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Phenotypic screening with primary neurons to identify drug targets for regeneration and degeneration

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Abstract

High-throughput, target-based screening techniques have been utilized extensively for drug discovery in the past several decades. However, the need for more predictive *in vitro* models of *in vivo* disease states has generated a shift in strategy towards phenotype-based screens. Phenotype based screens are particularly valuable in studying complex conditions such as CNS injury and degenerative disease, as many factors can contribute to a specific cellular response. In this review, we will discuss different screening frameworks and their relative utility in examining mechanisms of neurodegeneration and axon regrowth, particularly in cell-based *in vitro* disease models.

Keywords

high throughput screening; high content analysis; phenotypic analysis; axon growth; axon degeneration; axon regeneration

Introduction

Although drug screens based on cellular, organ, or whole-animal phenotypes once dominated the drug discovery landscape, the revolution in molecular biology and genomics resulted in this approach being supplanted by screens targeting defined proteins implicated in disease (Kotz, 2012). Over the past 15 years, the most common drug discovery approach has been the target-based screen, in which large numbers of compounds are screened for

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binding to or altering the activity of a single target protein. Subsequently, hit compounds are optimized through medicinal chemistry efforts to define structure-activity relationships (SAR) as well as to improve pharmacokinetic parameters (Khurana et al., 2015; Drews, 2000; Lee and Bogyo, 2013). Despite this dominance, phenotypic (cell-based) screening has recently made a comeback, as an approach to discover biologically active hits relevant to a biological process or therapeutic outcome (Lee and Bogyo, 2013). In particular, phenotypic screening of neural cells offers a way to find compounds or gene targets that modulate the key phenotypes of neurodegeneration and neuroregeneration, without the requirement for the detailed mechanistic knowledge that is often lacking in complex neurological disorders (Zhang et al., 2014; Swinney and Anthony, 2011; Khurana et al., 2015; Rosamond and Allsop, 2000). Multiple neurodegenerative diseases have been studied both with target-based and phenotypic-based screens, including Alzheimer's Disease (AD; Bettens et al., 2010), Parkinson's Disease (PD; Cookson and Bandmann, 2010), bipolar disease, autism, and schizophrenia (Haggarty et. al. 2016).

Target-based versus phenotypic drug screening

While both target-based and phenotypic based screens can be utilized for drug discovery, each methodology has distinct advantages and disadvantages (Figure 1). For compound screening, cell free, target-based assays are typically simpler to execute than phenotypic assays, provide quantitative results based on simple reporter systems, and clearly implicate a specific molecular target. Ideally, high-affinity compounds are identified with a known target and mechanism of action, but such targets must obviously be identified and validated before a screen can be developed; novel targets cannot be identified (Swinney and Anthony, 2011; Khurana et al., 2015). Well-designed phenotypic assays, in contrast, identify hits that are related to a biological process or disease state, and can also identify compounds that have undesirable features or effects, such as toxicity, poor cellular permeation and, of course, lack of biological (as opposed to biochemical) efficacy. An advantage of phenotypic screening compared to target-based screens is suggested by the relative numbers of drugs identified with novel molecular mechanisms of action (MMOA) that have been approved by the Food & Drug Administration (FDA) (Frantz, 2005). An important limitation of the phenotypic approach is that the cellular models require a robust and consistent phenotype that is clearly correlated with the pathology for which therapy is sought, and is measurable by automated microscopy (Swinney and Anthony, 2011; Khurana et al., 2015).

Once a therapeutic target has been identified, it needs to be validated, both *in vitro* and in animal models (Hughes et al., 2011; Tardiff and Lindquist, 2013). Target-based biochemical screening depends on knowing the exact target and the MMOA associated with a well-defined pathology (Sams-Dodd, 2005), and hypothesizes that varying the target activity will also modify the disease (Tardiff and Lindquist, 2013; Khurana, 2015). The process of target validation (Blake, 2007) could be done using structure-activity relationships (SAR) of analogs of a lead compound, generating a drug-resistant mutant of the presumed target, or knockdown or overexpression of the presumed target and monitoring the known signaling systems downstream of this target.

Evaluating SAR of small molecules is a key task in medicinal chemistry, following identification of a small molecule as a hit. Previously, SAR investigations were focused on individual compounds, but recently scientists interested in characterizing SAR have begun to investigate SAR by bioinformatic analysis, combining results from a large number of compound data sets and different target based screens (Hughes et al., 2011; Stumpfe and Bajorath, 2012; Keiser et al., 2009). In the future, this analysis will probably be enhanced by combining data from target-based and phenotypic screening (Stumpfe and Bajorath, 2012).

In the past 10 years, the scope of *in vitro* phenotypic screening has been expanded by the advent of “disease-in-a-dish” modeling offered by induced pluripotent stem cells (iPS; Takahashi and Yamanaka, 2006). iPS cells offer a source of disease-specific cells that can be differentiated and examined for molecular mechanisms, physiology, and screening (Unternaehrer and Daley, 2011) of the disease phenotype. This is particularly advantageous for neurodegenerative diseases, as it is often difficult to obtain primary, affected cells from patients, especially in high enough numbers to allow for screening procedures (Unternaehrer and Daley, 2011). Additionally, the use of iPS cells from human patients provides more direct clinical relevance for any screened targets that facilitate phenotypic changes. Indeed, the availability of human iPS cells has redefined the threshold for interest from the biotechnology industry in potential therapies relating to nervous system disorders. Results on a new compound or genetic manipulation, no matter how promising, must be demonstrated on human neurons to be worthy of serious interest.

In summary, the phenotypic approach provides a way to find compounds and genes active in the highly complex nervous system without assumptions about relevant molecular targets (Lee and Bogoy, 2013; Swinney, 2013). Therefore, we can hope to discover new therapeutic targets, new disease biology, and, at the same time, new lead compounds or genes (Rosamond and Allsop, 2000). However, phenotypic screens require a quantifiable phenotypic alteration, such as neurite length (Figure 2), neuronal survival or changes in fluorescently-tagged protein expression in order to generate a usable output. Here, we describe several methodologies used for phenotypic screening of mammalian primary neurons *in vitro*.

Gain of function screening: cDNA overexpression

Potential causal relationships between genes and diseases are most commonly studied using gain- or loss-of-function mutations. Complementary DNA (cDNA) overexpression increases the expression of a target gene above normal levels - a ‘gain of function’ approach that, by exaggerating a normal function, can implicate a particular gene in a given phenotype (Shalem et al., 2015). Overexpression is typically performed in one of two ways: transduction of the cDNAs by viral vectors or transfection through use of plasmid-based cDNA libraries (Agrotis and Ketteler, 2015). cDNA overexpression has been used to examine gene function in numerous contexts, including activation of DNA repair (Lovejoy et al., 2009), cancer regulation (Zhu et al., 2009), neuronal development (Brody et al., 2002), and neurite growth (Buchser et al., 2010).

Transfection is a gene manipulation process that occurs with the uptake of macromolecules such as plasmid DNA and RNA oligonucleotides (Luft and Ketteler, 2015). Transfection of

neuronal cells using cDNA vectors is possible, but controlling expression levels is extremely difficult (Pearlberg et al., 2005; Moore et al., 2009). Traditional transfection methods include lipid-based approaches and electroporation (Zeitelhofer et al., 2007), but both can be limiting by low efficiency, stressing or killing cells, and inducing an interferon response (Luft and Ketteler, 2015).

Use of cDNA overexpression has been key in the identification of general regulators of central nervous system (CNS) neurite outgrowth through high content analysis (Blackmore et al., 2010; Blackmore et al., 2012; Smith et al., 2011; Smith et al., 2009; Lerch et al., 2012; Dulin et al., 2015). One example is the identification of KLF transcription factors as regulators of axon regeneration in mammalian CNS neurons. Candidate cDNAs, identified by studies on differential gene expression, were screened by overexpression in hippocampal neurons, retinal ganglion cells and cortical neurons. KLF4 was found to be a negative regulator of axon regeneration while KLF6 and KLF7 promoted growth. When KLF7 was modified by adding a transcriptional activation domain, VP16, it could be shown to promote regeneration of the corticospinal tract in vivo (Blackmore et al., 2012). Sox11 was also found to promote CNS regeneration (Wang et al., 2015) after initially being identified in the same high content screening campaign as KLF6 and KLF7 (Blackmore et al., 2010). A different phenotypic screening campaign tested cDNAs of genes more highly expressed in a regenerating neuron (sensory neurons) than in a poorly regenerating CNS neuron (cerebellar granule cells). High content analysis confirmed that STAT3 was a positive regulator of neurite growth (Smith et al., 2011); the idea that STAT3 may enhance axon regeneration is also consistent with a number of other studies (Schwaiger et al., 2000; Fagoe et al., 2015; Nagata et al., 2014). Building on the STAT3 results and the VP16-KLF7 results, we have recently shown that overexpression in retinal ganglion cells of constitutively active STAT3 (STAT3CA) modified with VP16 increases optic nerve regeneration *in vivo* compared to STAT3CA alone (Mehta et al., 2016). Combinations of plasmids can be used to overexpress more than one relevant cDNA simultaneously; combinations of differentially expressed transcription factors have been shown in one focused screen to enhance neurite growth from cortical neurons (Lerch et al., 2014). Phenotypic screens with primary neurons may also be focused on classes of important signaling proteins; a screen involving overexpression of kinases and phosphatases in hippocampal neurons, for example, revealed that kinases in well-known cancer pathways also affected neurite outgrowth, primary neurite count, and neuronal branching (Buchser et al., 2010). These included ERK1, GSK3, and p38 as well as novel regulators of neurite outgrowth, including IRF6, activin, LRRN1, and the phosphatases PPEF2 and SMPD1.

Loss of function screening: RNA interference

The function of a gene may also be investigated using a loss-of-function approach, analyzing the phenotypic changes associated with its inactivation or down regulation. The main method for loss-of-function screening uses short hairpin RNA (shRNA), small interfering (siRNA), or micro RNA (miRNA) libraries in order to reduce mRNA transcript levels through sequence-specific targeting of mRNAs by RNA interference (RNAi) (Neumann et al., 2006; Bolle et al., 2011; Agrotis and Ketteler, 2015; Eulalio and Mano, 2015). Libraries of siRNAs and shRNA expression constructs may be used to target the entire genome or a

subset, such as protein kinases. The biological system and the choice of RNAi reagents determine the format of an individual screen. Both shRNAs and siRNAs are well suited to “one gene per well” screens, in which cells are transfected or infected in multi-well plates with each well containing RNAi reagents that silence one gene (Iorns et al., 2007; Ashworth and Bernards 2010). Standard practice in such screens is to include at least 4 different wells containing 4 distinct targeting constructs for each gene to be targeted; this helps to control for “off-target” effects of individual constructs.

RNAi methodologies provide a number of challenges. Although they are relatively easy to use, the knockdown of mRNAs is variable and often incomplete, turnover kinetics of the encoded protein(s) are variable and usually unknown, and both gene and signaling pathway redundancies exist (Mohr et al., 2014). RNAi methodologies are also prone to off-target effects, causing undesired knockdown of unrelated genes (Echeverri et al., 2006; Gerds et al., 2013). Lastly, effects of RNAi are often transient and restricted by the rate of cell division, inefficient delivery system(s) and limited functionality *in vivo* (Jain and Heutink, 2010). These methodologies can, however, be utilized with imaged-based assays for multi-parameter phenotypic screening (Mohr, 2014)

Gene “knockdowns” using RNAi are often used to identify potential targets for subsequent small molecule screening campaigns. While this can be a valid strategy, reduction or elimination of a gene’s expression does not always mimic pharmacological treatments that alter activity(ies) of the protein encoded by that gene (Fire et al., 1998; Cong et al., 2013). First, small molecules often target several members of a gene family, while knockdown specifically targets a single gene. Second, cellular signaling networks have shown an ability to compensate for knockdowns/knockouts of genes, which is likely to have different kinetics than for functional repression due to small molecules. Third, many proteins have multiple binding partners and/or functional activities, each of which is affected by knockdown, while only a single activity may be affected by a small molecule. Finally, small molecules may be agonists, antagonists, or inverse agonists, each of which has a distinct functional profile compared to simple knockdown.

RNAi-based, genome-wide screening methods have been used for more than a decade (Boutros et al., 2004; Boutros and Ahringer, 2008). Key genes responsible for controlling and modulating numerous cellular processes have been found using both cell and organism based RNAi screens (Mohr et al., 2010; 2014). RNAi screens have the same limitations as discussed above – off-target effects, incomplete knockdowns, and cellular redundancies (Collinet et al, 2010) and there is reason to believe they are more challenging in neurons than in other cell types (Sasaguri et al, 2009). Many efforts have focused on decreasing the impact of these limitations in genome-wide screens, both bioinformatically and experimentally (Mohr et al., 2014). Bioinformatically, tools such as the Genome-wide Enrichment of Seed Sequence (GESS, Sigoillot et al., 2012) and Haystack (Buehler et al., 2012) allow for examination of seed-sequence matches throughout the genome, assisting with reducing off-target effects. Experimentally, multiple RNAi sequences can be utilized to target a single gene or, more definitively, a knockdown phenotype being rescued by transduction of an interference resistant copy (Mohr et al., 2014). Regardless of these limitations, RNAi-based screens have been utilized extensively in phenotypic screens with

great success, including studying cancer cell gene interactions (Laufer et al., 2013), triggers for cell morphology alterations (Yin et al., 2013), and the genetic regulation of endocytosis (Collinet et al., 2010).

shRNAs cloned into viral vectors provide the advantage of infection-based delivery into most cells, including hard-to-transfect and/or non-dividing primary cells like neurons (Mohr et al., 2010). Lentiviral-based delivery approaches have been utilized extensively in phenotypic screening of neuronal cells for neurodegenerative diseases as well as in traumatic injury models. *Inpp5f* was identified as a novel suppressor of CNS axon repair using this approach (Zou et al., 2015). Silencing *Inpp5f* (*Sac2*) transcription in primary cortical cultures with shRNA lentiviral particles increased axon regrowth and growth cone formation after injury *in vitro*. These discoveries were subsequently validated *in vivo*, studying gene-targeted mice lacking *Inpp5f* (Zou et al., 2015; Zhu et al., 2009). The lentiviral approach has also been used in assays for axonal degeneration. Milbrandt and colleagues performed an RNAi-based screen in cultured mouse DRG neurons, injured by axotomy (Gerdtts et al., 2011), and found that shRNAs directed against SARM suppress axotomy-induced axon degeneration (Gerdtts et al., 2013). RNAi based phenotypic screening has also been utilized in the study of synapse formation regulation by examining changes in the abundance of Synapsin-1, Psd95, and Gephyrin following knockdowns of other genes (Nieland et al., 2014).

Many neurodegenerative disorders are associated with the expression of an aberrant protein or non-coding RNA; in such cases the induction of RNAi directed against an mRNA or a long non-coding RNA (lncRNA) may modify the disease progression (Harper et al., 2005; Martin and Caplen, 2007). Recently, Yao and colleagues showed that lncRNAs have important roles in peripheral nerve regeneration after sciatic nerve injury. Silencing *uc.217* via siRNA significantly increased neurite outgrowth in cultured DRG neurons (Yao et al., 2015). This study provided novel ideas about the roles of lncRNAs in peripheral nerve regeneration.

lncRNAs may bind to DNA or RNA in a sequence-specific manner and gene regulation may occur *in cis* (e.g. in proximity to the locus of the transcribed lncRNA) or *in trans* (at a distance from the transcription site). An antisense lncRNA, *FMR4*, is part of the Fragile X-associated FMR1 complex (Li et al., 2013; Peschansky et al., 2015). Loss- and gain of function of *FMR4* causes dysregulation in genes involved in important biological processes, namely proliferation and differentiation (Peschansky et al., 2015). *FMR4* function has been altered after knockdown by siRNA and by overexpression using lentiviral vectors, indicating that a perturbation in this lncRNA's expression could be associated with Fragile X. Additionally, it has been hypothesized that *FMR4* has a role in regulating gene expression through *trans*-activity in other neurological disorders (Peschansky et al., 2015).

Both humans and experimental animals naturally utilize miRNAs, which regulate post-transcriptional control of gene expression through mechanisms like those used in RNAi (Bartel, 2004; Ambros, 2004). While there are relatively few miRNA genes, a large portion of the genome is regulated by miRNAs, including during disease pathogenesis (Eulalio and Mano, 2015). While a miRNA screen can be done in a relatively short time, follow-up

experiments to understand the mechanisms by which a particular miRNA affects cellular phenotype can be extremely challenging (Lemons et al., 2013).

Loss of function screening: CRISPR-Cas

The clustered regularly interspaced palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system, first characterized in bacteria and archaea, acts as a primitive immune system by removing foreign DNA matching so-called protospacer RNAs derived from invading organisms (Barrangou et al., 2007). Over the past 5 years this system has rapidly been developed into a flexible gene editing technique, in which a guide RNA (gRNA) with complementarity to the DNA sequence to be edited, is introduced into cells together with the Cas9 nuclease that binds the gRNA and cuts the cell's complementary DNA (Jinek et al., 2012). CRISPR-Cas9 systems have come into wide use for directed genome editing in numerous contexts (Cong et al., 2013) in both pro- and eukaryotic organisms (Jako i nas et al., 2015). They are of particular use in genetic screening assays, as they are thought to have fewer off-target effects compared to RNAi-based techniques and are effective in non-proliferating cells (Boutros et al., 2015). Additionally, CRISPR-Cas9 and similar genomic editing systems are versatile, in that they can be used to activate/modify endogenously placed reporter genes for lineage/development tracing (Kimura et al., 2015), and/or to increase expression of or knock out specific genes either *in vitro* or *in vivo* (Lauschke et al., 2013). CRISPR-Cas9 systems can be utilized in high-content/high-throughput screening to examine numerous effects; either performing focused screens or using a genome-wide gRNA library to enable genome-scale CRISPR-Cas9 knockout (GeCKO; Shalem et al., 2015). In this way, screens can examine changes in responses of cell cultures for drug discovery/resistance, bacterial toxin resistance, and validation of drug targets (Wang et al., 2014; Koike-Yusa et al., 2014; Zhou et al., 2014; Kasap et al., 2014; Shi et al., 2015; Smurnyy et al., 2014; Carlson-Stevermer et al., 2016; Hsu et al., 2014). Wei and colleagues demonstrated the advantages of high-throughput phenotypic screening based on the CRISPR/Cas9 system, utilizing single guide RNA (sgRNA) library transduced HeLa cells to identify novel genes related to anthrax and diphtheria toxin toxicity (Zhou et al., 2014).

Compared to RNAi, CRISPR has the advantage of generally higher specificity and completeness of gene/protein removal, but proper gRNA sequence selection and construction can be challenging, particularly in mammals, (Cho et al., 2014; Fu et al., 2013). Similar genome editing systems such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) have similar gene-silencing results, but require more complex preparations to function (Wang et al., 2014). Genome-wide gRNA libraries provide the advantages of both target-based and phenotypic screening methods, as initial phenotypic responses can be gauged and putative target(s) determined simultaneously.

Gain versus loss of function screening processes

Gain-of-function genetic screens are a powerful method to identify genes sufficient to confer a particular cellular phenotype (Stark, 1999). In mammalian cells, most gain-of-function screens identify only the dominant phenotype-conferring genes. This approach is convenient, cost effective, versatile, and provides information complementary to loss-of-

function approaches based on RNAi, CRISPR-Cas9, or similar techniques (Paddison et al., 2004; Berns et al., 2004). However, loss-of-function screens may be more applicable for discovery of drug targets, as reduced levels of gene targets mimic drug effects more often than do increased levels (Mullenders and Bernards 2009). Because complementary information is obtained, the use of both gain- and loss of-function screening could be a great advantage in understanding putative target genes and their molecular networks responsible for controlling a phenotype (Pritsker et al., 2006).

Genome-wide screens reveal the contribution of each gene to the phenotypes being investigated (Friedman and Perrimon 2007); however, genome-scale screens entail considerable costs for reagents, instrumentation for automation, and data analysis (Echeverri et al., 2006). One way to minimize costs associated with the one gene, one well approach is to design assays that exploit cell proliferation, cell killing or rescue. In these cases, selection-based screening allows the delivery of an entire library of silencing molecules as one pool to a single, large population of cells, without the need for arrayed formats (Echeverri et al., 2006). Generation of libraries in which each gene in an organism is systematically deleted or mutated provide an invaluable tool for exploring the role of these genes in the complex cellular process (Alberts et al., 2002).

Chemical-based phenotypic screening

Cell-based *in vitro* screening systems allow for high-throughput testing of libraries composed of chemical compounds or naturally occurring biologics, including dose-response testing. The ability to test the effects of compounds on intact biological systems, rather than isolated components, gives more relevance to later steps within the drug discovery process, including examination of efficacy *in vivo* (Swinney and Anthony, 2011). Due largely to the focus on phenotypic outcome, rather than mechanistic cause, phenotypic screens with compounds can be better suited than target-based approaches for identifying candidates for further analysis. Importantly, drugs with higher phenotypic efficacies tend to have more than one target, especially in the nervous system, potentially compensating for pathway redundancies and similar mechanisms (Mencher and Wang, 2005; Al-Ali et al., 2015). This provides a rationale for screening compounds, rather than individual cDNAs or RNAi reagents, in assays aimed at neurotherapeutics. For example, overexpression screens conducted in our laboratory very rarely produce individual hits that increase neurite growth by more than 40 or 50% compared to controls (Blackmore et al., 2010; Buchser et al., 2010; Lerch et al., 2014; Mehta et al., 2016). In contrast, screens of small molecules (e.g., kinase inhibitors) have produced hits that increase neurite growth by 200-400% (Al-Ali et al., 2015).

A popular approach is to attempt to repurpose FDA approved drugs for new diseases. The NIH as well as several companies provide libraries composed of drugs and compounds with known targets and previously described IC₅₀s to guide screening parameters. Examples include the Library of Pharmacologically Active Compounds (LOPAC) and the NIH Clinical Collection (NCC; Developmental Therapeutics Program, NCI/NIH). Bryan Roth and colleagues screened 3,665 FDA approved and investigational drugs against known targets and identified thousands of unanticipated associations based on chemical similarities (Keiser

et al., 2009). Some were tested using phenotypic and *in vivo* assays, suggesting novel side effects and new indications. A focused High Content Screen on cortical neurons using topoisomerase inhibitors identified several that could unsilence the dormant allele of Ube3a, mutations of which cause Angelman syndrome (Huang et al., 2012). A recent phenotypic screening project using spiral ganglion neurons tested the NCC library with dissociated cultures in 384 well plates, measuring the longest neurite from each neuron (Whitlon et al., 2015). A member of the statin family, cerivastatin, was the sole compound to increase neurite length. Based on this finding, the investigators tested other statins, and found 4 more that increased spiral ganglion neurite growth.

High content screening of chemicals *in vitro* is most often performed on cells of interest using a single concentration for each compound, and examining the cells for the desired phenotypic response (Figure 1; Boutros et al., 2015). Once hit compounds are identified, dose-response experiments are performed to determine optimal concentration(s) for each.

Z'-Factor and Validation [Sidebar]

Experimental reproducibility is a problem in all scientific disciplines; therefore, a screen that is part of a campaign to identify a compound with therapeutic potential must be accurate, specific and reproducible. Poor reproducibility may be due to poor experimental design, variability in reagents or poorly controlled experimental technique, among other possibilities (Carp, 2012; Prinz et al., 2011). To assess reproducibility in a high throughput screen across plates on a given day, or across days, various measures have been proposed. Most commonly used in our laboratory is the Z' (Z-prime)-factor (Equation 1 and Figure 3).

$$Z' Factor = 1 - \frac{(3\sigma_+ + 3\sigma_-)}{|\mu_+ - \mu_-|}$$

Equation 1 – Calculation of Z' -factor statistic for high throughput screening assays. σ = standard deviation of upper (+) and lower (–) dataset, μ = mean of upper (+) and lower (–) dataset.

The Z'-factor quantifies the suitability of a particular assay by taking into account both the assay's dynamic range and the data variability; it is a measure of statistical effect size. A successful assay is characterized by a suitable Z' factor (typically >0.5), with good reproducibility across plates and days using positive and negative controls (Zhang et al., 1999; Bray and Carpenter, 2012). Hits from the screen need to be rescreened to allow confirmation of in secondary screens (Martin, 2012; Echeverri et al., 2006; Zhang et al., 2014; Hughes et al., 2011).

Experimental reproducibility depends on minimizing variability; compound collections should be managed, maintained, and delivered in a stable and reproducible way throughout screening campaigns (Hatzis, 2014). Factors include compound handling methods, compound solubility in the solvents used, the effect of solvents on the assay, compound storage conditions, and compound stability and replacement.

Induced pluripotency and phenotypic screening

iPS cells and their derivatives, such as neurons and astrocytes, provide a source of primary cells with increased relevance for human cell physiology and disease. Many primary cell types, and neuronal cells in particular, are difficult to maintain *in vitro*: they are non-proliferative, are damaged upon removal from the brain, difficult to transfect, do not survive long in culture conditions, and are often low in number, depending on the subtype being examined (Engel et al., 2016; Madonna, 2012; Unternaehrer and Daley, 2011; Mohr et al., 2010). The difficulty of obtaining large numbers of cells is an enormous limitation for most research applications, but especially for high throughput screening, as the number of cells required is often substantially higher than for other applications. iPS cells, like their counterparts, embryonic stem (ES) cells, can be propagated indefinitely in culture and are highly proliferative (Takahashi and Yamanaka, 2006). Directed differentiation enables researchers to generate numerous neuronal cell types from iPS and ES cells, including basal forebrain (Nilbratt et al., 2010), dopaminergic midbrain (Perrier et al., 2004; Chambers et al., 2009), motor neurons (Chipman et al., 2012), and astrocytes (Krencik et al., 2011), allowing iPS cells to act as a permanent and flexible source of cells for any experimental purpose. Additionally, neural stem cells can be derived from iPS cultures and maintained as an “in-between” stock, enabling faster and more efficient differentiation to mature cells.

Utilization of iPS cells is particularly advantageous for phenotypic screening of “disease in a dish” models, where cells cultured *in vitro* show phenotypes characteristic of a particular disease (Marchetto and Gage, 2012). Neurodegenerative disorders including AD (Ooi et al., 2013; Israel et al., 2012), PD (Devine et al., 2011), amyotrophic lateral sclerosis (Egawa et al., 2012), and mental disorders such as schizophrenia (Brennand et al., 2011, 2015) have been modeled using iPS cells; patient-derived neural cells show a distinct, disease-relevant phenotype in culture once differentiated. With this observed phenotype as a control, phenotypic screening can be performed for drug/gene target discovery, focused on finding conditions that restore a normal phenotype (Egawa et al., 2012; Ooi et al., 2013). At present, such studies are relatively rare, as consistency in differentiation procedures and characteristics of mature cells need to be standardized (Okano and Yamanaka, 2014).

Concluding Remarks

Two of the primary barriers in the development of therapies for neurodegenerative diseases and neural regeneration after trauma are the extensive numbers of cellular systems involved in the degeneration and the complexity and redundancy of signaling networks within cells. Phenotypic screening offers a means of recapitulating disease models *in vitro* using complex cellular systems. Hits from such screens can subsequently be tested with *in vivo* animal models. Rather than examining interactions of chemical compounds with individual molecular targets isolated from their cellular contexts, both compound-based and knockdown/overexpression approaches can be used with phenotypic screening to facilitate interrogation of complex biological systems. Target-based screening can yield probes for a specific molecular entity that can be used later with *in vitro* or *in vivo* systems, giving information regarding the precise MMOA, while phenotypic assays are focused on the overall cellular response, irrespective of the exact mechanism(s) involved. In the future, we

expect to see further advances in the utility and complexity of phenotypic screens. These could include the use of complex *in vitro* culture systems for automated screening (such as combining neurons, astrocytes, microglia and fibroblasts to better recapitulate *in vivo* disease responses), three-dimensional culture models such as human cortical spheroids (Pa ca et al., 2015), kinetic cellular models, and the widespread use of “primary” cell types derived from human iPS cells. This is predicted to result in both an increase in candidate compounds for *in vivo* testing and a lowering of drug candidate attrition rates during clinical trials.

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Highlights

- Phenotypic screening identifies targets and probes for complex biological processes
- Phenotypic screening is very successful at identifying first in class pharmaceuticals
- Target based screening can optimize compounds for therapeutics
- Combining phenotypic and target based screening is a power approach

Figure 1.

Target-based versus phenotypic screening approaches. Two major approaches dominate drug discovery. The first, target-based screening, seeks novel compounds to alter that activity of a validated target. In this approach, the compounds are typically tested in a biochemical assay against a single target and the compounds are prioritized based on the readouts of the assay. The biggest limitation in this process is that the targets must be identified and validated before a screen can be considered. In a phenotypic screen, the perturbagens are screened in cellular models and they are prioritized based on phenotypic readouts. It doesn't require a target-based hypothesis, allowing discovery of highly effective perturbagens in an agnostic approach. However, targets may be difficult to identify and sometimes they remain unknown. If the perturbagens are compounds, an effort is then made to determine their molecular targets.

Figure 2.

Example of phenotypic tracing during screening. A and B) β -III tubulin staining (A) and automated tracing (B) of rat cortical neurons infected with a virus expressing doublecortin (DCX). C and D) staining (C) and tracing (D) of cortical neurons expressing oxidation resistance 1 (OXR1). Automated tracing of neurites by ThermoFisher Cellomics Neuronal Profiling Bioapplication (version 3.5). Highlighted sections in B and D indicate tracing by the computer program. E) Example measurements by automated microscopy for average neurite count and average branch points in DCX and OXR1 transduced cells. F) Measurements of average neurite total length and max neurite length in DCX and OXR1 transduced cells. Scale bar = 100 microns.

Figure 3.

Example Z'-factor calculations from phenotypic screening of neurite outgrowth. Tightly clustered, high-quality assay data are shown in A and B, with Z'-factor values > 0.5 . C and D show lower-quality data with high standard deviations and Z'-factor values < 0.5 .