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Drug discovery using neurons derived from patients:

Studying Human Disease Using Human Neurons

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Abstract

Utilizing patient derived cells has enormous promise for discovering new drugs for diseases of the nervous system, a goal that has been historically quite challenging. In this review, we will outline the potential of human stem cell derived neuron models for assessing therapeutics and high-throughput screening and compare to more traditional drug discovery strategies. We summarize recent successes of the approach and discuss special considerations for developing human stem cell based assays. New technologies, such as genome editing, offer improvements to help overcome the challenges that remain. Finally, human neurons derived from patient cells have advantages for translational research beyond drug screening as they can also be used to identify individual efficacy and safety prior to clinical testing and for dissecting disease mechanisms.

Drug discovery for neuronal disorders - why traditional approaches have failed

A patient diagnosed with Parkinson's disease (PD) in 1967 would have been introduced to the first symptomatic treatment, namely dopamine replacement therapy with Levodopa. A PD patient diagnosed in 2015 would receive the same treatment. Even as basic neurobiology research has yielded tremendous advances in understanding of the brain, disorders of the central nervous system (CNS) remain pervasive, debilitating, and largely without effective therapeutic options. PD is only one example of a neurological disorder but CNS diseases, which include neurodevelopmental, psychiatric, and neurodegenerative disorders, represent a large and growing disease burden, with few effective treatments (Bloom, 2011; Insel et al., 2013; World Health Organization [WHO], 2006). Indeed, the prevalence of neurological disease is increasing due especially to the aging population. There is an urgent need to develop new drugs that can do more than treat the symptoms of a disease. Rather, we need to identify therapeutics that can cure disease, or at least halt disease progression. It is necessary to gain a detailed understanding of the mechanisms underlying disease in order to develop effective intervention strategies.

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The identification of small molecules to treat disease is a labor-intensive, expensive, and risky process. It often begins with a high-throughput screen (HTS) of small molecules on a target protein or cellular phenotype of interest. Hits identified at this stage are iteratively modified through chemical analog synthesis and tested in secondary and tertiary assays to assess efficacy and safety. Lead compounds with desirable effects in all assays are moved forward toward clinical testing in humans. Despite growing clinical need and striking advances in other fields, there have been few drugs approved in recent years for CNS indications. In fact, CNS drugs have almost half the chance of approval as all other drugs, with nearly twice the cost of development (Hurko and Ryan, 2005; Kaitin, 2012; Milne, 2014). These statistics reflect the underlying problem that CNS drug discovery is marked by unique scientific challenges (Dawson et al., 2011; Enna and Williams, 2009; Hurko and Ryan, 2005; Pangalos et al., 2007).

First, the nervous system is complex. In CNS disorders, often only a restricted region of the brain or subpopulation of neurons is disturbed, but the underlying mechanism of this specificity and the best way to optimize therapeutic strategies are not known (Enna and Williams, 2009; Insel et al., 2013; Tye and Deisseroth, 2012). The genetic underpinnings and pathology of most neurological disorders do not explain the cell-type specific differences that mediate the catastrophic effects of each disease. It remains unclear, for example, why in Alzheimer's disease (AD) neurons of the entorhinal cortex carry the heaviest burden of plaque and tangle formation (Duyckaerts et al., 2009; Gomez-Isla et al., 1996). In addition, a number of the FDA approved drugs for CNS indications have pleiotropic biological activities and numerous attempts to develop target-selective CNS drugs have been ineffective in humans (Roth et al., 2004). Thus, the traditional drug discovery approach of molecular-target based screening has been mostly ineffective. Additionally, since neurons are post-mitotic and very diverse, generating large quantities of the correct neurons that are necessary for any type of neuron-based HTS efforts remains difficult. Finally, animal models of brain disorders have shown little ability to predict efficacy in humans, making the transition to clinical trials even more risky (Hurko and Ryan, 2005; Kola, 2008; Pangalos et al., 2007; Schmid and Smith, 2007). While rodent models of neurological disorders can recapitulate some aspects of disease, important differences remain and can severely limit their utility. For example, in Spinal Muscular Atrophy (SMA), an early onset degenerative disease of motor neurons caused by mutation of the Survival of Motor Neuron 1 (SMN1) gene, disease severity is determined by the number of copies of the SMN2 gene, which is not present in mice, (Monani et al., 2000). The standard mouse model of a familial form of Amyotrophic Lateral Sclerosis (ALS), a late-onset motor neuron degenerative disorder, was generated by overexpressing a mutated form of the human superoxide dismutase (SOD1) gene (Gurney et al., 1994). In spite of the fact that these mice have ALS-like symptoms, positive preclinical mouse results have not translated into humans, as evidenced by 30 large, unsuccessful, clinical trials (Gordon and Meininger, 2011). In both the SMA and ALS models, significant differences between the human and animal form of the disease exist, including the mechanism of death and the high level of expression of the human protein required. While for other disease areas (oncology, for instance), it is often possible to compare animal findings with characteristics of primary tissues of human patients, this is not feasible for many neurological diseases. For example, the most affected

cell type in the progression of Parkinson's disease (PD) is the dopaminergic neurons found in the midbrain of patients in the substantia nigra (Fearnley and Lees, 1991). As these neurons are lost during the course of disease, post mortem studies are based on tissues lacking the cell type of most interest, and tissue biopsies cannot be obtained from living patients.

Using neurons derived from human pluripotent stem cells (hPSCs) offers advantages over traditional drug discovery methods. Human PSC-derived neurons provide an unparalleled opportunity to study the pathogenesis of affected cell-types in a controlled environment. In particular, it is possible to differentiate hPSCs into specialized neuronal populations, such as motor neurons or dopaminergic neurons. hPSCs allow for the design of HTS assays using large numbers of specific neuronal types, with the goal of identifying modulators of the relevant disease phenotype rather than focusing on a single target or tangentially related cellular process. In principle, studying the genetics, biochemistry, and cell-biology of CNS disease in human cells can provide important insight beyond animal models.

In this review, we discuss current attempts to employ stem cells for CNS drug discovery and discuss the potential of applying this technology in the future. To highlight the promise of the approach, we focus on recent efforts to use hPSCs to model disease, as the basis for validating new therapeutic approaches, and for HTS assays. We also discuss current barriers to developing hPSC-based assays and compare to more traditional approaches to drug discovery. Beyond screening, hPSC-derived neuron models offer the potential to dissect disease mechanisms, assess safety and toxicity, and develop more effective clinical strategies.

Acquisition of hPSCs with genotypes of interest

By definition, hPSCs have the ability to self-renew indefinitely in culture while retaining their ability to differentiate into most and potentially all cell types of the human body (Yu and Thomson, 2008). The reprogramming of somatic cells into a pluripotent state through overexpression of a cocktail of transcription factors, achieved by Takahashi and Yamanaka, is one of the biggest advances in modern biology (Takahashi and Yamanaka, 2006). Cells reprogrammed in this way are termed induced pluripotent stem cells (iPSC). The field of reprogramming has been advancing at an incredible speed and today it is the norm to derive cells from any patient using integration free methods like adenovirus (Zhou and Freed, 2009), Sendai virus (Fusaki et al., 2009), episomal vectors (Yu et al., 2009) or through delivery of modified RNA (Warren et al., 2010). Because hPSCs maintain the ability to undergo differentiation to all cell lineages, they provide a unique opportunity to study diseases in the most affected cell types in vitro. This is of particular importance for CNS diseases where there are no other means for isolating the affected cells. Patient-specific iPSCs have been generated from hundreds of donors representing most CNS diseases and detailed reviews on specific disease models, such as motor neuron disease, frontotemporal dementia, AD, autism spectrum disorders, psychiatric disorders and schizophrenia, Huntington's disease, Niemann pick's disease and Fragile X syndrome can be found in this special review edition (Sarkar et al., Gerhardt et al., Lee et al., Mattis et al., Beltrao-Braga et al., Sullivan et al. in this issue).

Despite this tremendous progress, many hurdles have to be overcome before the full potential of hPSCs is realized. One issue that has garnered attention recently is the variability that has been found between iPSCs derived from different patients and even from single individuals (Bock et al., 2011). One important limitation of hiPSC studies has been the inability to perform experiments under genetically defined conditions where cell lines are genetically matched (isogenic) with the exception of the mutation of interest. This is particularly relevant for late onset disorders with complex genetic contributions, as the expected disease phenotypes can be subtle, and disease manifestation may rely on interplay among other genes, pathways and environmental factors.

The utilization of genome editing technology overcomes some of these limitations. Further, it circumvents the difficulties of finding patients with desired mutations and the lengthy process of generating hiPSCs. Genome editing in hPSCs was achieved through zinc finger nucleases (Urnov et al., 2005) and transcription activator-like effectors (TALEs) (Ding et al., 2013a; Miller et al., 2011). The most recent and most successful genome editing technology is the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system CRISPR (Cong et al., 2013; DiCarlo et al., 2013). Many labs have reported the use of the CRISPR system to generate knockout mutations of genes of interest or to introduce specific mutations through homologous recombination (sgRNA) (Ding et al., 2013b; Musunuru, 2013). A recent study, focusing on the LRRK2 gene implicated in PD, demonstrated that the isogenic model can be superior to patient iPSC models and showed that it can be highly advantageous especially for complex diseases. The authors derived control IPS cell lines from unaffected donors as well as from 2 patients homozygous for the G2019S mutation in the LRRK2 gene. They corrected the G2019S mutation and created isogenic lines. They also edited one of the original unaffected control cell lines to harbor the G2019S mutation. All lines were differentiated into midbrain dopaminergic neurons and phenotypically characterized. They found that only isogenic lines differing at just the LRRK2 site, but not comparisons of nonisogenic control and patient derived LRRK2–iPSCs, showed statistically significant differences in a variety of disease relevant phenotypic assays. Thus potential phenotypes were masked by the inherent variability in nonisogenic cultures. (Reinhardt et al., 2013).

Nonisogenic iPSC models might overcome the problem with variability if enough disease and control cell lines are used. On the other hand, patient-derived cell lines provide an advantage when considering sporadic forms of disease, where no causative genes have been identified or where disease development might rely on the dysfunction of several genes or gene networks. Such complex genotypes would best be captured by deriving iPSC lines from afflicted patients. The choice of the culture system will heavily depend on the questions being asked as well as on available knowledge about genetic contributions or disease mechanism. Figure 1 illustrates the general approach to developing and utilizing hPSC-based disease models.

Patient derived neurons as disease models

One big hurdle to disease modeling using hPSCs is the availability of protocols that can efficiently and reliably yield the type of cells that are affected in a specific disease. However

neurons were among the first cells that were successfully obtained through morphogen based differentiation strategies (Munoz-Sanjuan and Brivanlou, 2002; Reubinoff et al., 2001; Stern, 2006; Wichterle et al., 2002; Zhang et al., 2001). Ever since, many protocols have been developed to derive specific neuronal cell types. In recent years there has been a switch from morphogens to small molecules, and with a gain in our understanding of the developmental pathways came increases in specificity and efficiency of the protocols. Generally, all neuron differentiation protocols follow a neural induction step through the inhibition of SMAD pathways (Chambers et al., 2009) followed by patterning of neural progenitors along the rostro-caudal and dorso-ventral axes using a small set of morphogens or small molecules that inhibit or activate the morphogenic pathways for example protocols utilizing Nodal/Activin, WNT and BMP pathways have been devised to derive diverse populations such as dopaminergic neurons, excitatory and inhibitory cortical neurons or pyramidal neurons (Chambers et al., 2009; Espuny-Camacho et al., 2013; Hansen et al., 2011; Kriks et al., 2011; Smith et al., 2008).

There exist a great variety and complexity of subpopulations of neurons in the nervous system and one difficult problem is to devise a differentiation strategy using morphogens that results in a specific neuronal sub-type. A recent approach to derive specific neurons of interest is the programming of hPSCs into neuronal cell types through the overexpression of cell type specific transcription factors, and many studies have shown that this programming approach can greatly enhance the efficiency and the specificity of the differentiation protocols. For example, many good protocols exist to differentiate hPSCs into motor neurons. A recent study has shown that overexpression of FOXP1 in differentiating motor neurons programs these cells toward bona fide lateral motor column (LMC), a subset of motor neurons that innervate limb muscles at cervical and lumbar levels (Adams et al., 2015). In principle, utilizing this general strategy, virtually any desired cell fate could be derived.

Lineage reprogramming, the overexpression of specific transcription factors in adult cells has been proposed as a fast and efficient alternative method to generate patient-specific induced neuronal (iN) cells that could be used in drug development or screening efforts (Vierbuchen et al., 2010). This approach has been successfully applied to derive for example specific neuronal populations such as dopaminergic neurons (Caiazzo et al., 2011; Liu et al., 2012), motor neurons (Son et al., 2011) striatal medium spiny neurons (Victor et al., 2014) and nociceptive neurons among others (Wainger et al., 2015). Very recently it has been shown that direct lineage reprogramming can be achieved by small molecules alone by disrupting the somatic transcriptional program and activating neuronal-specific genes (Hu et al., 2015; Li et al., 2015). Unlike in hPSCs models, the source cell types in this approach typically have poor proliferative ability and thus cell numbers are limited. However lineage reprogramming can also lead to proliferative cell types such as neural stem cells (Han et al., 2012; Kim et al., 2011; Meyer et al., 2015; Ring et al., 2012; Thier et al., 2012) that could after proliferation be terminally differentiated into neurons. Today it remains to be seen whether or not iN cells will be utilized in drug screening.

Other problems of hPSCs derived neurons involve the maturity of the derived cells as well as the problem of aging of the cells which might be necessary to model late onset diseases such

as PD, AD, and Huntington disease (HD). This is discussed in the accompanying review article on artificial aging (Cornacchia et al. in this issue). In Hutchinson-Gilford progeria syndrome (HGPS) the production of progerin a short pathogenic version of lamin A protein causes accelerated aging and early death. A recent paper presented the surprising finding that progerin treatment of PD-iPSC derived dopamine neurons revealed a disease phenotype that was not seen without the artificial aging treatment. Probably other manipulations that lead to cellular stress could also uncover specific phenotypes that are necessary to establish disease phenotypes (Miller and Studer, 2014; Miller et al., 2013).

A variety of studies show that patient derived neurons can serve as disease models. Drugs that have been identified by more traditional approaches have been assessed in hPSC derived neuronal disease models, including Gaucher's disease (Tiscornia et al., 2013), Niemann Pick type C (NPC) (Soga et al., 2015; Swaroop et al., 2012), and SMA (Naryshkin et al., 2014). In these studies, patient iPSC derived neuron experiments add confidence to drug candidates that have been assessed in other cellular assays and in animal models. This is especially important since neurons are often more sensitive to toxic effects of drugs. For NPC, which is caused by mutation of the lysosomal cholesterol binding proteins NPC1 or NPC2, assays that measure neuronal viability and cholesterol accumulation in iPSC derived neurons were used to evaluate drug candidates that had previously shown efficacy in fibroblasts and mouse models (Soga et al., 2015; Swaroop et al., 2012). These experiments demonstrated the utility of the therapeutic strategy in human neurons. In fact, one compound, hydroxypropyl- β -cyclodextrin, was found to be more potent and efficacious in NPC1 mutant neural stem cells derived from iPSCs than in fibroblasts from the same patient (Yu et al., 2014).

For schizophrenia (SCZD), a mental disorder characterized by a number of neurological changes, researchers assayed the effects of Loxapine, an anti-psychotic drug, on patient derived neurons (Brennand et al., 2011). In this case, the SCZD iPSCs were differentiated into mixed neuronal cultures that included a broad array of sub-types and assayed for neural connectivity. Decreased neuronal connectivity was found in the SCZD patient cells, and this defect was partly rescued through administration of Loxapine. The exact neuronal sub-types affected in SCZD have not been well characterized, although dopaminergic, glutamatergic and GABAergic neurons have all been implicated. Thus, this disease is perhaps well-suited to modeling using these mixed cultures.

Other CNS diseases require more selective differentiation into specific neuronal subtypes. For example, SMA is a neurodegenerative disorder that mainly affects motor neurons, leaving the rest of the nervous system intact. A recent study identified small molecule splicing modulators that act on the human SMN2 gene to increase SMN protein levels (Naryshkin et al., 2014). These splicing modulators were identified through a targeted biochemical screen, but were shown to be effective at increasing SMN protein levels in motor neurons derived from SMA patient iPSCs. In complementary experiments, these splicing modulators significantly extended the lifespan in the mouse model of the disease that expresses the human SMN2 transgene. However, not all effects of drug candidates in human neurons reflect results seen in other model systems. For studies of AD, the effects of gamma secretase modulators were markedly different when tested on iPSC derived neurons carrying presenilin-1 mutations compared to APP-overexpressing cell lines and APP

transgenic mice (Liu et al., 2014): in the iPSC-derived neurons, the A β 42:40 ratio was affected, whereas in other models, the total A β levels were altered. As iPSC derived neurons reveal cell-type and disease specific phenotypes, it will be increasingly important to evaluate the effects of drug candidates in these models in parallel to other approaches. Human derived neurons offer the ability to assess the effects of a drug on the cells most relevant to treatment of the disease.

Disease Model in a dish, Considerations for Phenotypic Assays – Examples of drug screens

Traditionally HTS approaches have involved biochemical assays, or made use of established cellular systems like Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells, accompanied by the exogenous expression of the molecular target and a reporter system. This targeted approach is very scalable with a strong signal-to-noise ratio, and it certainly has led to the development of drugs that have made it into the clinic (Swinney and Anthony, 2011; Swinney, 2013). Yet the target-based approach is poorly suited to capturing the relevant mechanisms of complex diseases so often present in CNS disorders, and it has led to failures in recent clinical trials (Dawson and Carragher, 2014). Between 1999 and 2008, 75 new drugs, listed as first-in-class drugs with new molecular mechanism of action were approved by the FDA. Of these, 75 drugs 28 (21%) were discovered in phenotypic versus 17 (12.75%) in target-based screens (Swinney, 2013). This is particularly striking as target-based screens are simpler and thereby more common. The biggest critique concerning target-based screening is related to the exogenous expression of the molecular target leading to artificial results/leads and subsequently high rates of failure in clinical trials of drug candidates. hPSCs can serve as a superior platform for reporter-based screening as they allow the study of a target in a much more relevant cellular context. A HTS reporter assay was developed for Familial Dysautonomia (FD), which is caused by IKBKAP mis-splicing (Lee et al., 2012). In this screen, IKBKAP transcription using quantitative RT-PCR was measured in neural crest precursors derived from iPS cells from FD patients (Lee et al., 2012). One of the hits identified from this screen of 6,912 compounds was shown to act by modulating intracellular cAMP levels and PKA dependent CREB phosphorylation in secondary assays.

As disease models, a large value of hPSCs is for their use in phenotypic screens. While this approach holds great promise to drug discovery, many challenges have to be overcome independently for each disease, namely the differentiation of the neurons into the affected cell type, the establishment of a relevant disease phenotype, and the development of a meaningful and reproducible readout. Robust and specific phenotypes have to be observed in order to achieve a large enough signal to noise ratio for screening purposes.

The key determinants for designing a HTS with hPSCs are the capacity for differentiation, scalability and phenotypic discovery (Figure 1). Once these challenges have been overcome, several avenues can be taken to develop a phenotypic assay that is compatible with testing chemical or small compound libraries. Similarly, screens that disrupt gene expression in the

phenotypic assay, using genetic libraries (siRNA's, miRs, expression of ORF's), can be valuable for developing new therapeutic approaches.

Multiple cellular phenotypic assays have been developed with the goal of screening small molecules for effects on motor neurons derived from ALS-patient iPSCs. These cells display an array of phenotypes compared to motor neurons derived from healthy control iPSCs, which might be linked to the neurodegeneration observed in patients. ALS patient motor neurons have reduced neurite length, abnormal electrophysiology, and increased sensitivity to arsenite (Egawa et al., 2012; Kiskinis et al., 2014; Wainger et al., 2014). Another pathological feature of ALS is the aggregation of Tar DNA-binding protein 43 (TDP-43), which has been described in post mortem tissue from sporadic ALS patients. Neurons derived from sporadic ALS patients recapitulate this phenotype, which can be observed as insoluble aggregates biochemically or visualized as punctate accumulations with antibody staining (Burkhardt et al., 2013; Egawa et al., 2012). Both cell number after 16 hours of arsenite treatment and TDP-43 puncta per cell have been developed into high-content screening assays using ALS patient neurons (Burkhardt et al., 2013; Egawa et al., 2012). So far these assays have only been applied to a small number of compounds, but molecules that reverse the ALS-specific phenotypes have been identified. Additionally, a cell survival screen in motor neurons derived from both mouse wild-type and SOD1 mutant ES cells of approximately 5,000 compounds identified kenpaullone, an inhibitor of GSK and HGK kinases. In follow-up assays, kenpaullone promoted survival of iPSC derived motor neurons from ALS patients. Importantly, the effect of kenpaullone was larger than dexamipexole and olesoxime, compounds that have failed in ALS clinical trials (Yang et al., 2013). This highlights an advantage of phenotypic screens in uncovering disease relevant signaling pathways, by remaining agnostic to the molecular target and focusing on a readout that is directly relevant to the pathophysiology of the disorder.

Advances in technology have enabled screens that include elements of reporter-based and phenotypic assays. Many excellent reviews have been written on screening technologies, miniaturization, library management and related technologies (Janzen, 2014; Mayr and Bojanic, 2009). In this review we will focus on some of the most recent and successful examples conducted in the field and on novel developments that could be used to effectively screen in hPSCs derived neuronal cells. A schematic of different screening approaches is shown in Figure 2. These include screening technologies that have already been successfully employed in hPSC disease models, as well as novel techniques that have been used as assays in hPSC disease models but not yet in screens. For example, high-content screening can be utilized to not only query at a cellular level, but even subcellular compartments. This can be achieved in 384 well formats utilizing high content screening systems like the Opera or Operetta (Perkin Elmer) or ArrayScan (Thermo Scientific). A wide variety of parameters can be assessed through high-content imaging, including impaired morphology, neurogenesis, migration, synaptic dysfunction, increase or decrease in levels of protein, toxic protein accumulation and selective neuronal cell death. These parameters can be assayed through immunocytochemistry or dye staining and are mostly carried out as end-point assays. In addition, genetic reporters, such as adding a luminescent or fluorescent tag to a protein of interest can also be used, and have the added advantage that they can give kinetic, live-cell image information. In general, genetic reporters greatly enhance the application and

scalability of screens. High content imaging and reporter gene assays are the primary methods used for the handful of iPSC derived neuronal cells screens that have been executed to-date, summarized in Table 1.

For SMA, the therapeutic strategy is to increase levels of the SMN protein, and thus screens have been developed to measure SMN levels in tandem with motor neuron survival in human motor neurons derived from SMA patient iPSCs using high-content imaging (Cherry et al., 2014). Importantly, SMA patient derived motor neurons recapitulate many aspects of the disease, including low SMN levels and premature death of motor neurons. It will be interesting to compare the hits identified from screens of SMA patient-iPSC derived neurons with hits resulting from screening the same library of compounds on human patient fibroblasts and mouse SMA ES cell derived motor neurons (Makhortova et al., 2011). If different classes of compounds emerge, it may serve to highlight the advantage of screening in human neurons derived from iPSCs, which require substantially more investment and effort than traditional methods. Fragile X Syndrome (FXS), an intellectual disability disorder, is another disease where low levels of the mutated protein, in this case FMRP, lead to a pathological effect. A high-content imaging based screen to measure FMRP levels in the cytoplasm, as well as other subcellular compartments has been performed on more than 50,000 compounds in neural progenitor cells derived from FXS patient iPSCs (Kaufmann et al., 2015).

Screens have also been developed to identify compounds that activate pathways implicated in disease. For example, Huntington's disease (HD), a neurodegenerative disease caused by a genomic repeat in the Huntington gene, leads to death of neurons in the striatum, and a molecular feature is increased expression of the repressor element-1 silencing transcription factor (REST). A cell-based screen was developed to identify inhibitors of REST activity using RE1-containing reporter plasmids in neural cells derived from human iPSCs. 6,984 compounds were tested in this assay, and the most potent compound was shown to also be effective in neural cells derived from iPSCs of a HD patient, but not in the medium spiny neurons, which degenerate in the disease, and in a HD mouse model (Charbord et al., 2013). In another example, researchers aimed to identify new strategies for treating neuropsychiatric disorders by focusing on the WNT/ β -catenin signaling pathway (Zhao et al., 2012). In this case, researchers utilized a luciferase reporter of TCF/LEF expressed in neural stem cells derived from wild-type ES cells. For PD, a careful comparison of α -synuclein mutant iPSC derived dopaminergic neurons revealed MEF2/PGC1 α as an important transcriptional regulator in disease pathogenesis, and a screen in wild-type hPSC derived neural progenitor cells assaying MEF2 luciferase activity was performed to identify potential therapeutics (Ryan et al., 2013).

Outlook and Future Directions

Screening in hPSC derived neurons is still in its infancy and technological improvements will be needed to fully realize the potential of drug discovery in hPSCs. Two technologies that offer promise for future neuronal iPSC based screens are imaging based readouts of electrophysiology and next generation sequencing (NGS). A recent development merges high content imaging with electrophysiological assessment of the cells. To this end, calcium

imaging can be used through overexpression of a calcium indicator such as GCaMP. To our knowledge no screens using hPSC derived neurons has been performed but the feasibility has been shown in other model organisms (Larsch et al., 2013) or cell lines (Honarnejad et al., 2013). Another approach to consider relates to the use of multi-electrode arrays embedded in cell culture dishes. For instance one group showed that hyperexcitability of SOD1^{A4V} IPS derived motor neurons (Wainger et al., 2014), a phenotype that precedes the death of these neurons, could potentially be exploited as a therapeutic target. High throughput electrophysiology platforms like the PatchXpress and IonWorks have also been developed (Castle et al., 2009). Approaches like these offer the unique opportunity to screen for compounds that affect the unique electrophysiological properties of distinct neuronal populations that may affect diseases development and progression.

NGS has dramatically improved capabilities and led to a sharp decline in costs of DNA sequencing. NGS has been used in many applications like whole genome sequencing, resequencing, single nucleotide polymorphism (SNP), and RNA (mRNA, noncoding) profiling (Yadav et al., 2014). This has led to remarkable insights into the molecular biology and pathobiology of cells. For this review, we focus on NGS as a novel tool to detect hits in chemical screening. NGS can capture the expression of the whole transcriptome, or, with a targeted library design, will capture the expression of a defined subset of genes. This approach, in conjunction with barcoded or tagged multiplexed libraries, allows for high-throughput hit detection without the need for difficult image based-assays or even a specific cellular phenotype. This offers a large advantage as a relatively simple approach with an unbiased means for scoring hits. Small molecules, siRNA, miRs and ORF clones can be used to treat the cells and the read-out captures gene changes in the whole well. One group utilized this approach in a functional genomic screen that revealed pathways involved in neurodevelopment and neurodegeneration. The authors used pooled shRNA libraries in hESCs, differentiated the cells into the neuronal lineage and utilized PSA-NCAM expression as an early neural differentiation marker. Depletion and enrichment of shRNAs was measured via deep sequencing (Zhang et al., 2013).

We discussed earlier the role that CRISPR plays in genome editing, but it is important to note that CRISPR can also be used as a novel tool for screening in hPSCs in conjunction with NGS. Recently CRISPRs have been utilized in functional genomics screens. Shalem et al. generated a genome-wide CRISPR knockout library and used it to understand the resistance of melanoma cells with the BRAF V600E mutation to the cancer drug vemurafenib (Shalem et al., 2014). They identified 2 genes previously reported to result in resistance but also several novel genes. Another group generated a CRISPR library containing 73000 sgRNAs and performed two successful loss-of function screens in human cells (Wang et al., 2014). Genome wide loss-of function screens have previously been performed using RNAi but only relative knockdown is achieved, off-target effects are high and the screens are limited to transcribed regions. The CRISPR approach, on the other hand, can be used for regulatory regions as well. It is important to note that this genome wide screening method can be conducted in a pooled manner. All sgRNAs are transfected or transduced in the hPSC disease model; the read-out relies on a defined phenotype. Comparison of the abundance of sgRNAs in the cells with the desired phenotypes via massively parallel sequencing allows for the hit detection. Applying this powerful

technology to iPSC derived neurons could be quite informative in particular for the mechanistic understanding (Figure 2). Further dCas9 can be fused to other effector domains like transcriptional activators to yield genome wide gain of function studies or epigenetic modifiers that would allow us to screen for the effects that for example methylation states have on disease development (Hsu et al., 2014). The potential applications are discussed in depth in a recent review (Shalem et al., 2015).

It is important to note that there are many steps to drug discovery after HTS and hit identification, including evaluation in secondary assays, safety and toxicity assessment, and developing a clinical strategy. Neurons derived from hPSCs can play an important role in each of these stages. The utilization of secondary assays for lead optimization is a critical step toward identifying the therapeutic that will ultimately be tested in man. While HTS assays mostly focus a few critical parameters, careful and detailed analysis of the diverse phenotypes displayed by patient neurons compared to control can help identify compounds with optimal properties. For example, in ALS, it may be necessary to identify compounds that not only prevent TDP43 aggregation but also correct other cellular phenotypes, such as reduced neurite length and altered electrophysiology.

Safety is a critical issue during drug development. Despite the extensive toxicity testing in rodents and other animal models that must be performed prior to clinical trials, more than half of drugs fail due to safety concerns (Cook et al., 2014). The ability to predict unwanted CNS effects is especially poor. While it may never be possible to completely predict the safety profile of a clinical candidate, using human cells to de-risk this process may be beneficial. In fact, comparison of effects in neurons from different species, including rat and human, has revealed species-specific toxicology profiles (Malik et al., 2014). It is possible to imagine that, in the future, it will be standard to assess the effects of clinical candidates in a diverse hPSC derived cellular assays, including electrophysiology and neuronal survival.

Finally, iPSC-based approaches offer great promise for personal medicine or the customization of medical treatment to the individual patient. By studying the effects of clinical candidates on neurons derived from many individual patients, it may be possible to predict who is most likely to respond to a given treatment. Such a so-called in vitro clinical trial has already revealed patient specific response to different drug treatments in ALS motor neurons (Kim and Lee, 2013; Yang et al., 2013). Defining patient populations that are more likely to respond to a particular therapy may lead to optimization of clinical strategy and a higher chance of success at clinical stages. It may one day be commonplace to utilize iPSC models to predict patient specific safety and efficacy, or perhaps even to screen on each patient's cells to identify truly personalized therapeutics.

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Highlights

- Drug discovery for neuronal disorders
- Patient derived neurons as disease models
- Considerations for Phenotypic Assays – Examples of drug screens

Figure 1.

hPSCs with Genotypes of interest can be generated in 2 principle ways. hPSCs can be derived through ectopic expression of reprogramming factors in somatic cells from a patient known to carry the disease genotype. In this model control IPS lines are compared to patient derived iPS cell lines. Isogenic disease lines can be derived through the introduction of disease loci of interest through genome editing (knockout/homologous recombination). In this model donor lines are compared to the derived isogenic lines harboring the genetic locus. Alternatives might exist in miss-expression models. Independent of the source, the cells have to be differentiated into the affected cell types and a phenotype has to be established. This phenotype can just manifest itself in the derived neurons (a good example of this is the low expression of SMN in SMA-IPS cell lines) or specific treatments, like aging/oxidative stress/mitochondrial stress that have to be employed in order to establish a specific phenotype. It is of importance to note that the window of screening is established through the difference between the control lines and the disease lines. Several other controls can be employed in the pre-screen phase to ensure a specific phenotype. For example in our scheme the disease lines can be differentiated into a neuronal subtype that is not affected in the disease and should not show the specific phenotype found in the affected cell type. Once the disease model is validated, an assay has to be developed that can capture rescue/reversal or alleviation of the phenotype. A screen has to be conducted and analyzed. Potential hits have to be identified, confirmed and follow-up experiments have to be conducted to guide the discovery process towards the development of new therapeutics.

Figure 2.

Screening in hPSC disease models relies on the established disease model and an assay that can be used to read-out the effect of treatments. High-Throughput assay read-outs are used to quantify the fluorescence of a whole well using a plate-reader (left). Reporter genes containing fluorophores, luciferase assays, fluorescent dyes or immunocytochemistry can be employed. High-Content-Screens employ the same fluorescent dyes but can also be used to capture bright-field morphology changes. Automated image capture and analysis are used to capture variables on a cell-by-cell basis or even changes in cell organelle structure (middle left). A novel approach is the use of calcium imaging or microelectrode arrays that can capture physiological parameters in response to small molecules (middle right), in the future sequencing of the transcriptome will be used to a.) capture the effect of single compound/ gene function on the disease model and b.) whole genome functional genomics and phenotype/tag enrichment strategies (right).

Table 1

Examples of screening assays using neuronal cells derived from hPSCs.

Disease	Readout	Cell-type assayed	Genetic Defect	Reference
Reporter assays				
PD	MEF2 luciferase reporter screen	Neural cells	wild-type	(Ryan et al., 2013)
HD	luciferase assay measuring REST activity	neural stem cells	wild-type ES	(Charbord et al., 2013)
FXS	FMRP antibody staining	Neuronal Progenitor Cells	Fmr1 mutant	(Kaufmann et al., 2015)
FD	qRT-PCR of IKBKAP expression	neural crest precursor cells derived from FD-iPSC	IKBKAP mutation	(Lee et al., 2012)
Reporter assays in affected cell-type				
SMA	SMN Protein levels and motor neuron survival	Motor neurons derived from patient iPSCs	SMN1 mutation	(Cherry et al., 2014)
SCZ	TCF/LEF reporter assay for WNT/ β -catenin signaling	Neuronal Progenitor Cells	wild-type	(Zhao et al., 2012)
Phenotypic assays				
NPD	lysosomal cholesterol accumulation	neural stem cells	NPC1 mutant	(Yu et al., 2014)
AD	A β 142 toxicity	Neurons (with forebrain markers)	wild-type	(Xu et al., 2013)
Phenotypic assays in the affected cell-type				
ALS	TDP-43 aggregate and motor neuron (hb9::gfp) number	Motor neurons	Sporadic ALS	(Burkhardt et al., 2013)
ALS	survival after arsenite treatment	Motor neurons	TDP43-mutant	(Egawa et al., 2012)