiated from iPSCs such as astrocytes, oligodendrocytes or microglia may improve physiological relevance (Douvaras et al., 2017; Duan et al., 2015; Haenseler et al., 2017; Kawabata et al., 2016; Li et al., 2015; Misumi et al., 2013) and image analysis could be used to study the different cell types separately. Astrocyte co-culture has been shown to improve synaptic activity, and also the number of dendritic branches in co-cultured neurons (Tang et al., 2013). However, the effects of co-culturing neurons and astrocytes of different genotypes should be taken into account. Wild-type astrocytes can have a positive effect on diseased neurons whilst mutant, or reactive, astrocytes can have a negative effect on wild-type neurons (Du et al., 2018; Haidet-Phillips et al., 2011; Kunze et al., 2013; Toli et al., 2015; Tripathi et al., 2017). Since the role of astrocytes have been implicated in various neurological diseases, it would be more relevant to co-culture neurons and astrocytes from the same patient iPSCs. The available protocols

for glial differentiation would enable this. Cell autonomous phenotypes could potentially be identified using this type of co-culture system alongside high-content screening techniques.

Furthermore, cells could be grown in three dimensions (3D) to improve physiological relevance, using scaffolds or gels (reviewed recently in Kim et al., 2015; Korhonen et al., 2018; O'Rourke et al., 2017; Pasca, 2018). One published protocol detailed a 3D system of growing human neural progenitor cells within a Matrigel matrix (Kim et al., 2015). The 3D culture improved neuronal differentiation and promoted extracellular deposits of amyloid-β in cells with Alzheimer's disease-related mutations. This demonstrates that 3D culture could exacerbate disease phenotypes. Recent advances in protocols to culture brain organoids, or 3D co-cultures of brain cells, may further improve the physiological relevance of *in vitro* models. For example recent studies have shown that 3D co-cultures rapidly produce synapses (Krencik et al., 2017), and recapitulate transcriptional patterns of cell types in the human brain (Tekin et al., 2018). 3D co-culture has recently been used to assess toxicology (Sirenko et al., 2018) and can reveal toxic effects on processes such as cell migration which cannot be determined in two dimensional culture (Hellwig et al., 2018). 3D culture systems make analysis much more complex for high-content screens but image analysis technology is also developing rapidly. Recent advancements in analysis of 3D images and live cell imaging have been extensively reviewed elsewhere (Kriston-Vizi and Flotow, 2017; Nketia et al., 2017). Furthermore, advances in microscopy such as superresolution and image processing techniques will be integral in analysing images of complex 3D co-cultures (Almada et al., 2015; Culley et al., 2018; Gustafsson et al., 2016). In addition. data analysis techniques such as machine learning have advanced rapidly in recent years (Cao et al., 2018), and will likely greatly assist in determining phenotypes from high-content screens of complex cell culture systems.

Developments in the field of microfluidics may also play an important role in improving the physiological relevance of *in vitro* models for neurological diseases and the use of iPSC-derived neurons in drug screening (reviewed recently in Neto et al., 2016; Nys and Fillet, 2018; Regnault et al., 2018). The field of microfluidics is relevant to drug screening for several reasons. Miniaturisation of fluid movement can greatly reduce the quantities of reagents needed in high-throughput screens, thus reducing the cost of the screen. Several analytical techniques can also be combined into a microfluidic device such as fluorescence, enzymatic and electrical measurements. Microfluidics provide many opportunities to improve the physiological relevance and complexity of neuronal models (reviewed in Karimi et al., 2016). These include the control of the flow of nutrients, application of sheer stress and the compartmentalisation of different cell types. Furthermore, coupling microfluidic systems with 3D culture can improve differentiation and enhance neural migration. Recently

a microarray chip-based platform was used to grow 3D cultures of neural progenitor cells and assess the effects of soluble factors on neuronal differentiation (Nierode et al., 2018). This work demonstrates that this technology could not only improve differentiation protocols but similar assays could be used to screen for drugs. Interestingly, microfluidics and 3D coculture have recently been combined to grow motor neurons alongside endothelial cells. This improved neurite elongation and neuronal connectivity and may provide an improved model to test drugs for motor neuron disease (Osaki et al., 2018).

The field of optogenetics has also provided exciting developments that may contribute to increased use of iPSC-derived neurons in high-throughput screens (reviewed recently in Agus and Janovjak, 2017; Zheng et al., 2018). Light can serve as a universal trigger for membrane depolarization or activation of ion channels which can be used for the measurement of neuronal excitability and synaptic transmission and could facilitate high-throughput screens for these readouts in iPSC-derived neurons. Indeed, such systems have already been used in 384-well plates in cultured neuronal cells and even combined with fluorescent readouts (Agus et al., 2015; Zhang and Cohen, 2017).

For screening platforms it is important to find a balance between improved physiological relevance of the system used, and increased complexity of the screen. However, with some imaginative automation and analysis systems it could be possible to perform high-throughput screens on physiologically relevant 3D co-cultures of patient-derived cells to more accurately identify new therapies for neurological diseases.

A major benefit of using iPSC-derived neurons for drug screening is the possibility of using cells with the exact genetic profile of patients with a particular disease. This provides an opportunity to test target compounds on a panel of different patients, for example with familial or sporadic disease, or patients with different genetic mutations. Using a panel of cells from a variety of patients could enable the identification of different compounds that are beneficial for different subgroups of patients. It may also be possible to identify novel subgroups of patients, classified as responders and non-responders to particular compounds. This system of using a panel of different patient lines could therefore facilitate precision medicine for neurological diseases. Only a small number of the screens cited here (five) have used patient lines, perhaps in part owing to limited access to patient cells. To overcome this, consortia are currently generating and banking large numbers of iPSC lines from patients with different diseases for the use of the academic community and the pharmaceutical industry. These include: StemBANCC, HipSci and EBISC in Europe, GAiT, IPSCORE and the CRIM iPSC Repository in the USA, the MNI depository in Canada and the RIKEN Bioresource Center Cell Bank in Japan. These large collaborative projects will

improve the availability of iPSCs, increasing the use of these cells in research and increasing the likelihood of using them in drug screening. Wide availability of large numbers of patient iPSC lines will also enable researchers to perform screens on a panel of lines from multiple patients, possibly with different mutations. This could help identify subgroups of patients who respond differently to different treatments that could then inform clinical trial design. It is important to note that many of these banks involve partnerships between academia and industry. These partnerships are an important way of combining expertise in academia and industry to develop new technologies.

4. Summary and Conclusions

Many cell lines have been made from patients with different neurological diseases, but robust phenotypes still need to be established that can be screened against. Some screens have been performed using stem cell-derived neurons, as detailed above, showing that it is possible to use these cells for drug screening applications. However, it is still more favourable to use other cell types, which are easier to grow for large-scale screens, and then use iPSC-derived cells for confirmation of positive hits. Technological improvements are making complicated screens more feasible, but cell production needs to be easier and more reliable before they are commonly used. Interestingly, many studies using iPSC-derived neurons opted to use immature neuronal stem cells rather than maturing cells for longer. This reduces the time taken to grow the cells, and these immature cells may also be easier to use in screening assays, however it may be necessary to use more mature cells to reveal disease phenotypes. Recent reports demonstrate a real interest in using these cells in drug screening, however it may continue to be a small part of the drug development process. This technology is clearly accessible, and useful, but its universal use in high-throughput screening may not be favourable until the generation of mature neurons is more efficient and more reliable, if not quicker. Therefore, these cells may be favoured as a secondary screening method for the time being, providing an additional step in the drug discovery pipeline.

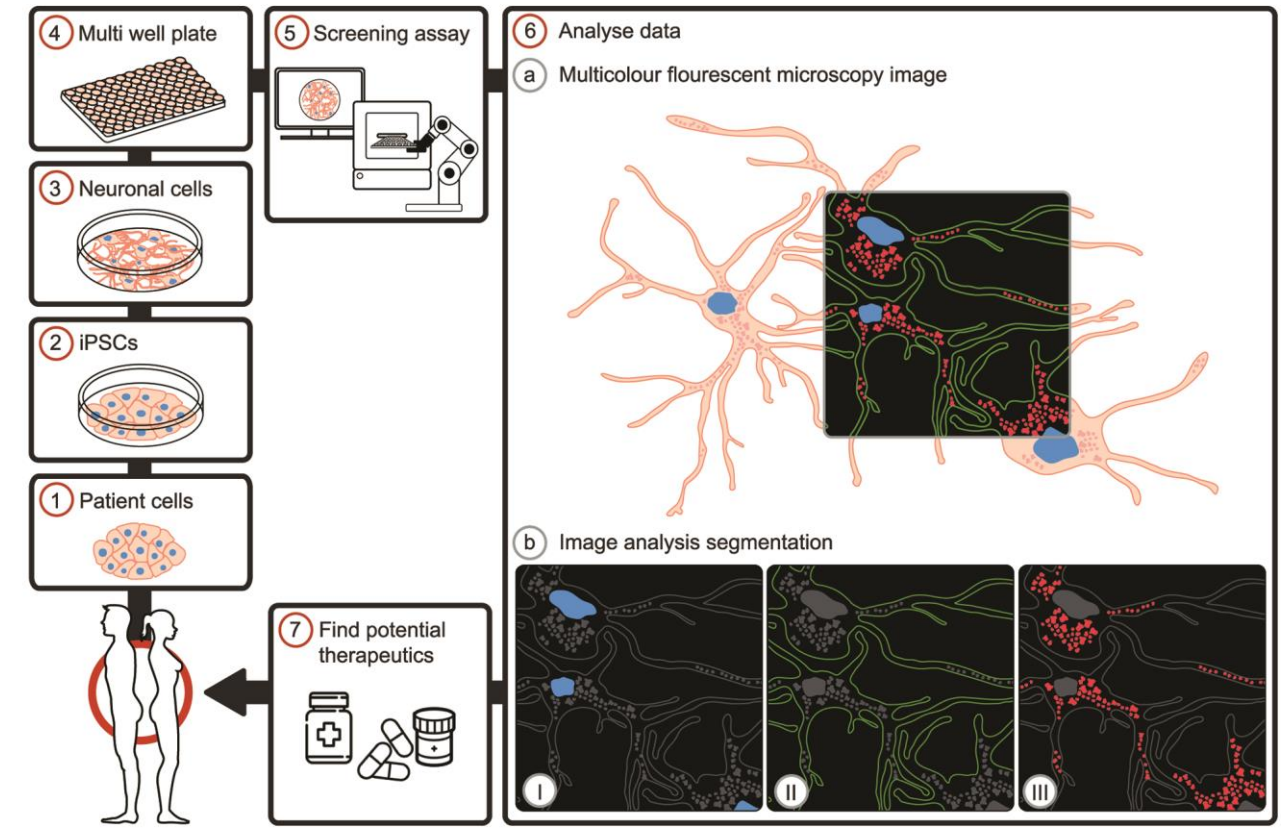

Figure 1 Workflow of high-content screen using iPSC-derived neurons

Somatic patient cells are transformed into iPSCs and then into neuronal cells before being transferred to multi well plates to perform the screening assay (1-4). Multicolour fluorescent microscopy images are acquired automatically using a screening microscope and these images are then segmented using image analysis software (5-6)**.** Data analysis then identifies disease-relevant cellular phenotypes and responses of these phenotypes to test compounds to identify potential therapeutics (7).

Target of screen	Cell type used	Compounds screened	Hit criteria	Outcome of screen	Industry involvement	Reference
Neurite outgrowth	Commercial human iPSC-derived neural precursors (Clonetics)	899 commercially available compounds and 25,000 proprietary compounds (data not reported)	Compounds that increased neurite outgrowth measured by high content image analysis in Tuj1 positive and GFAP positive cells	12 confirmed hits in Tuj1+ cells: 2-chloro-ATP, 1,3-dipropyl-8-*p*-sulfophenylxanthine, quinine, CGP20712A, ambenonium, bethanechol, BayK8644, pergolide, 1-aminocyclohexane-carboxylic acid HCl, U50488, ceramide, and bicuculline and 2 confirmed hits in GFAP+ cells: palmitoylcarnitine and Y27632.	MSD	Richards et (2006)
Neurotoxicity	Human ESCs and human ESC-derived neural stem cells	720 FDA-approved drugs	Compounds that caused a reduction in cell viability, measured by changes in ATP levels	9 drugs identified as toxic to NSCs: pirenzepine HCl, amiodarone HCl, selamectin, clofoctol, perhexilline maleate, griseofulvin, chloroactoxyquinoline, menadione and hexetidine	None	Han et al. (2009)
SMA	Human iPSC-derived motor neurons	200,000 compounds	Compounds that increased SMN gem number in SMA patient cells expressing motor neuron markers, measured by high content image analysis	Not published	iPierian	Reviewed in Grskovic et (2011) , screen not published
Parkinson's disease	Human ESC-derived	2000 compounds	MEF2 luciferase reporter plasmid	6 hits; Isoxazole tested further	None	Ryan et al. (2013)

	neural precursor cells					
Alzheimer's disease	Commercial human iPSC-derived neurons (iCell)	GSK proprietary compound library (several hundred compounds)	Compounds that protected against Aβ1-42 toxicity, measured by luminescent cell viability assay	19 hits; one was a Cdk2 inhibitor	GlaxoSmithKline, Cellular Dynamics International	Xu et al. (2013)
ALS	Human iPSC-derived motor neurons	1757 compounds	Compounds that reduced TDP-43 aggregates in sporadic ALS cells, measured by high content image analysis	38 hits reduced percentage of cells with aggregates. Confirmed hits classed as cyclin-dependent kinase inhibitors, c-Jun N-terminal kinase inhibitors, Triptolide and cardiac glycosides	iPierian	Burkhardt e al. (2013)
Neurotoxicity	Human iPSC-derived neural stem cells & rat cortical neurons	2000 compounds	Compounds that affected cell viability, measured by MTT assay	43 compounds identified for follow up study including sanguinarine, pyrithione zinc, amiodarone, digoxin, lanatoside C and ouabain	None	Malik et al. (2014)
Alzheimer's disease	Human iPSC-derived cortical neurons with trisomy of chromosome 21	Prestwick Chemical library, 1200 compounds	Compounds that reduced Aβ38, Aβ40 or Aβ42 (increased in TS21 cells), measured by immunoassay	55 compounds identified; validated hits included (R)-flurbiprofen and ivermectin	None	Brownjohn e al. (2017)
Alzheimer's disease	Human iPSC-derived cortical neurons	1258 pharmaceutical compounds	Compounds that altered Aβ40 and Aβ42 production, measured by ELISA. BSI-IV used as a positive control for Aβ40 and JNJ-	27 hits after secondary testing, 6 lead compounds chosen: bromocriptine, cilostazol, cromolyn, fluvastatin, probucol and topiramate	None	Kondo et al (2017)

			40418677 as a positive control for Aβ42 alteration			
Studies using mouse cells						
Cognitive impairment	Mouse ESC-derived neurons	Over 2.4 million compounds	Compounds that did not evoke a calcium signal in the absence of AMPA and enhanced calcium signal with AMPA	5911 screening hits, 37 confirmed hits, including known AMPA potentiators and 2 novel molecules, one named CE-382349	Pfizer, ArunA Biomedical	McNeish et (2010)
Motor neuron disease	Mouse-ESC-derived motor neurons cocultured with ESC-derived astrocytes.	10,000 compounds	Compounds that inhibited toxicity induced by BV2-derived microglia determined by total neurite length per cell	37 compounds from initial screen, 26 confirmed including one inhibitor of nitric oxide synthase and Nrf2 activators	None	Hoing et al. (2012)
ALS	Mouse ESC-derived motor neurons (wildtype and $SOD1^{G93A}$)	5000 compounds at 3 concentrations	Compounds that increased the number of surviving motor neurons after trophic factor withdrawal and preserved cell morphology. Trophic factor removal induced 80% death, Cycloheximide (protein synthesis inhibitor) used as a positive control	Total number of hits not reported; 9 compounds that inhibit protein or DNA synthesis, MMP inhibitor 1,10-phenanthroline monohydrate, cannabinoid receptor agonist CP55940, calpain inhibitor MDL 28170, ligands for neurotransmitter receptors A77636 hydrochloride and 3-Tropanylindole-3-carboxylate methiodide, calcium agonist FPL-64176, and kinase inhibitors including Kenpaullone	None	Yang et al. (2013)

Table 1: Details of screens performed using iPSC- and ESC-derived neurons.

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