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Chemical Genetic Screening Approaches to Neurobiology

Minireview

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Chemical genetics is an emerging technology for revealing the signaling networks that regulate biological phenotypes using exogenous reagents such as small organic molecules. To study neurobiology using chemical genetics, high-throughput cell and organismal assays can be created to identify compounds and proteins that regulate diverse neuronal phenotypes, such as cell viability, gene expression level, protein association, protein aggregation, glutamate uptake, membrane polarization, mitochondrial function, neurite outgrowth, and growth cone composition. This powerful set of tools will enable the molecular dissection of complex processes that occur within the nervous system.

Introduction

Chemical genetics is a process that uses exogenous ligands to reveal the molecular circuitry controlling biological phenotypes (Schreiber, 1998; Stockwell, 2000a, 2000b). Analogous to classical forward genetic screens in model organisms, this approach makes use of high-throughput, phenotypic assays to identify small molecules that disrupt gene product function in a way that alters a phenotype of interest. Although most efforts and definitions of chemical genetics limit the approach to strategies using small organic compounds, a wider definition would include other exogenous reagents, such as small interfering RNAs (Elbashir et al., 2001) or peptide aptamers. Once such phenotype-modifying reagents are identified, they can be used to identify their macromolecular targets, usually DNA, RNA, or protein. Using this process, it is possible to identify genes and proteins that comprise the pathways and networks that regulate biological systems. In effect, this method allows, for the first time, the use of an unbiased genetic approach in diverse mammalian systems, i.e., the chemical equivalent of saturation mutagenesis. Moreover, it is possible to use these small molecule probes, identified through the chemical genetic screening process, to reveal the various cellular functions of such genes and proteins once they have been identified. Although little has been published on the topic of chemical genetic screens related to neurobiology, the field is ripe for dissection using this new set of tools. This review focuses on the discovery and use of small molecules to explore basic biological processes, but it is surely the case that many small molecules discovered in phenotypic screens could be drug candidates, providing another impetus for undertaking a search for such compounds.

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Compound Library Selection

The most widely used probe reagents for chemical genetic studies are small organic molecules, which are available from a number of commercial suppliers and include compounds from historical archives, natural sources, or those produced using combinatorial chemistry. Typically, purveyors will supply compounds in 96- or 384-well plates and provide structure data (SD) files with electronic chemical structures and quality control data on each compound's purity. The major challenge in procuring compounds for chemical genetic screens lies in selecting the small molecules to use. Quantitative analyses using molecular descriptors can differentiate between various classes of small molecules for the purpose of library procurement. For example, polar surface area is a molecular descriptor that correlates with penetrability through the blood-brain barrier and is a useful filter when selecting compounds for neurobiological applications (Root et al., 2002). For more information on the blood-brain barrier and strategies for penetrating it, see the accompanying minireview by Pardridge (2002 [this issue of *Neuron*]).

Cell and Organismal Model Systems for Studying Neurobiology

In order to test a large number of compounds within a reasonable period of time, it is necessary to make use of high-throughput screening (HTS) technology, which enables the performance of tens of thousands of discrete assays each day. This powerful new tool can be brought to bear on many cellular and organismal phenotypes of relevance to neurobiology. Such cell or organismal models must be adapted to a plate-based HTS environment. One class of tractable model systems consists of yeast-based screens, which allows for the performance of many tens of thousands of assays per day but is limited by the facts that yeast cells are less permeable to many compounds than human cells, that only 10%–20% of human genes have a yeast ortholog, and that many neuronal processes do not have a counterpart in yeast cells. A second class of model systems consists of non-neuronal mammalian tumor cell lines, which allow for assays involving protein aggregation and general cell biological functions, such as apoptosis, transcription, translation, splicing, protein degradation, and mitochondrial function. A third class of model systems consists of immortalized neuronal cell lines, such as rat PC12 cell, which can be induced to differentiate into neuronal cells by treatment with nerve growth factor (Greene and Tischler, 1976), murine N2A neuroblastoma cells, which can be induced to differentiate into neurons using a variety of methods (Olmsted et al., 1970), ST14A cells, which express a temperature-sensitive variant of the simian virus 40 large T oncoprotein and can be induced to differentiate at 39°C (Cattaneo and Conti, 1998), or NSC34 cells, which are immortalized cells that constitutively manifest characteristics of motor neurons (Cashman et al., 1992). Finally, a fourth class consists of organismal model systems, such as worm, fly, zebrafish, and mouse models, which are obviously less amenable to high-throughput assays but more accurately reproduce

neurobiological processes. For example, expression of a huntingtin N-terminal fragment containing 128 glutamines in the six touch receptor neurons of *Caenorhabditis elegans* causes touch insensitivity, forming the basis for a medium-throughput screening assay (Parker et al., 2001). Expression of mutant α-synuclein, TAU, or ataxin-3 in *Drosophila melanogaster* causes age-dependent progressive neurodegeneration, providing models for Parkinson's Disease, Alzheimer's Disease, and Spinocerebellar Ataxia type 3, respectively (Muqit and Feany, 2002). With creative thinking, it should be possible to convert many existing cellular and organismal assays into robust, automated screens that can accommodate the testing of hundreds of thousands of compounds.

Phenotypic Readouts and Assay Technologies

The majority of high-throughput screens utilize an assay whose end product is a spectroscopic change, such as a change in fluorescence, luminescence, or absorption. A recent trend in high-throughput screening is the development of high-content screens that capture more information per sample, such as microscopy-based screens that detect changes in the subcellular localization of proteins or in the morphology of cells.

The presence of live, viable cells is a useful phenotypic readout for those cases in which a disease allele causes cellular toxicity. Viability dyes exist that selectively stain live cells or dead cells and therefore enable detection of disease allele-induced toxicity in a high-throughput fashion. For example, a mutant allele of the *hungtingtin* gene causes cell death in differentiated ST14A neuronal cells (Rigamonti et al., 2000), and mutants of *superoxide dismutase-1* (*SOD1*) that cause amyotrophic lateral sclerosis also cause cell death in the presence of oxidative stress in differentiated N2A cells (Pasinelli et al., 1998). In such cases, it is possible to perform screens for enhancers and suppressors of this toxicity to identify those pathways and processes that functionally interact with the toxic allele.

In addition, it is possible to make use of viability assays to perform synthetic lethal screens when a disease-causing allele does not directly cause cell death (Stockwell et al., 1999). In this approach, one searches for pathways that, when inhibited or activated, cause cell death only in the presence or absence of an allele of interest. For example, glutamate causes selective toxicity in the presence of mutant *SOD1*, suggesting that glutamate-induced signaling is involved in the mechanism of mutant *SOD1*-mediated cell death (Roy et al., 1998). Of course, it is important to determine that such synthetic lethality does not arise because two weak cell death stimuli are simply additive in terms of their gross toxicity. A counter screen for selectivity relative to other weakly lethal stimuli (toxic alleles or small molecules) must be performed. For example, it would be important to determine that glutamate does not cause increased toxicity in the presence of unrelated toxic proteins (such as mutant huntingtin, β amyloid, etc.) or other weakly toxic compounds, such as tubulin inhibitors. If such counter screens reveal that glutamate only causes increased toxicity when mutant SOD is present, it is likely that there is a functional connection between glutamate and mutant SOD.

The expression level of a specific gene product can also be a useful phenotypic readout. For example, the expression level of the excitatory amino acid transporter 2 (EAAT2) is altered in astrocytes in the spinal cord of mice and humans with ALS (Lin et al., 1998). Thus, it would be of interest to identify pathways and proteins that affect EAAT2 expression in order to better understand its regulation via transcription, translation, splicing, and degradation.

Protein aggregation has emerged as a common event in neurodegenerative diseases such as Creutzfeldt-Jakob Disease (CJD), Alzheimer's Disease (AD), Huntington's Disease (HD), Parkinson's Disease (PD), and amyotrophic lateral sclerosis (ALS). Assays that report on protein aggregation are of broad interest for the study of these neurodegenerative diseases, as they may shed light on the connection between protein aggregation and disease pathogenesis. Aggregates can be observed in diverse systems ranging from yeast cells to human brain sections, suggesting they form via conserved, fundamental processes. Chemical genetic screens can be used to shed light on the intracellular pathways that regulate aggregate formation and clearance. A recent chemical screen for inhibitors of α-synuclein fibril formation identified numerous dopamine-like catecholamines that cause accummulation of α-synuclein protofibrils (Conway et al., 2001). This screen revealed that dopamine can be oxidatively ligated to α-synuclein and suggests a possible basis for the dopaminergic selectivity of α-synuclein-associated neurotoxicity in Parkinson's Disease.

Many neuronal processes can be detected most effectively using microscopy. Recent developments in automated microscopy have stimulated an interest in high-content, or imaging-based, screens. In this paradigm, an image is obtained of cells or organisms treated with each test compound and scored for the relevant phenotype. Such microscopy-based screens are slower than conventional platereader-based screens but offer the opportunity to detect more complex phenotypes. For example, neurite outgrowth of PC12 cells can be used as a phenotypic marker for neurotrophic signaling (Wu and Bradshaw, 2000). In addition, detection of specific proteins in the growth cone of a neurite can be used as the basis for an imaging-based screen. The Survival Motor Neuron (SMN) protein has been shown to localize to growth cone and filopodia-like structures in neuronal cells (Fan and Simard, 2002). Compounds that disrupt or promote neurite extensions or SMN localization could be used to shed light on the pathways and proteins that regulate these processes (Figure 1). Although subjective assessment of images obtained in such a high-content screening mode is possible, a quantitative and automated method for analzying such images is more reliable and allows for greater throughput.

Development of High-Throughput Assays

Once a model system and phenotypic readout are selected, it is necessary to develop a robust, miniaturized assay that can be adapted to robotic instrumentation, typically in 96-well or 384-well plates. This involves optimizing assay parameters such as number of cells, composition of growth medium and assay buffers, washing conditions, length of incubation, plate type, and plate coating method. During the optimization of these assay parameters, the separation between the positive control

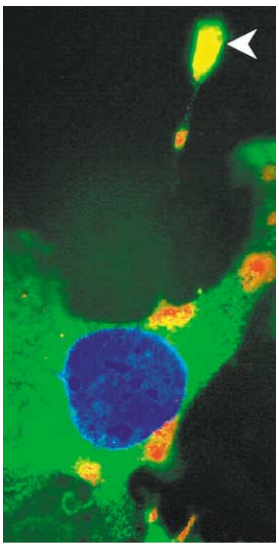

Figure 1. An Example of an Imaging-Based Assay that Could Be Adapted for High-Throughput Screening

Survival Motor Neuron (SMN) is shown to be localized in the growth cone of retinoic acid-treated mouse P19 embryonal carcinoma cells. SMN is stained in green, GAP-43 (a growth cone marker) in red, the nucleus in blue, and colocalized SMN and GAP-43 in yellow. The arrowhead indicates a growth cone-like structure (Fan and Simard, 2002). Figure reprinted by permission of Oxford University Press (L. Fan and L.R. Simard, Human Molecular Genetics *11*, 1605–1614).

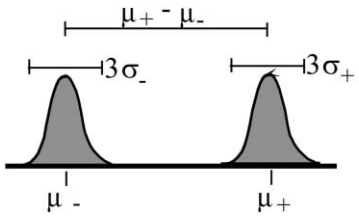

Figure 2. Optimization of the Z Factor during Assay Development

A positive control and a negative control are used to optimize assay conditions such that the separation between the mean of the positive signal (μ_+) and the mean of the negative signal (μ_-) is maximized. Simultaneously, the variability of the positive (σ_+) and negative (σ_-) signal is minimized. The Z factor, which is defined as $Z = 1 - \{[3 \times (\sigma_+ + \sigma_-)]/(\mu_+ - \mu_-)\}$, incorporates all of these parameters and is used as a quantitative score of assay quality (Zhang et al., 1999).

signal and negative control signal is maximized, while signal variability is minimized. A quantitative index that incorporates all of these parameters, known as the Z factor, has been developed and is widely used in these optimization studies (Figure 2). Typically, an assay must have a Z factor greater than 0.2 in order to be sufficiently robust for high-throughput screening (Zhang et al., 1999).

Once the assay is developed, compounds are typically screened at one concentration, such as 10 μM, ideally in replicate. Active compounds are selected for retesting in a dose-response series to determine the maximum achievable signal and the concentration at which 50% of this maximum is achieved (EC_{50}). These are quantitative parameters for the activity and potency of each compound in the assay and allow for a relative ranking of the active compounds.

Target Identification and Mechanistic Elucidation

The most potent and most active compounds are studied in secondary assays that serve to characterize further the activity profile of the compounds. Ultimately, it is desirable to identify the molecular bases governing the phenotypic effects of these compounds, ideally by identifying protein targets and downstream effectors of active compounds. There are a variety of methods for studying the mechanism of action of small molecules, including affinity chromatography, protein array profiling (testing the ability of a compound to bind to each member of a protein array), mRNA or phage display, transcription profiling, proteomic profiling, classic genetic screens, and RNA interference.

A conventional and well-validated method for identifying protein targets of small molecules is affinity chromatography, which has its origins in the identification of the protein targets of dopamine and acetylcholine in the 1960s and 1970s. However, newer methods of target identification are being developed that are simpler and faster. Protein array profiling and mRNA and phage display, for example, are emerging methods used to identify specific binding proteins for a small molecule of interest. Protein arrays consist of proteins deposited in a regular grid, typically on a microscope slide. Such arrays have been used in proof-of-principle experiments to identify binding proteins for small molecules (MacBeath and Schreiber, 2000; Ziauddin and Sabatini, 2001). Messenger RNA display, phage display, and expression cloning are methods for displaying proteins derived from a cDNA library to test their interaction with a candidate small molecule. For example, an expression cloning strategy revealed that the protein target of capsaicin, the active ingredient in hot chili peppers, is vanilloid receptor subtype 1, a heat-activated ion channel present in sensory neurons (Caterina et al., 1997).

There are a variety of additional emerging tools for studying the mechanism of action of a small molecule. Cellular profiling methods, including transcription and proteomic profiling, have been used to view the global molecular changes induced by a compound in cells or organisms. In some cases, the pattern of transcriptional or protein-level changes induced by a small molecule illuminate the mechanism by which the small molecule acts. In cases where the phenotype of interest is seen in a model organism, classic genetic screens in yeast, worms, and flies can be used to identify genes that, when mutated, cause resistance or sensitization to a small molecule. Finally, RNA interference can be used to test the effects of knocking down specific transcripts that are implicated in these experiments. If a target protein is involved in mediating the effects of a small molecule, directly or indirectly, then reducing the concentration of the target protein should either cause sensitization or resistance to the compound, depending on whether the mechanism of action involves a gain of function or a loss of function. Any one of these methods may fail for a specific compound, but in aggregate, they represent a powerful arsenal of tools for dissecting the

molecular pathways that mediate the phenotypic alterations induced by exogenous reagents.

Conclusion

Many areas for innovation remain for chemical genetic studies of neurobiology. There is a need for better and more diverse cell models of relevance to neurobiology that are compatible with high-throughput screens. Cell lines that can be inducibly and rapidly differentiated from an immortalized cell type represent attractive solutions to this problem. However, in such cases it is critical to determine which aspects of neuronal function are recapitulated in the differentiated model system. Detection of novel phenotypes with relevance to neurobiology and neurodegeneration also remains a critical issue in this emerging field. New phenotypic readouts that are amenable to high-throughput testing will undoubtedly result in the discovery of interesting new compounds and targets regulating neurobiological processes. Finally, faster and better methods of target identification would be invaluable in accelerating the final stages of the chemical genetic process, providing valuable new tools for the dissection of neurological processes.

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