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Leveraging large-scale behavioral profiling in zebrafish to explore neuroactive polypharmacology

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Abstract

Many psychiatric drugs modulate the nervous system through multi-target mechanisms. However, systematic identification of multi-target compounds has been difficult using traditional *in vitro* screening assays. New approaches to phenotypic profiling in zebrafish can help researchers identify novel compounds with complex polypharmacology. For example, large-scale behavior-based chemical screens can rapidly identify large numbers of structurally diverse and phenotype-related compounds. Once these compounds have been identified, a systems-level analysis of their structures may help to identify statistically enriched target pathways. Together, systematic behavioral profiling and multi-target predictions may help researchers identify new behavior-modifying pathways and CNS therapeutics.

Keywords

in vivo high throughput screening; phenotypic screening; zebrafish behavioral drug screening; neuropharmacology; polypharmacology; multi-targets; systems pharmacology; Similarity Ensemble Approach; Enrichment Factors

Introduction

Polypharmacology is both a challenge and an opportunity in central nervous system (CNS) drug discovery^{1–3}. Although drugs are frequently associated with ‘magic bullets’ against single targets, most CNS drugs act on multiple targets simultaneously². Polypharmacology complicates drug discovery efforts because compounds with complex mechanisms are difficult to identify, understand and optimize. Despite these challenges, polypharmacology also represents an opportunity to discover new compounds with mechanisms of action that have not already been exhaustively exploited *in vitro*. Here, we review two approaches to large-scale behavior-based chemical biology—phenotypic profiling and predictive multi-target enrichments—that can help researchers identify novel compounds with complex polypharmacology.

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Psychiatric disorders such as psychosis, depression and mood disorders are thought to have multigenic and multifactorial etiologies^{4–6}. Typically, drug discovery paradigms focus on identifying compounds with single target mechanisms. This single-drug-single-target approach is very effective for diseases caused by a single mutated gene or deregulated protein. However, most psychiatric drugs including antipsychotics, antidepressants and anxiolytics are thought to exert their therapeutic effects via multiple targets^{2,7,8}. As shown by the Psychoactive Drug Screening Program (PDSP), these compounds tend to have complicated target interaction profiles and complex mechanisms of action⁹. For example, antipsychotic drugs are thought to exert their efficacy through a constellation of multiple targets. Attempts to improve these compounds by maximizing single target selectivity have been largely unsuccessful². For CNS drug discovery, more promiscuous compounds are often more effective^{2,7,8}.

High content compounds

How can multi-target compounds be identified? Unlike *in vitro* assays that identify compounds acting on single predefined targets, phenotypic assays encompass a broader target-space. For example, high-content cell-based screens are powerful tools for studying intracellular signaling pathways¹⁰. Similarly, behavioral screens in whole-organisms are a powerful approach to understand neuronal phenotypes that require integration of a multitude of cell types, sensory systems and neuronal circuits across an entire organism^{11,12}.

Behavioral screens are an effective way to identify neuroactive compounds, but understanding their mechanisms is a major challenge. Once identified, how can these compounds be understood? One approach may be to leverage the hit compounds themselves.

Different types of screening assays identify hit compounds with varying extents of biological information. (The term “hit compounds” can have different meanings in different contexts. Here we use the term “hit” to refer to compounds that have been identified in a screen. In some cases we may also use the terms “primary hit” and “confirmed hit” to refer to these compounds pre-and post validation, respectively.) Hit compounds from target-based assays are expected to primarily contain information about the target they were screened against (Figure 1a). *In vitro* assays can be used to screen millions of compounds and identify hits with maximum potency and selectivity^{13,14}. However, since the screening assays are performed in a simplified system (and typically are aimed against a single target) the hit compounds from *in vitro* assays do not contain meaningful information about anything except the original screening target.

By contrast, compounds identified *in vivo* benefit from more biological context and information than those identified *in vitro*. These compounds are likely to act on multiple targets and pathways to cause a given phenotype (Figure 1b). Although some phenotypes may depend on a single target¹⁵, many phenotypes depend on multiple targets. This is one reason why target identification is such a major challenge in phenotypic screening and why phenotype-based assays are so effective at identifying compounds with complex binding profiles. Any single confirmed hit compound can provide some clues about its target pathways. However, large numbers of phenotypically related compounds contain more information than the sum of their parts. Using large numbers of structurally diverse primary

hit compounds, it should be possible to enrich for clues about the signaling pathway networks that underlie complex multi-target signaling pathways *in vivo*. How can large numbers of phenotypically related hits be identified? This requires a model organism that is well suited for large-scale chemical biology.

Behavior based drug screening in zebrafish

Zebrafish are uniquely suited to whole-organism phenotype-based chemical screening^{16,17}, and therefore to the discovery of new drugs with polypharmacological effects. Zebrafish exhibit a wealth of complex behaviors including anxiety/fear^{18–21}, mating^{22,23}, feeding^{24,25}, pain^{26–28}, sensory^{29,30}, and sleep behaviors^{31–33} (over 190 catalogued³⁴). The majority of these responses are robust, conserved and in some instances resemble those of mammals^{35,36}. For example, the acoustic startle response is a defensive reaction to potentially threatening stimuli in multiple species of vertebrates, including zebrafish^{37,38}. Compounds that modify a particular phenotype, such as zebrafish acoustic startle response, could act on common targets. Alternatively, they could act on different, or multiple, targets in the same, or parallel, signaling pathways. Although this relatively simple zebrafish behavior does not directly simulate human CNS pathologies, the response is controlled by evolutionarily conserved neurotransmitter signaling pathways³⁸. As a result, this kind of simple reflex can be useful for identifying compounds with the potential to modify neuronal signaling and behavioral circuits in humans.

Studies investigating how psychoactive compounds affect zebrafish behavior have been successfully conducted in both adult and larval animals^{31,39–46}. Many classes of psychoactive compounds can modulate zebrafish behavior including hallucinogens, stimulants, sedatives, anti-psychotics, alcohol as well as other drugs of abuse^{36,42,44,47–50}. Similar to their effects on humans, low doses of alcohol and amphetamine can increase zebrafish locomotor behavior, while higher doses of alcohol reduce locomotion^{47,51}. Benzodiazepines and barbiturates are sedatives in humans and also reduce motor activity in larval zebrafish^{12,40,48,52,53}. In addition, hallucinogens such as ibogaine change adult behavior in light/dark preference assays, promote novel tank exploration, and mirror exploration⁴². These findings suggest that zebrafish are an effective means to identify and characterize psychoactive compounds.

The first high-throughput behavior-based screens in zebrafish assayed thousands of compounds to determine their effects on the photomotor response (a stereotyped motor behavior in early zebrafish embryos initiated by high intensity light⁵⁴) and sleep/wake cycles^{31,55}. Behavior-based screens do not require a priori knowledge of precise neurological mechanisms, but rather utilize change in animal behavior as a read out. This is advantageous because much of neuronal network signaling in both healthy and pathological conditions is still unknown. Systematic behavioral profiling enabled identification of unique phenotypes. Importantly different neurological circuits control similar behavioral phenotypes^{56,57}, indicating various mechanisms of action through which screening compounds could act to alter behavior. Subsequent clustering of known and novel compounds by phenotypic similarity was then used to predict mechanisms of action^{31,55}.

Although any single assay may access a limited number of signaling pathways, a large battery of assays may provide a higher-resolution readout of many neurological pathways. Animal behavior is the output of a complex network of neurological signaling pathways^{18,29,58}. As a result, many behaviors can be used to identify neuroactive compounds and study their mechanisms¹¹. A high-resolution behavioral battery would increase the scope of neurological systems being assayed as well as further increase the multidimensional behavioral profile generated for specific small-molecules. Because zebrafish screens are scalable, they can be used to generate large databases of behavioral information. The more behavioral and chemical space covered in a screen, the greater the predictive power of the system to classify new neuroactive compounds and identify their mechanisms of action.

Identifying phenotypically-related compounds

Phenotypes, and their relationships, can be challenging to measure. As more compounds are profiled against an expanding set of assays, the predictive power of the resulting databases will likely increase. However, as the number of phenotypic dimensions increase, the relationships between phenotypes may become more challenging to identify. To systematically identify the target pathways that give rise to a given phenotype, it is first necessary to identify and categorize phenotypically related compounds from a screen. Here, we describe three approaches to phenotype classification: manual annotation, similarity ranking, and cluster analysis.

One way to classify compounds with robust phenotypes is by manual annotation. For example, assume a researcher is looking for drugs that modify animals' acoustic startle response. Whereas control animals do not respond to a particular acoustic stimulus (Figure 2a, upper panel), a subset of screened compounds causes the animals to respond in a robust and reproducible way (Figure 2a, lower panel). Expert analysis of recorded movies is one way to identify compounds that cause this behavior. Manual phenotyping can be a beneficial first step because the human brain is a powerful pattern recognition tool and the experience gives the researcher a first-hand appreciation for the entirety of the screening data set. However, despite the power of expert classification, there are many limitations to this method, such as the time constraints of scoring increasingly large datasets, user fatigue, and lack of scalability.

Objective quantification of behavioral features can help researchers to identify phenotypically related compounds. Many different similarity metrics can be used to represent phenotypic similarity (Figure 2b). Approaches to identifying phenotypically related compounds include feature extraction, hierarchical clustering and calculating distances between time series^{31,55}. For example, a plot of distances between a query profile and the entire dataset would typically show a normal (or bimodal) distribution, where a few compounds in the dataset may be defined as hit compounds depending on how closely they match the query profile (Figure 2c). Specific behavioral features can also be used to identify subsets of related hit compounds (Figure 2d). And, clustering approaches can be used to identify major clusters of phenotypically related compounds (Figure 2e). All of these approaches can be used to organize hit compounds into phenotypically related sets to

identify large sets. No single method is best in all situations. One can expect that each of these approaches will result in a large but not complete set of overlap (Figure 2f). A combination of approaches may give the most comprehensive and useful results.

Mining phenotypically related compounds for multi-target mechanisms

Phenotype-based screens can identify hundreds of primary hit compounds and it may be impractical to follow up on all of them. Once phenotypically related compounds have been identified, an important question is how to make sense of them. Common questions include: Are the primary hits reproducible? How should compounds be prioritized? What are their mechanisms of action? Here, we review two approaches for understanding hit compounds and their targets— structure-based clustering and computational target predictions— and introduce the concept of multi-target enrichment factors.

Structure-based clustering is one way that biologists can quickly organize their primary hit compounds into meaningful groups. Structurally related compounds are often prioritized for follow up studies because chemical substructures that are identified multiple times are likely to indicate truly reproducible hit compounds. However, while structure-based clustering can help researchers to focus on specific compounds and compound series, this alone does not always bring the investigator any closer to understanding the biological mechanisms about how these compounds are working.

Computational approaches from the field of systems pharmacology have been successfully used to predict single targets of single compounds. For example, the Similarity Ensemble Approach (SEA) has been used to identify both off-target interactions^{59,60}, and predict targets of novel compounds identified from in-vivo phenotypic screens in *C. elegans*⁶¹ and zebrafish⁶². Given the power of SEA to predict targets of single compounds, it may also be possible to identify predicted targets that are statistically enriched among the most phenotypically related primary hit compounds. This approach could enumerate testable hypotheses about how poorly understood and novel compounds affect behavioral phenotypes (Figure 3c). For example, in a recent study, we predicted targets for compounds found to modify *C. elegans* feeding behavior⁶¹. In the first stage, a screen yielded 84 phenotypically-related but structurally-diverse compounds, which we compared against more than two thousand human targets. Of the 84 compounds, SEA thereby predicted 79 to have one or more human targets in ChEMBL, with 572 compound-target pairs in total. Sixteen of these pairs were tested *in vitro* to validate their putative mammalian targets, of which 9 had strong activity. These mechanistic hypotheses were then analyzed *in vivo* against both biologically and pharmacologically homologous proteins through combined genetic and pharmacological perturbations. Together, these data illustrate the use of simple model organisms with target predictions to gain mechanistic information from phenotypic screening compounds. Notably, the pharmacological target predictions arising from SEA were articulated by ligands known to human targets, but it was possible to map the results back to *C. elegans* biology.

In the future, large databases of structurally-diverse compounds and their behavioral profiles may enable researchers to identify multi-target drugs with complex pharmacological profiles⁶³. Beyond identifying novel molecules, one can leverage the entire set of

phenotypically related compounds in order to gain insight into single targets by which hit compounds modify the system. For drug side-effect prediction, enrichment factors (EFs)⁶⁴ have been used to link target singletons to drug adverse events. Perhaps even more exciting is the possibility of being able to use clusters of targets to determine multi-target mechanisms^{62,65–67}. For example, by looking at how hit compounds cluster with their predicted targets (Figure 3c), one may be able to predict potential combinations of targets that form mechanistic hypotheses. Joint enrichment factors could be developed to predict combinations of targets that underlie a given phenotype (Figure 3d). These kinds of chemoinformatic target predictions can help prioritize compounds and identify targets or target combinations that would be difficult to identify by any other means.

There are many reasons why neuroactive drug phenotypes may not translate from zebrafish to humans including issues related to target engagement, the blood brain barrier, and the evolutionary conservation of specific receptor and neural pathways. Neuroactive compounds are expected to interact with different targets at different concentrations and saturating levels may engage targets above the clinical dose thus complicating phenotypic predictions. In zebrafish, many compounds are frequently tested in the μM range (high compound doses $> 100 \mu\text{M}$ are often toxic while low doses $< 100 \text{ nM}$ frequently have no effect^{31,55}). Although these concentrations may differ from the clinical dose, full dose response curves can be generated to identify behavioral profiles at specific concentrations. For example, if one wanted to identify novel antipsychotic-like compounds it might be more useful to focus on phenotypes caused by relatively low doses (because high-dose phenotypes may be caused by toxic side effects.) Ultimately, compounds identified in zebrafish should be tested in rodent and other mammalian models to fully understand their pharmacokinetics and brain penetration.

Another issue that pertains to dose is the existence of a blood brain barrier in the larval animal. Markers for the presence of a protective layer exist in zebrafish as early as 3 days post fertilization including epithelial cells and the presence of tight junctions^{68–71}. The presence of a functional blood brain barrier in the screening model may increase the chance that any hit compounds will also cross the blood brain barrier in humans. However, some zebrafish receptors may differ from their human and rodent orthologs in functionally important ways. For example, delta opioid receptors in frogs and zebrafish are insensitive to high affinity ligands at the human delta opioid receptor due to a single amino acid variation⁷². While studies like this one do raise concern for the translatability of compounds identified from a zebrafish screen, other compounds do work on human and zebrafish receptors. For example, a reversible TrpA1 ligand, optovin¹⁵ was discovered in a zebrafish behavioral screen and found to work on both mouse and human orthologs *in vivo* and *in vitro*, respectively. Thus, confirmed hit compounds identified in a zebrafish screen can translate to mammalian neuropharmacology.

A hypothetical multi-target drug discovery workflow might work as follows: First, an interesting behavioral phenotype is defined and a method of quantification is established (Figure 3a). Next, *in vivo* phenotypic screens are performed; hit compounds are identified and clustered into phenotypically related sets (Figure 3b). These related hit compounds are then analyzed via target-prediction algorithms to determine the potential target space, and

joint EFs are calculated to generate hypotheses of target combinations and larger network pathways acting in concert to produce the phenotype (Figure 3c,d). To validate these hypotheses one could then use combinations of small molecules with established pharmacology to test these hypotheses *in vivo* (Figure 3e). In this way, researchers could use structurally diverse yet phenotypically related compounds to gain insight into cellular pathways, neurological networks, and how neuroactive compounds with complex multi-target mechanisms affect the brain and behavior in intact living organisms (Figure 3f).

Phenotypically related compounds contain a wealth of biological information. By combining *in vivo* high throughput screening with multidimensional phenotyping, it should be possible to identify large sets of diverse compounds with enough statistical power to drive target prediction and identification. Examination of large and diverse chemical libraries against an extensive repertoire of zebrafish behavioral assays could identify novel neuroactive compounds with complex target interaction profiles, while systems pharmacology methods can predict mechanisms of action for these novel compounds to generate hypotheses for further *in vivo* validation. Together, this approach may describe target-signaling pathways within the cell, circuitry within neuronal networks, and even reveal mechanisms of whole animal behavioral neuropharmacology.

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Glossary

Phenotypic screening

A screening approach used in biological research to identify molecules or genes that change a cell or animals phenotype in a desired or interesting way.

Neuropharmacology

The study of small molecules or peptides that affect the nervous system, and how these interactions alter behavior.

Polypharmacology

The identification and implementation of compounds that interact with multiple biological targets and or disease pathways.

Primary hit compound

A compound that has been identified in a screen but has not been validated.

Confirmed hit compound

A primary hit compound that has been validated in subsequent assays.

In vivo

Studies performed on whole organisms, Latin for “within the living”.

Photomotor response

A stereotyped sensorimotor behavior in zebrafish provoked by visual light but is not transduced by the eyes or pineal gland.

Acoustic Startle Response

An evolutionarily conserved defensive behavioral response to adverse acoustic stimulus.

Enrichment Factor

A “guilt-by-association” metric that relates a set of compounds to a protein target, after correction against a distribution of random sets. In this review the sets are specifically groups of compounds selected from a phenotypic screen by their ability to trigger a certain behavior.

Similarity Ensemble Approach

Statistical method to predict biological targets for compounds. It uses chemical “fingerprints” to rapidly compare the 2D structure of a query compound against structures of ligands experimentally known to bind to approximately 2500 protein targets (using ChEMBL), and outputs an E-value for each association.

Systems pharmacology

A network view of drug action, rather than the canonical “one drug, one target” view. Seeks to answer questions such as which groups of targets or pathways a drug must modulate in order to achieve a therapeutic effect, as well as to understand how drug or protein target combinations can work by triggering different nodes in a multiscale biological network.

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Figure 1. Hit compounds from phenotype-based assays have more target-content

(a) Hit compounds from target-based assays are expected to contain biological information about only the target they are screened against. **(b)** Hit compounds from phenotype-based assays may contain information about multiple targets and pathways that contribute to the phenotype. As a result, sets of structurally diverse and phenotypically-related compounds are a valuable tool for understanding complex phenotypes.

Figure 2. Manual and objective quantification methods to identify phenotypically related compounds

(a) Manual observation of behavioral phenotypes, for example from an acoustic startle response assay, can be used to identify neuroactive compounds in zebrafish. (a') A sub-threshold acoustic stimulus does not initiate the acoustic startle response in vehicle treated zebrafish, (a'') but does cause a startle response when in a subset of compound treated animals. (b) An example of behavioral quantification. The motion index (*y*-axis) is plotted against time (*x*-axis). Black rectangles represent the timing and duration of the stimulus.

Vehicle treated controls (light blue) display no significant change in motion index in response to the stimulus. By contrast, animals treated with some hit compounds (dark blue) show large changes in motion index due to a sensitized startle response. **(c)** Normal distribution of phenotypic distances relative to a query profile. A small number of related compounds will match the query phenotype (outlined in red). **(d)** Scatter plot of two separate phenotypes; related compounds group together as having high magnitude in phenotype 1, but low magnitude in phenotype 2. **(e)** Hierarchical clustering algorithms can be used to group together screening compounds with similar behavioral phenotypes, potentially revealing patterns not recognized by other methods. **(f)** Venn diagram illustrating potential overlap of phenotypically related compounds identified from different methods.

Figure 3. Proposed workflow for identifying phenotypically related neuroactive drugs and their targets

(a) Example of a behavioral phenotype used to identify hit compounds from a phenotypic screen; magenta bars represent acoustic stimulus, motion index in black. (b) Similarity ranking, phenotypic features, or clustering methods can then be used to identify related hit compounds. (c) SEA algorithms used to predict hit compound-target space, where subsets of compounds could be predicted to interact with certain targets. (d) Joint enrichment factors (EFs) are calculated from full sets of phenotypically related screening compounds to predict

multiple targets potentially required for the phenotype. Plot reads outward: Inner wedges represent the first target of the enriched target-pair, and outer wedges the second target. **(e)** Validation of the single or multi-target predictions by *in vivo* treatment and phenocopy of animals with drugs of known pharmacology. Experimentally validated target pairs are colored green in (d), while pairs that do not validate are orange. Untested pairs are light blue. **(f)** Information gained from complete sets of phenotypically-related compounds, multi-target predictions, and subsequent phenocopy validation will aid in understanding networks of neuronal circuits, cellular signaling pathways, and whole animal neuropharmacology. Abbreviations: 5-HT1-2, serotonin receptor 1-2; ACh, acetylcholine; CNR1, cannabinoid receptor 1; D1-4, dopamine receptor 1-4; DAT, dopamine active transporter; GABA, gamma-aminobutyric acid; H1-3, histamine 1-3 receptor; μ 1-2, mu-opioid receptor 1-2; M1-2, muscarinic acetylcholine receptor 1-2; σ 1-2, sigma receptor 1-2; Nav, sodium ion channel; NMDA, N-methyl-D-aspartate receptor; P2X, P2X purinoreceptor; SERT, serotonin transporter; V(1a,1b,2), vasopressin(1a,1b,2)