

# Neurobiological Applications of Small Molecule Screening

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## 1. Introduction and Scope

Chemical genetics, like classic forward genetic approaches to neurobiological questions, relies on high-throughput phenotypic screens to decipher mechanisms underlying phenotypes. Unlike classical genetics, chemical genetics uses small molecule probes to effect changes in cellular components responsible for producing phenotypes.<sup>1</sup> Such phenotype-modifying compounds are discovered in high-throughput assays; they may act by disrupting<sup>2</sup> or restoring<sup>3</sup> the functions of specific macromolecular targets in cells. The target proteins are identified and validated after the original phenotype-modifying activity is known. Small molecule probes, while more often more resource-intensive to create than classical genetic mutations, possess a number of advantages. Biologically active small molecules enable reversible, temporally controlled perturbations that can be used readily in multiple model systems. The latter attribute is of particular importance in neuroscience, owing to the diversity of model systems for many neuronal processes and neurodegenerative diseases.

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The study of many neurobiological phenomena has been intricately linked to small molecules, such as neurotransmitters. Compounds directly targeting receptors of small molecules native to the central nervous system have provided insight into both the mechanism of neural development and the pathological mechanisms underlying conditions such as schizophrenia and depression. This review attempts to focus on screening methods that can ultimately expand the number of small molecule targets in neuroscience; for example, small molecules discovered in Alzheimer's and Parkinson's disease models may aid in this endeavor. We also discuss potential applications of chemical genetic tools in the field of neurobiology.

In recent years, the definition of chemical genetics has been broadened to include reagents other than small organic molecules, such as siRNAs,<sup>4</sup> peptidomimetics,<sup>5,6</sup> and peptide aptamers.<sup>7</sup> This review will not discuss these methods but will concentrate on the development of high-throughput assays and the validation and study of small organic molecules emerging from such screens (Figure 1).

In chemical genetics, the outcome of a project is often decided months or years before any screening takes place: selection of small molecule libraries to be tested is crucial to finding selective and effective ligands. Synthesis strategies for such compound libraries follow one of two general approaches, focusing around a known molecular scaffold or exploring as much scaffold diversity as possible.<sup>8</sup> The former strategy, also known as focused library synthesis or target-oriented synthesis, is most often employed against known cellular targets and is considered a lower risk approach to finding active compounds.<sup>9</sup> This approach is also used to optimize properties of existing hit compounds, usually by creating a large number of molecules exploring variations of a chemical scaffold. To date, Focused Library Synthesis has been the predominant strategy in neuroscience-oriented screens, yielding high affinity, specific agonists and antagonists to many neurotransmitter receptors and have had a profound effect on both psychopharmacology and neuroscience.

The second approach, diversity-oriented synthesis, attempts to generate maximal scaffold diversity, often using entirely novel scaffolds. Diversity-oriented synthesis<sup>10</sup> provides access to a large number of complex and diverse compounds that may be suitable for modulating a larger set of targets. As phenotype-based screens invariably involve complex cellular or organismal processes, the nature of the target macromolecule for an active compound is not known. Thus, compound libraries need to be capable of affecting a large variety of potential binding partners.

In addition to elegant synthesis strategies, numerous techniques have been developed in the past decade to improve the biological utility of these libraries. Unsuitable and toxic compounds have posed a considerable challenge



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in both chemical genetics and drug discovery; their poor pharmacokinetic properties render the compounds less valuable, creating a need for techniques that predict these properties prior to synthesis. Studies of ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties<sup>11</sup> are especially important in neurobiological models, as the existence of the blood–brain barrier<sup>12</sup> (BBB) in vertebrate systems severely impairs the ability of compounds to reach their desired targets. In past years, much attention has been directed toward correctly measuring small molecules' BBB penetrating capabilities,<sup>13</sup> though their cumbersome nature has led to development of numerous *in silico* predictive techniques.<sup>14</sup>

The development of robust<sup>15</sup> high-throughput assays capable of testing thousands of compounds per day is essential for exploiting the potential of large compound libraries. Such assays are varied, in terms of both model systems and detection methods. Assays using purified cellular components, such as *in vitro* protein binding<sup>16,17</sup> or enzymatic<sup>18</sup> assays, are useful in the discovery of modulators of known protein and RNA targets; more complex cell-based assays are crucial in defining pathways and networks. Cell-based model systems include immortalized and cancer-derived cell lines, providing a platform for assays observing basic cellular processes, such as viability,<sup>19</sup> protein aggregation,<sup>20</sup> or mitochondrial function. These cell lines, though readily available, are not suitable for observing some of the complex processes of interest in neurobiology, especially morphological traits such as neurite number, growth, and branching. The development of immortalized neuronal cell lines,<sup>21</sup> created from primary tissue cells or neuroblastomas, made it possible to explore these complex phenotypes in high-throughput, for example by using automated microscopy. Through such an approach, cell morphology can be visualized and evaluated in a reasonable amount of time, allowing the monitoring of such processes as endocytosis,<sup>22</sup> nuclear translocation,<sup>23</sup> and receptor trafficking.<sup>24</sup>

In contrast to mammalian cell-based screens, yeast-based screens make use of a model organism for large-scale genetic screens;<sup>25</sup> its usefulness as a screening tool is limited by the fact that many genes present in vertebrate genomes have no functional counterpart in yeast. Nonetheless, many basic cell



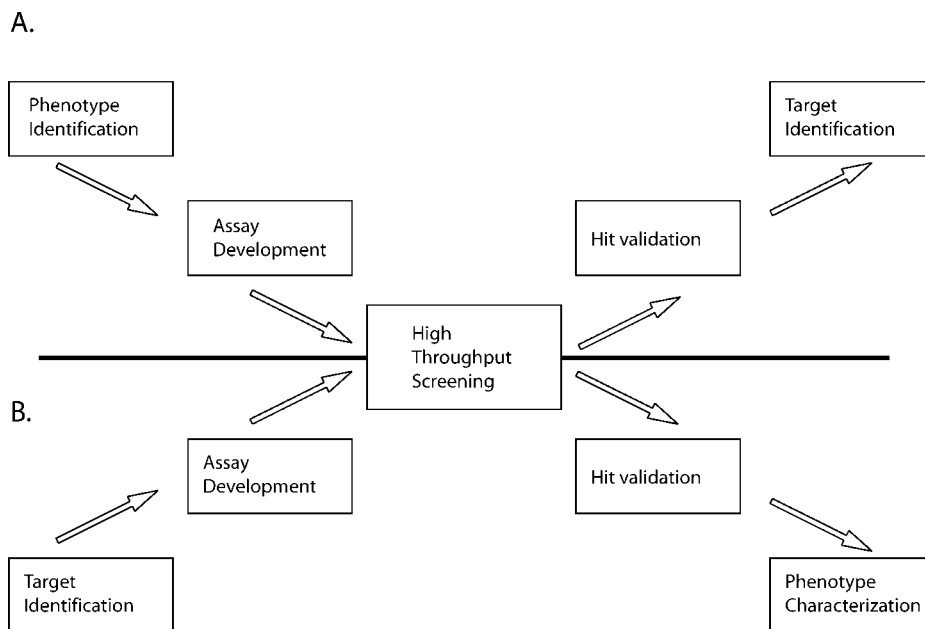
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biological processes, including some relevant to neurobiology, can be effectively studied in yeast.

While cellular phenotypes can be screened for with reasonable ease in cell culture, others, such as context-dependent cell death or diseases with pathological mechanisms involving the cells' environment, require more complex systems. One approach that is more practical than whole animal models is screening in tissue explants. Tissue-based screening holds particular promise for neurobiological applications, by providing a model for tissue-dependent pathological mechanisms, such as ischemic stroke.<sup>26</sup>

Finally, worm,<sup>27</sup> fruit fly,<sup>28</sup> mouse, and zebrafish models have been used for lower throughput screens. Their unwieldy nature and inherent cost is offset by the accuracy with which neurobiological phenotypes may be reproduced, as was shown in the case of neurodegenerative disease models. To date, few examples exist of high-throughput screens with whole organism models, but recent results with *C. elegans* and *D. melanogaster*<sup>29</sup> indicate the feasibility<sup>30</sup> of such efforts.

Most high-throughput assays rely on automated spectroscopic methods for detection of a compound's activity. These methods can be used to detect changes in both *in vitro* and *in vivo* assays and often make use of instruments capable of reading 96-well or 384-well microplate formats. Detection of absorption and fluorescence changes has been used with great efficiency in many enzymatic assays, both *in vivo* and *in vitro*. With the availability of fluorescently labeled substrates that respond to enzymatic cleavage with a change in absorption or fluorescence, these assays are versatile and efficient ways of screening for high affinity inhibitors of known enzyme targets.



**Figure 1.** Development scheme of forward (A) and reverse (B) chemical genetic screens. Forward genetic screens require the identification of a phenotype of interest and then adoption of a model where high-throughput detection of the phenotype is possible. Any hits yielded by the subsequent high-throughput screen are then traced back to their targets and their effect is validated. (B) Reverse chemical genetic screens focus on known target molecules of interest. While assay development is fairly similar to forward screens, postscreening studies generally serve to validate and characterize the phenotype obtained by hit compound treatment.

Direct binding of compounds can be measured using fluorescence resonance energy transfer (FRET) and fluorescence polarization assays, expanding the range of available targets to more exotic systems, such as protein–protein and protein–DNA interfaces, or to the study of protein aggregation, a process that is observed in many neurodegenerative diseases,<sup>31</sup> such as Huntington’s Disease, Alzheimer’s disease, and amyotrophic lateral sclerosis.

Viability can be detected using dyes that are reduced within live cells but not dead or dying ones. Some dyes can only enter cells with compromised cell membranes, giving an indication of the cell’s integrity.

A valuable contribution to detection methods is high-content screening. High-content screening involves detection of morphological changes in intact cells through the use of multiple fluorescent probes, which in turn are analyzed by automated software tools.<sup>32</sup> Such an approach allows systematic analysis of cellular phenotypes at a scale that is compatible with genome-wide screening of gene functions. In addition to cell-based models, use of high-content screening has expanded in recent years to more complex models, such as brain-slice assays and screening in whole-animal systems. These screens allow for early detection of off-target effects of small molecules, such as cytotoxicity, and produce low false positive and negative ratios.

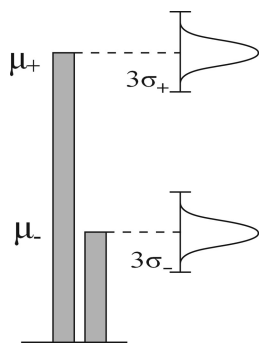
The purpose of a chemical genetic screen is to discover new macromolecules regulating phenotypes, by finding probes that target specific macromolecules. In order to identify or validate these macromolecular targets and eliminate false positives, secondary assays divining the nature and mechanism of action are required. One of the most successful approaches to target identification is affinity chromatography, which uses derivatives of small molecule hits tethered to a solid-phase resin to purify potential targets.<sup>33</sup> This approach was further perfected by the synthesis of pretagged compound libraries, facilitating target identification.<sup>34</sup> The limited availability of high-affinity small molecule ligands restricts

the use of this approach, though advances in small molecule tagging methods may provide improved means of identifying protein targets.

A higher throughput means of identifying binding partners for both hit compounds and candidate proteins is through the use of protein or small molecule microarrays.<sup>35,36</sup> Arrays of proteins, antibodies, and small molecules linked to solid substrates offer high speed detection of binding through displacement of labeled probes or through sandwich ELISA (enzyme-linked immunosorbent assay) methods, providing a rapid means of identifying hit compounds and their targets;<sup>37–39</sup> however, this requires purifying or expressing in situ large numbers of proteins. Classical genetic screens can also complement small molecule screens, by introducing mutations in genes that reproduce the small-molecule-induced phenotype. Mutagenesis screens can also generate cells resistant to the original hit compound, providing information not only on the target protein but also on a specific binding site.<sup>40,41</sup>

As hit compounds profoundly alter gene expression patterns in living organisms, expression profiles have been demonstrated to be of use in identifying the mechanism of action of new hit compounds. Microarray data sets of cells treated with known compounds can serve as templates for identifying mechanisms of action for new hit molecules with unknown target pathways.<sup>42</sup>

Protein expression levels can also be monitored by two-dimensional electrophoresis, a technique capable of separating thousands of proteins in cell lysates.<sup>43</sup> By comparing protein expression levels of untreated and compound-treated samples, changes in expression levels are monitored and provide information about the systemic effects of a hit compound. Finally, a potential way to find target genes of interest is through RNA interference.<sup>44</sup> Using short RNA molecules to induce gene silencing via the RISC complex, this method allows genomewide screens for target genes that



**Figure 2.** Definition of the Z-factor. The aim of assay development is to optimize the difference between the mean of the positive ( $\mu_+$ ) and negative control ( $\mu_-$ ). The Z-factor represents the degree of signal separation, defined as  $Z = 1 - \{[3(\sigma_+ + \sigma_-)]/(\mu_+ - \mu_-)\}$ , where  $\sigma_+$  and  $\sigma_-$  represent the variability of the positive and the negative control, respectively.

modulate a compound's activity and provides insight into the mechanism of action.

## 2. High-Throughput Assay Development

Development of high-throughput assays most often relies on adaptation of existing assays into a 96-well or 384-well microplate format. This usually means optimizing the assay to give a suitable signal-to-noise ratio, as well as clear signal separation between the positive and negative controls. One descriptor of signal separations was described by Zhang et al. as the Z-factor<sup>15</sup> (Figure 2), while other groups reported the use of other statistical tools, such as *t* tests to define hit criteria.<sup>45</sup>

### 2.1. Compound Library Selection

#### 2.1.1. Focused Library Synthesis

The synthesis of focused chemical libraries through combinatorial chemistry has gained prominence in recent years because of the ability to create candidate molecules with improved activity over nonbiased libraries. Focused libraries are usually developed against a class of defined target proteins, often with known scaffolds available. While not ideally suited to classical forward genetic screens, these libraries have nonetheless provided an abundance of small molecule probes in investigating CNS receptor signaling pathways.

Kinase-targeted libraries are a classic example of target-defined library synthesis. Kinases have generated much interest in recent years, both because of their importance in cellular processes<sup>46</sup> and because of the existence of already validated kinase inhibitors.<sup>47–49</sup> Also, the existence of 30 X-ray crystallographic structures for members of the kinase family revealed the similar nature of the ATP binding sites,<sup>50</sup> leading to a rush to discover the basis for selective kinase<sup>51,52</sup> inhibitors. Examples of structure-based library design include the discovery of nanomolar inhibitors for Factors Xa and VII and adenosine receptor A3,<sup>53–55</sup> indicating the versatility of this approach.

The other approach to focused library synthesis relies on the availability of an existing, albeit suboptimal (e.g., nonspecific, low affinity, endogenous ligand) hit molecule. This ligand-based approach relies on the creation of large compound libraries exploring chemical ligand space<sup>56</sup> to explore the putative binding site.<sup>57</sup>

Glycogen Synthase Kinase 3 (GSK3) has been the subject of great interest owing to its role in the pathogenesis of Alzheimer's disease,<sup>58</sup> and over the past decade, numerous groups and companies tried to exploit its possible therapeutic potential. For example, Coghlan and colleagues screened a library of maleimide compounds designed based on the Protein Kinase C inhibitor staurosporin to discover inhibitors with nanomolar  $IC_{50}$ .<sup>59</sup>

Libraries targeting G protein-coupled receptors (GPCRs) have to rely on ligand-based synthesis strategies, as, to date, only two GPCR structures have been published, those of bacteriorhodopsin<sup>60</sup> and the  $\beta_2$ -adrenergic receptor.<sup>61</sup> However, the presence of small molecule ligands for these proteins has provided a core structure for library synthesis. This approach has in recent years led to the identification of novel compound classes in the treatment of neurological disorders, such as schizophrenia and depression. In a study by Campiani et al. the authors have reported a technique for identifying novel, selective 5-HT<sub>2A</sub> receptor antagonists based on previously existing, nonspecific compounds. Using a core structure derived from known antipsychotics, they created a small library of benzothiazepines to discover receptor-subtype specific, subnanomolar inhibitors.<sup>62</sup> Other small focused libraries have been compiled and screened for other receptors, such as 5-HT<sub>6</sub><sup>63</sup> and LTD<sub>4</sub>.<sup>64</sup>

#### 2.1.2. Diversity-Oriented Synthesis

One vision of chemical genetics is that a small molecule modulator can be created ultimately for every protein.<sup>1</sup> The discovery of ligands for many proteins is challenging and may require a degree of diversity from screening libraries that is difficult to achieve. One major source of diverse compounds are natural products<sup>65</sup> or libraries mimicking natural compounds, for example those created using diversity-oriented synthesis (DOS).<sup>66–68</sup> The goal of diversity-oriented synthesis is to populate currently unexplored regions of chemical space,<sup>69</sup> for example through creating different chemical skeletons from the same small-molecule precursors.<sup>70</sup> Such libraries have recently been created by Spring and co-workers yielding highly diverse biaryl-containing medium ring compounds<sup>71</sup> and by Kubota et al.<sup>72</sup> These libraries have been used for a variety of different screens in model systems ranging from yeast to zebrafish and have discovered active compounds with nanomolar effective concentrations. Libraries created by DOS have been used to identify inhibitors to NAD<sup>+</sup>-dependent deacetylases in a high-throughput phenotypic screen performed in yeast,<sup>73</sup> as well as a new class of Phosphorothioate di- and trinucleotides, acting as viral replication inhibitors for hepatitis virus B.<sup>74</sup> While use of DOS libraries in neurobiological screens is not yet widespread, existing examples in zebrafish neural development<sup>71</sup> indicate the usefulness of this synthesis approach.

#### 2.1.3. Suitability Filters

Many hit compounds with significant activity *in vitro* never become useful probes, mainly because poor physicochemical properties prevent them from reaching their required molecular targets *in vivo*. These properties affect pharmacokinetic traits, including absorption, distribution, metabolism, excretion, and toxicity,<sup>75</sup> or ADMET. While these properties can be predicted in some cases,<sup>11,76</sup> they are almost impossible to fix through follow-up compound modifications.<sup>77,78</sup>

It is thus no surprise that considerable efforts have been made in recent years to improve the quality of screening libraries by removing unsuitable compounds. These methods involve *in vitro*<sup>79</sup> or, more often, *in silico* methods for probing ligand solubility. *In silico* attempts at compound characterization revealed that, most often, ADME properties correlate with simple whole-molecule descriptors, such as the number of hydrogen-bond donors or acceptors, the total polar surface area, or the octanol–water partition coefficient ( $\log P$ ),<sup>80</sup> as well as other, more complex methods involving quantum theory-based semiempirical methods.<sup>81</sup> A number of methods have been published on descriptor-based prediction models.<sup>82–84</sup> In addition to ADME and toxicity prediction,<sup>85</sup> numerous examples exist of compound-pooling, that is, selecting or classifying smaller subsets of existing libraries according to their suitability for a specific target. These pooling methods are all the more important in selection of compound from commercial vendors whose chemical libraries contain compounds unsuitable for *in vivo* screening.<sup>86</sup>

One example of pooling compounds was performed by Hann and colleagues, who have constructed a set of practical filters for removing toxic and unsuitable compounds as well as classifying compounds based on their reactive properties.<sup>87</sup> Their approach to removing unsuitable compound scaffolds, as well as reactive functional groups, made use of hard filters for removing a set of common unsuitable and reactive moieties defined using the Daylight SMARTS language.<sup>88</sup> An example of pooling compounds for specific molecular targets is provided by Balakin and co-workers.<sup>89</sup> By training a neural network on a pool of 13000 compounds, they constructed a filter to recognize potentially GPCR-targeting compounds, based on a set of 2D descriptors.

#### 2.1.4. Blood–Brain-Barrier Penetration

In addition to having druglike ADMET properties, small molecule compounds targeting the central nervous system must also have the ability to penetrate the blood–brain barrier (BBB). The term BBB refers to a layer of endothelial cells linked by tight-junctions surrounding the central nervous system, acting both as a passive and an active<sup>90</sup> obstacle to small molecule compounds. While the passive component of the barrier is due to the tight-junctions, allowing for considerably reduced paracellular transport for small molecules, the active nature of the barrier is mainly the work of the carrier P-glycoprotein, also known as the human multidrug resistance protein (MDR).<sup>91</sup> A member of the ABC transporter family, MDR is responsible for actively secreting a wide range of small molecule substrates from cells; playing a key role in the development of multidrug-resistant cancers.<sup>92</sup> As the substrate specificity of this enzyme is wide and ill-defined,<sup>93</sup> strategies to predict potential substrates and inhibitors have had mixed success.<sup>94</sup> As experimental determination of a compound's pBB ( $\text{pBB} = \log(c_{\text{brain}}/c_{\text{blood}})$ ) is difficult and time-consuming,<sup>95,96</sup> *in silico* prediction methods are now common. One class of methods use neural networks or multiple-regression methods to find correlation between physicochemical traits, such as total polar surface area, and octanol/water partition and pBB values based on known BBB-penetrating compounds.<sup>97,98</sup> Recently, other, quantitative structure–activity relationship methods and structural similarity-based methods have been reported. Cuardano and colleagues proposed the use of QSAR-like

similarity models built around compounds with known pBB values to predict unknown compounds' penetration capabilities.<sup>99</sup>

## 2.2. Model Systems for High-Throughput Screening

### 2.2.1. High-Throughput Screening *in vitro*

The simplest of model systems target a macromolecule of interest directly using *in vitro* methods. These assays invariably use purified proteins of interest in hopes of finding high affinity binders or inhibitors. This approach can reveal new biophysical mechanisms for inhibition of proteins or protein complexes, as well as new scaffolds for targeting a specific protein.

One large class of proteins easily targeted *in vitro* is that of enzymes,<sup>100</sup> as their activity is usually easily detectable by assaying either for product synthesis<sup>101</sup> or for substrate depletion.<sup>100</sup> In many cases, however, products or substrates of a reaction are not readily detectable. In such cases, the reaction of interest can be coupled to other, more detectable reactions.<sup>102,103</sup> These approaches, with some modification, can also be used to find probes restoring or activating enzyme function.<sup>18</sup>

In addition to enzymatic assays, small molecules disrupting protein–protein or receptor–ligand interactions can also be assayed *in vitro*, usually by assaying for displacement of the protein<sup>104</sup> or native ligand binding partner.<sup>105</sup> Being capable of targeting proteins without enzymatic activity or small molecule ligands, these assays expand the number of available small molecule targets, and thereby the druggable genome,<sup>106</sup> considerably. Targeting protein–protein interactions, despite its challenging nature, is seen by many as a promising way of developing small molecule tools.<sup>107</sup>

One successful example of this is a set of small molecules targeting the  $\beta$ -catenin–tcf4 interface, discovered in a displacement assay where  $\beta$ -catenin was tethered to microplate wells, incubated with compounds and affinity-tag labeled tcf4. After removal of the unbound compound and excess protein, residual tcf4 was detected.<sup>2</sup> Though very few examples exist of screens targeting protein–protein interactions for neurobiological phenomena, the existence of previously known compounds such as FK506, a protein modulator inducing neuronal differentiation and regeneration,<sup>108</sup> and Ro 08-2750, a nerve growth factor (NGF) receptor ligand,<sup>109</sup> show their potential.

*In vitro* assays can also be used to screen for compounds affecting protein stability. As the formation of protein aggregates plays a crucial role in neurodegenerative diseases,<sup>110</sup> several *in vitro* methods were developed to discover compounds preventing protein aggregation.<sup>111–113</sup> *In vitro* screens for compounds preventing aggregation make use of pH or temperature changes to trigger aggregation of target proteins incubated with small molecules. The amount of precipitate produced under controlled conditions is assessed to find compounds that stabilize the protein. For example, Heiser and colleagues used a filter-retention assay to screen 183,000 compounds for hit molecules that inhibited the formation of mutant–huntingtin aggregates. In the assay, molecules inhibiting protein aggregation would be identified by filtering the assay mixture and analyzing the amount of aggregate retained.<sup>111</sup> Though easy to create, these assays assume (sometimes falsely) that precipitation conditions and triggers in cells have the same mechanism as protein

precipitation triggered by chemical or physical means,<sup>114</sup> and, moreover, that aggregation of proteins is toxic to the cell; both of these assumptions can be questioned.

### 2.2.2. Cell-Based Screening Methods

While *in vitro* assays offer a simple method for identifying ligands for proteins of interest, they fail to take into account many other factors required for biological activity. Cell-based assays offer a more complex model system, providing the capability to control for potential pitfalls in compound development, such as nonspecific binding interactions. Immortalized neuronal cell lines, though similar in many aspects to non-neuronal cell lines, will be discussed separately to reflect the focus of this review.

**2.2.2.1. Non-neuronal Cell-Based Screens.** Most non-neuronal cell-line-based screens are conducted in tumor-derived<sup>115</sup> or engineered<sup>116</sup> cancer cell lines, as they provide a more-or-less defined genetic background required for chemical genetic screens,<sup>19</sup> though screens in primary cell lines such as fibroblasts have also been reported.<sup>117</sup> Such an assay was described by Chen and colleagues in an effort to develop a high-throughput method for evaluating the *in vivo* efficacy of kinase inhibitors by using a microplate adapted Western blotting, also known as a cytoblot.<sup>117</sup>

Cell-based reporter gene assays take advantage of this background to find active compounds against known targets, much in the manner as *in vitro* assays. These assays involve the stable expression of a reporter-fused gene construct in tumor-derived or fibroblast cell lines and use expression levels of the transfected protein as an indicator of small molecule function.<sup>118–120</sup> While these screens do not take into account the different context of neuronal cells, they provide useful probes for studying neurodegenerative diseases. In a study to find compounds increasing the levels of Survival Motor Neuron 2, a protein whose depletion leads to the condition Spinal Muscular atrophy, Lunn et al. expressed luciferase-linked *smn1* and *smn2* constructs in a tumor cell line and then screened 47,000 compounds for molecules selectively upregulating SMN2 levels. Treatment with the hit compound, indoprofen, showed upregulation in SMA-patient fibroblasts as well as a less severe phenotype in the SMA mouse model.<sup>121</sup> Reporter-gene assays can also be used in screens against unknown targets. Hong and co-workers have used primary keratinocytes carrying a luciferase reporter construct containing a marker protein for cell differentiation to screen for compounds inducing terminal differentiation in skin cells.<sup>120</sup> Chemical genetic screens in mammalian cell lines are also suitable for genome-wide screens for disease-related phenotypes.<sup>122</sup>

**2.2.2.2. Neuronal Cell-Line-Based Screens.** The difficulty of creating cell lines that display neuron-like characteristics is apparent from the small number of cell lines currently available. The limited ability to culture primary neuronal cells after removal from their native tissue limits their use in high-throughput screens. One solution to this problem is the creation of immortalized neuronal cell lines that can be induced to differentiate into functional neurons in a number of ways. PC12 cells, derived from a rat pheochromocytoma, can be induced to grow neurites and differentiate using nerve growth factor,<sup>123</sup> are one example of such cell lines. PC12 cells have been used for high-throughput assays in a number of cases,<sup>124</sup> for example in an assay aimed at discovering small molecules that suppress the toxicity of mutant huntingtin,<sup>124</sup> a protein involved in the pathogenesis of Hun-

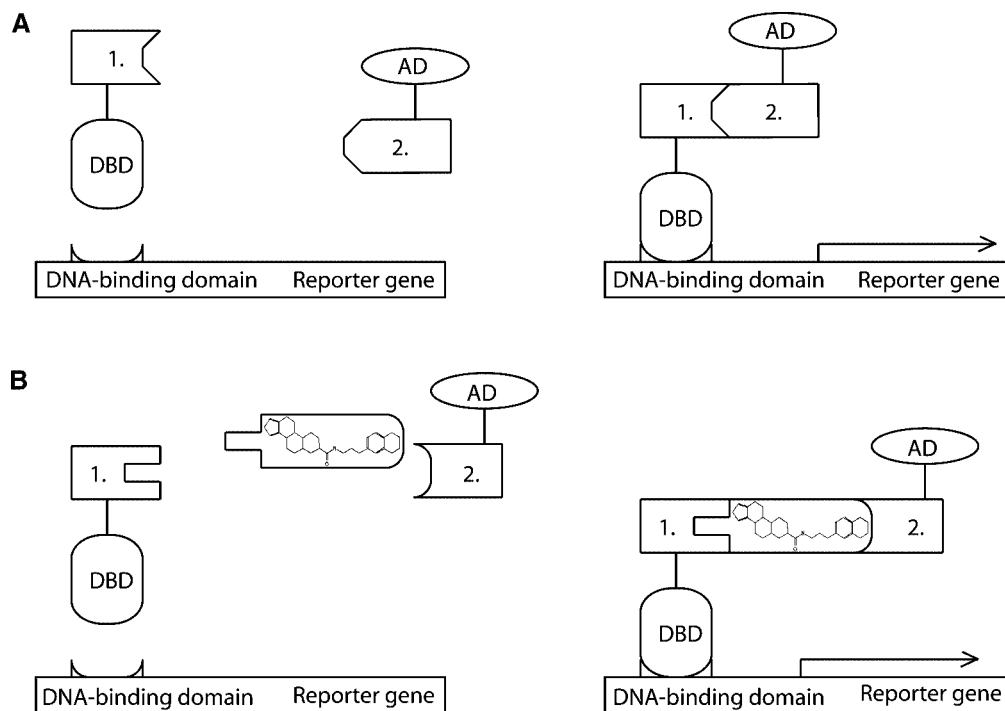
tington's disease.<sup>125,126</sup> PC12 cells have also been used to discover compounds that inhibit toxicity and subsequent depolarization caused by aggregated A $\beta$  1-42 peptide, a  $\beta$ -fibril forming peptide upregulated in Alzheimer's disease. In this study, Blanchard and colleagues cotreated differentiated PC12 cells with compounds and aggregated peptide and then used a voltage-sensitive dye to detect membrane depolarization.<sup>127</sup> Several hits of the screen have previously been described as ion channel modulators, highlighting the potential of such efforts in understanding ion channel function in neurodegenerative diseases.

NSC cell lines were developed by fusing embryonic mouse spinal chord cells fused with neuroblastoma cell lines to create a model of developing motor neurons.<sup>128</sup> Such a cell line served as a good model in a screen for compounds increasing the expression of Survival Motor Neuron 2 (SMN2) protein, whose low expression levels are responsible for the neurodegenerative disease SMA.<sup>129</sup> NSC34 cells were transfected with a BLA reporter construct containing SMN2 and part of its promoter to drive the expression of SMN2, and then they were subjected to a screen with 580,000 compounds to identify compounds effective at submicromolar concentrations.<sup>130</sup>

Finally, ST14A cells are rat striatal neurons containing a conditional mutant of the SV40 large T antigen. These cells act as immature neuronal cells and retain the ability to divide, while subjecting them to higher temperatures inactivates the viral oncogene, causing ST14A cells to differentiate.<sup>21</sup> These cells have recently been used in high-throughput assays to find compounds decreasing mutant huntingtin-induced cytotoxicity.<sup>131</sup> In a screen conducted by Varma and colleagues, serum-deprived ST14A cells expressing mutant huntingtin were screened to find compounds inhibiting cell death. Hit compounds from this screen were then validated in *C. elegans* and other models<sup>131</sup> of HD, serving as proof that cell-based assays can be used for the development of probes useful in more complex systems.

**2.2.2.3. Yeast-Based Assays.** Yeast-based forward genetic assays have for a long time provided useful genetic information in neuroscience.<sup>132</sup> Requiring less maintenance than mammalian cell lines, they have been used for numerous chemical library screens,<sup>133–135</sup> though a major drawback lies in the passive and active barrier function of the yeast cell wall, hindering compound permeation into cells. As well as serving as a target for chemical genetic screens targeting the yeast-genome,<sup>136</sup> they are also amenable to experiments with three-hybrid systems.<sup>137</sup> Primary small molecule screens in yeast have recently yielded compounds with therapeutic potential in mammalian and *Drosophila* models of Huntington's disease.<sup>138</sup> Another recent example of a successful screening project was the discovery of the compound C2-8 by Zhang and colleagues. Discovered in a yeast-based protein-aggregation assay, the compound inhibits the formation of mutant huntingtin expressing cells.<sup>134</sup> Although the target and mechanism of action of the compound are not yet known, it has demonstrated its effectiveness in preclinical studies in mice.<sup>139</sup>

While a good model system, yeast can also serve as a host for expressing exogenous genes in reporter gene assays,<sup>134,140,141</sup> such as two- and three-hybrid systems (Figure 3). Reverse two hybrid systems have been used by multiple groups to identify small molecule compounds targeting protein–protein interactions of selected macromolecules.<sup>140</sup> One such example was using a reverse yeast two-hybrid



**Figure 3.** Yeast two- and three-hybrid systems. (A) The classic yeast two-hybrid system. Transcriptional activation of the reporter gene is accomplished by bringing the activation domain (AD) in close proximity of the DNA-binding domain (DBD) through direct interaction between two proteins of interest, labeled 1 and 2. (B) The yeast three-hybrid system contains an additional component, a small molecule made by covalently coupling the ligands of proteins 1 and 2. For this system, reporter gene activation indicates binding of proteins 1 and 2 to their respective ligands.

system to find novel calcium channel blockers. One fragment of the calcium channel was expressed linked to the GAL4 DNA binding domain, while its binding partner was fused to the GAL4 transcriptional activation domain, while the yeast was transfected with a cycloheximide resistance gene with a GAL4 promoter. Any compound disrupting the binding of the two calcium channel fragments conferred sensitivity to the drug cycloheximide.<sup>140</sup>

### 2.2.3. Tissue-Explant-Based Screens

In many cases, primary cell culture or cell-line-based screens fail to produce good quality results, owing either to the lack of tissue environment or to intercellular interactions in the model system. One attempt at remedying these shortcomings is the use of tissue explants, such as brain slices in high-throughput assays.<sup>142</sup> In this model system, more complex pathological processes can be subjected to medium to high-throughput screens, such as models of ischemic stroke<sup>26</sup> or Alzheimer's disease.<sup>143</sup> Wang and colleagues have developed such a high-throughput assay to screen a library of FDA-approved compounds for neuroprotective molecules in a model of ischemic stroke.<sup>26</sup> By introducing GFP into pyramidal neurons of cortical tissue explants after subjecting them to oxygen and glucose deprivation, a population of sentinel neurons was created which allowed the observation of neuronal cell death over a three-day time period in both the presence and the absence of compound. A neuroprotective compound, neriifolin, was found to be related to a class of  $\text{Na}^+/\text{K}^+$  ATPase inhibitors, originally used in congestive heart failure, recently discovered to be neuroprotective in other model systems.<sup>144</sup> The development of robust assays for a number of complex neurobiological processes, such as nerve sprouting,<sup>145</sup> neurogenesis,<sup>146</sup> or

huntingtin aggregation,<sup>134</sup> demonstrates the future potential and feasibility of small molecule screening in organotypic models.

### 2.2.4. Organismal Model Systems

High-throughput screening in complex model organisms, while more resource intensive, offers unprecedented opportunities in exploring complex phenotypes that cannot be recreated with *in vitro* models or in cell-based models.<sup>147</sup> While their greatest advantage lies in visualizing complex processes, these model systems also offer a better approximation of compound toxicity and distribution. Though a fairly new field, examples exist of high-throughput chemical genetic screens conducted on two model organisms, *C. elegans*<sup>148</sup> and zebrafish.<sup>149</sup> Burns and colleagues have described a screening procedure enabling the screening of thousands of compounds for bioactivity in *C. elegans*. The assay involved compound treatment of worms in 24-well dishes with bioactive compounds, followed by detection of phenotypes by automated microscopy. The advantage of the assay is the possibility of target identification in the affected worms and their offspring through mutagenesis screens for compound-resistant individuals.<sup>148</sup> This technique has already been put to use in finding new calcium-channel antagonists in *C. elegans*.<sup>27</sup>

Zebrafish models have also been developed for high-throughput assays.<sup>150–152</sup> Burns and colleagues have developed a screen to identify chemical probes affecting zebrafish-embryo heart rates. They expressed green fluorescent protein (GFP) in zebrafish myocardium and then detected heart rates by automated fluorescence microscopy in response to compound treatments.<sup>152</sup> One great advantage of zebrafish model systems is that they can be used to discover small molecules influencing vertebrate development, as done in a high-

throughput developmental screen by Peterson et al.<sup>151</sup> While to date there is no literature on large-scale small molecule screens in a fruit fly disease model, genomic RNAi screens show the feasibility of such screens in the future.<sup>153</sup>

## 2.3. Assay Detection Methods

### 2.3.1. Spectroscopic Methods

To date, most high-throughput small molecule screens use some form of spectroscopy as a means of detection. Luminescence, absorbance, or fluorescence detection is easily done in high-throughput and provides robust detection of even small changes.

Detection of bio- or chemoluminescence can be used both *in vitro* and *in vivo*. Among chemoluminescent methods, enzyme-linked immunosorbent assay (ELISA) detection<sup>154</sup> is the most commonly used reaction. It relies on the oxidation of luminol by horseradish-peroxidase, an enzyme that, when linked to an antibody, will give a luminescence signal proportional to the amount of targeted antigen. Such screens have been known to be used in protein-binding assays<sup>155</sup> as well as cyto blot assays.<sup>156</sup> Bioluminescence is most often used in cell-based reporter gene assays, such as the enzyme luciferase that, when provided with its substrate, catalyzes a reaction generating visible light.<sup>157</sup> Several groups have described methods using luciferase-fused constructs for high-throughput cell-based screens.<sup>119,158</sup> Yen and colleagues have used a luciferase-reporter-based assay to identify molecules inhibiting ribozyme self-cleavage. Luciferase was fused to the ribozyme of interest and then transfected into cells. Compounds inhibiting ribozyme self-cleavage were found by screening for increased luciferase mRNA translation detected through luciferase activity.<sup>159</sup> Based on differential substrate specificity observed among luciferase isoforms isolated from different species, Nieuwenhuijsen and co-workers have developed a technique allowing the multiplexing of different luciferase assays.<sup>119</sup>

Colorimetric and absorbance-based assays are a convenient way to detect small molecule products or substrates produced by fungal<sup>160</sup> or bacterial as well as microsomal<sup>161</sup> enzymes. The enzyme alkaline phosphatase, linked to antibodies, is widely used in ELISA assays in a manner similar to that of horseradish-peroxidase (HRP).<sup>2</sup> Colorimetric readouts can also be used in reporter gene assays, such as  $\beta$ -galactosidase assays. Originally a staining technique for gene expression,<sup>162</sup> the cleavage of the galactose derivative X-gal yields galactose and the dye 5-bromo-4-chloro-3-hydroxyindole. This assay has been adapted into a high-throughput format in a number of studies.<sup>163,164</sup> In one case, Naqvi and colleagues have described a galactosidase enzyme fragment complementation assay to screen for the enzymatic activity of specific substrates. By coexpressing a  $\beta$ -gal acceptor enzyme with a cyclic  $\beta$ -gal donor peptide containing a cleavage site for the enzyme of interest, they created a cell-based model system where the enzymatic activity of the target protein results in the linearization of the  $\beta$ -gal donor peptide, resulting in galactosidase activity.<sup>164</sup>

Cell viability is a readout often utilized in screens. The development of absorbent or fluorescent dyes that allow differentiation between live and dead cells based on intracellular reduction such as Alamar Blue<sup>165</sup> has allowed high-throughput screening for compounds affecting cell viability<sup>19,166</sup> both in absorbance and in fluorescence-based assays.

Fluorescence-based assays offer the possibility of more complex, quenching and energy transfer-based detections methods often needed for detection of macromolecular association. These techniques are readily adaptable to high-throughput format, both *in vitro* and in cell-based screens. The use of fluorescently labeled peptides and proteins in protein–protein binding assays usually exploits a direct increase in fluorescence anisotropy,<sup>167</sup> the quenching of the fluorophore,<sup>168</sup> or fluorescence resonance energy transfer (FRET).<sup>20</sup> FRET assays can also serve to detect protein–protein interactions. In a screen for compounds inhibiting mutant-huntingtin-based aggregation, Pollitt and co-workers expressed mutant huntingtin linked to cyan and yellow fluorescent proteins (CFP, YFP) in HEK293 cells and found compounds yielding a higher CFP/YFP fluorescence intensity ratio,<sup>20</sup> indicating decreased protein aggregation. Their hit compound, Y-27632, turned out to be a Rho-kinase inhibitor that was proven neuroprotective in *Drosophila* models of Huntington's disease.

### 2.3.2. High-Content Screening

In recent years, efforts have been made to recover more information from screens. In contrast with simple detection methods that detect one-dimensional readouts, the development of automated microscopy systems adds the possibility of looking at spatial arrangements, as well as signal intensity. In addition to allowing for complex screens such as high-throughput localization<sup>23</sup> and cell motility,<sup>169</sup> it also enabled screens in whole organisms<sup>170</sup> or tissue samples.<sup>26</sup> Examples of high-content, high-throughput screens in neuroscience include the discovery of neuroprotective molecules in ischemic stroke models<sup>26</sup> (see above for discussion), as well as screens in zebrafish<sup>151</sup> and worm models<sup>148</sup> (see above for discussion). While the predominant detection method for explant-based and whole-animal-based screens, high-content screening (HCS) can also be efficiently utilized in cell culture models to detect more complex phenotypic or molecular changes. In a study published by Lundholt and co-workers, a microscopy-based screen was conducted for p53–Hdm2 interaction inhibitors. Both proteins were expressed with EGFP and PDE4A affinity tags, respectively. The cells were then treated with a compound known to localize PDE4A into foci and detected aggregation of EGFP using microscopy. Hits were detected through nuclear EGFP localization in the absence of Hdm2 binding.<sup>23</sup>

Wound healing can also be assayed for in the cell-based format, as described by Yarrow and colleagues. Cells were seeded in microplates, and then wounds were produced in each of the wells using a multipin probe, then assaying for cell motility using fluorescent microscopy. Because of the amount of data obtainable from these screens, high-content screening has produced a whole new set of parameters requiring optimization in assay development (e.g., imaging time, assay length).<sup>169</sup> For example, though the wound healing process occurred over a time period of 24 h, the observed time window for the assay would be reduced to only 1 h, a trade-off between content and throughput.<sup>169</sup>

Though usually associated with microscopy, other methods, such as MRI, have also been reported<sup>170</sup> as viable means of small molecule screening. This method, in addition to its noninvasive nature, also enables tracking of compound distribution in a whole organism model.



### 3. Target Validation

#### 3.1. Affinity Chromatography

Affinity chromatography is one of the most conventional and best defined methods for identification of small molecule targets.<sup>171–173</sup> Originally used to find receptors for endogenous ligands<sup>174</sup> such as neurotransmitters,<sup>175</sup> it has been extensively used in identification of small molecule binding sites, by extracting target proteins with matrix-tethered small molecule ligands. One example by Ding and colleagues used a derivatized version of their small molecule hit TWS119 identified in a screen for compounds regulating stem cell differentiation. This compound was linked to an agarose matrix, incubated with cell lysates, and then bound proteins were removed by denaturation and subsequently identified by mass spectrometry<sup>173</sup> to identify the compound's target, GSK3.

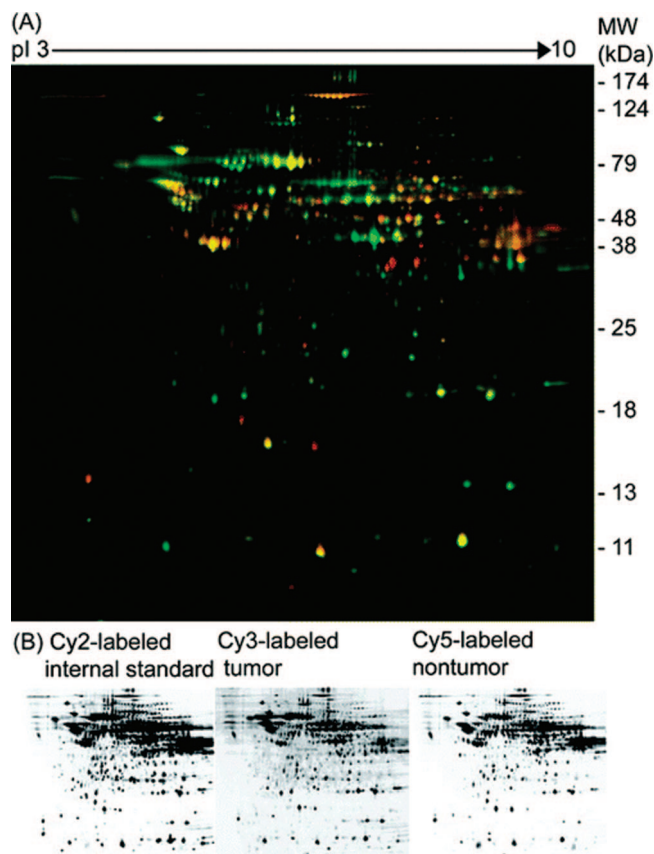
One drawback to this method is the need for high-affinity, high-specificity ligands. Efforts to deal with this challenge resulted in the use of chemical cross-linking agents and moieties designed to bind probes to their targets irreversibly.<sup>176</sup> This approach has been used by Barrington and colleagues in the identification of the ligand-binding domain of the A<sub>2</sub> adenosine receptor.<sup>176</sup> By using a radiolabeled adenosine analogue and cross-linking it with the bifunctional photoaffinity-cross-linker *N*-succinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (SANPAH), they successfully identified the subunit of the A<sub>2</sub> receptor.

Affinity tag modification of hit compounds can be a very challenging task, as any chemical modification of the hit compound can disturb the compound's ability to bind its target. To overcome this problem, chemical libraries containing uniform affinity tagged compounds have been developed, reviewed here.<sup>34</sup> One outstanding example was reported by Khersonsky and co-workers, who accomplished the synthesis of a library of triazine analogues with chemical linker molecules attached. This library was screened in zebrafish embryos, and compounds with interesting developmental phenotypes were investigated. By chemically linking the hit compound to an affinity matrix, target proteins were identified as 40S ribosomal proteins. Further mutagenesis studies of the targets revealed new, extraribosomal functions for these proteins in zebrafish development.<sup>177</sup>

An approach similar to tagged libraries is the use of "click" chemistry in target identification.<sup>178</sup> This approach requires the addition of an alkyne group on the hit compound. This compound is then incubated with the target protein and then is reacted in a Cu<sup>+</sup>-catalyzed reaction with an azide-containing tag molecule. The resulting tagged compound is then used for affinity column-based purification of the target protein.<sup>178</sup>

#### 3.2. Protein and Small Molecule Microarrays

A high-throughput approach to target identification is the use of small molecule or protein microarrays and a series of multiplexed protein–ligand binding assays. Using technology originally developed for DNA arrays,<sup>179</sup> these microarrays contain small molecule compound libraries or protein collections tethered to a solid substrate. These arrays can be used for protein or small molecule binding assays.<sup>35,180</sup> Small molecule microarrays can be used to find and validate ligands of target proteins. In one screen published by Bradner and co-workers, a compound library containing nucleophilic



**Figure 4.** 2D-DIGE analysis of a HepatoCellular Carcinoma patient tissue sample using a pooled internal standard. Tumor proteins were labeled with Cy3 (green), nontumor proteins were labeled with Cy5 (red), and an aliquot of internal pooled standard lysates was labeled with Cy2. IEF was performed on 24 cm IPG strips, pH 3–10, and proteins were further separated by SDS-PAGE (12.5%) in 2D. (A) Image overlays of Cy3- and Cy5-labeled proteins. (B) Three separated Cy-dye images from pooled internal standard, tumor, and nontumor samples. Reprinted with permission from ref 195. Copyright 2005 American Chemical Society.

groups was linked to an electrophilic scaffold and then used to find FKBP-binding small molecules. Affinity tag-labeled, FKBP-containing whole cell lysate was incubated with the tethered small molecules and then removed, and residual FKBP binding was detected by fluorescently labeled antibodies directed against FKBP, showing that small molecule probes can be isolated, without the need for purified protein targets.<sup>35</sup>

In addition to identifying cellular targets of hit compounds, microarrays can also be used to map cellular pathways affected by small molecule probes. One elegant study was performed by Huang and colleagues to identify probes affecting rapamycin toxicity in yeast. Coupled to a small molecule screen for suppressors of rapamycin induced toxicity, hit compounds were linked with biotin and then incubated on microarrays containing 5800 open reading frames from the yeast genome.<sup>181</sup> Binding was detected by fluorescently labeled streptavidin.<sup>180</sup> Recently, protein microarrays consisting of classes of proteins, such as G protein coupled receptors, were reported.<sup>182</sup> These libraries will be a useful addition to efforts to find ligands for orphan receptors in the future.<sup>183</sup>

### 3.3. Classical Genetic Screens

Forward genetic approaches are also valuable tools in target validation. By introducing point mutations in putative target binding sites, target proteins' affinity toward their small molecule ligands can change profoundly. Using techniques such as point mutagenesis and alanine scanning,<sup>184</sup> binding characteristics of small molecules can be determined. Mutagenesis screens were used to decipher the substrate specificity and binding site characteristics and partners of many receptors<sup>184–186</sup> and enzymes.<sup>187</sup>

Forward genetic screens can also be used as counterscreens to identify other components involved in the pathway targeted by a small molecule.<sup>188</sup> Nass and colleagues have reported the discovery of dopamine transporter mutants in *C. elegans* resistant to the effects of 6-hydroxydopamine<sup>188</sup> (6-OHDA) by performing a standard ethyl methanesulfonate (EMS) screen<sup>147</sup> and then exposing F2 offspring to 6-OHDA to find resistant mutants.

### 3.4. Transcription and Proteomic Profiling

Profiling methods, both transcriptional and proteomic, provide a tool for systematic analysis of small molecule effects on cells, enabling the elucidation of mechanisms of action for small molecules.<sup>189</sup> By establishing the underlying expression changes, probes targeting a given pathway can be clustered based on their effect on transcription levels, helping with the identification of target pathways for novel hit compounds.

Connectivity maps were established by Lamb and colleagues in MCF7 breast cancer cells in an effort to link small molecules and genes involved in human disease to pathways. Using 164 compounds with known biological activity, microarray data sets were compiled of compound-treated cells and then clustered for similarities in gene expression signatures. The results showed coclustering of molecules with similar mechanisms of action and allowed the target identification of a previously uncharacterized small molecule, gedunin.<sup>190</sup> A similar approach was used to find modulators of EWS/FLI, an oncoprotein involved in Ewing sarcoma. By analyzing microarray data from RNAi-mediated knockdown EWS/FLI and then comparing it to that of compound-treated samples, Stegmaier and colleagues identified cytosine arabinoside, a compound with a similar transcriptional profile to that of the original knockdown.<sup>189</sup>

Another method of monitoring changes in protein expression levels is two-dimensional difference gel electrophoresis (DIGE).<sup>191</sup> Two-dimensional gel electrophoresis allows a high degree of separation between proteins of different charge, separated by isoelectric focusing (IEF), and size, separated by gel electrophoresis. Difference gels use a mixture of two samples, prepared under different conditions and labeled with different fluorescent probes to visualize the differential in protein levels between samples.<sup>43</sup> As the samples are separated on the same gel, the same protein occupies the same spot in both gels; the difference in fluorescence intensity correlates with the amount of protein in the different samples. An additional benefit of two-dimensional DIGE is the possibility of detecting posttranslational modifications in proteins, adding a new layer of information on small molecule mechanisms. DIGE analysis has been used to profile protein expression levels in a number of cancer cell lines<sup>192–195</sup> (Figure 4). Though not widely used

in small molecule target validation, the method has great potential to become a key tool in the field.

### 3.5. RNA Interference

First discovered in the nematode *C. elegans*, short interfering RNAs have become a useful tool in abolishing the expression of genes in higher eukaryotic systems. A natural extension of small molecule probes, RNAi uses short strands of single or double stranded RNA sequences to target endogenous mRNA molecules for early degradation using the RISC enzyme complex, originally part of the cell's defense system against RNA-based viruses. In recent years, many different techniques based on RNAi have been developed, for example, short-hairpin RNAs (shRNA)<sup>4</sup>, double-stranded RNAs (dsRNA),<sup>196</sup> or short interfering RNAs (siRNA).<sup>45</sup> Though using different delivery systems and targeting different organisms, all these techniques make use of the RISC complex for knocking down gene expression. Through the use of computational genomics, the creation of genomic siRNA libraries became possible, enabling researchers to silence each gene specifically.

RNAi has been used to discover targets of small molecules<sup>197,198</sup> as well as the function of genes affecting small molecule targets.<sup>45</sup> Jiang and colleagues have used siRNA to locate the target of PTCEM, a small molecule inducing caspase-3 activation in cancer cells. By knocking down levels of putative target proteins involved in the apoptosis pathway, they have identified ProT, a factor whose loss of function leads to sensitization to apoptotic stimuli.<sup>197</sup>

A recent genomic RNAi screen led to the discovery of several siRNAs sensitizing lung cancer cell lines to paclitaxel, a cytotoxic agent. By incubating NCI-H1155 cells with sublethal concentrations of paclitaxel, Whitehurst and co-workers discovered a set of genes involved in microtubule organization whose knockdown significantly enhanced paclitaxel sensitivity,<sup>45</sup> showing the use of RNAi in the elucidation of small molecule mechanisms of action.<sup>4</sup>

## 4. Concluding Remarks

Though chemical genetic approaches have yet to gain widespread use in neuroscience, the number of successes using small molecule tools suggests potential for this strategy in the field. The lack of diverse and neurobiologically relevant cell models amenable to high-throughput screening is a bottleneck that has only been partially addressed with the development of inducibly differentiated neuronal cell lines; the phenotypic models in these systems must be carefully investigated to assess their relevance to *in vivo* models. The advent of new, validated phenotypic models may enable the discovery of novel small organic molecules targeting previously unexplored components of developmental and disease pathways in neuroscience.

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