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# Rapid behavior—based identification of neuroactive small molecules in the zebrafish

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## Abstract

Neuroactive small molecules are indispensable tools for treating mental illnesses and dissecting nervous system function. However, it has been difficult to discover novel neuroactive drugs. Here, we describe a high—throughput (HT) behavior—based approach to neuroactive small molecule discovery in the zebrafish. We use automated screening assays to evaluate thousands of chemical compounds and find that diverse classes of neuroactive molecules cause distinct patterns of behavior. These `behavioral barcodes' can be used to rapidly identify novel psychotropic chemicals and to predict their molecular targets. For example, we identify novel acetylcholinesterase and monoamine oxidase inhibitors using phenotypic comparisons and



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**AUTHOR CONTRIBUTIONS** D.K. designed and performed the research, analyzed the data, and wrote the manuscript. J.B., C.L., R.W., and B.S. analyzed and interpreted the data and contributed to the manuscript. CY.J.C., R.M., D.H., and S.K. performed experiments. A.A.W. contributed to hardware design. S.J.H, and C.A.M. contributed reagents. R.T.P designed the research, analyzed the data and wrote the manuscript. All authors contributed to data interpretation and commented on the manuscript.

For chemical treatments, light stimuli, data processing, statistical methods, and enzymatic assays, see Supplementary Methods.

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Summary sentence: Systems—level behavioral phenotyping in zebrafish efficiently identifies novel neuroactive compounds and their mechanisms of action.

computational techniques. By combining HT screening technologies with behavioral phenotyping *in vivo*, behavior—based chemical screens may accelerate the pace of neuroactive drug discovery and provide small—molecule tools for understanding vertebrate behavior.

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Neuroactive drugs discovered in the 1950s revolutionized our understanding of the nervous system and the treatment of its disorders1. Most of these drugs were discovered serendipitously when they produced unexpected behavioral changes in animals or humans. Elucidation of the targets of these behavior—modifying compounds led to major new insights into nervous system function, and many of the drugs used for treating nervous system disorders today were derived from those same, serendipitous discoveries.

Unfortunately, few novel classes of neuroactive molecules have been discovered in the last 50 years, in part because pharmaceutical discovery efforts are currently dominated by simple, *in vitro* screening assays that fail to capture the complexity of the vertebrate nervous system2. Current drug discovery approaches are typically target—based, meaning they seek to identify compounds that modify the *in vitro* activity of a specific protein target. These approaches benefit from being systematic and high—throughput. However, they generally lack the ability to discover drugs that modify nervous system function in novel ways.

Unlike target—based approaches, phenotype—based screens can identify compounds that produce a desired phenotype without a priori assumptions about the optimal target. Phenotype—based screens in cultured cells and whole organisms have identified powerful new compounds with novel activities on unexpected targets *in vivo*3. However, it has been difficult to combine chemical—screening paradigms with behavioral phenotyping, perhaps because many well—studied behaviors are too variable or occur in animals that are too large for screening in multi—well format.

A common limitation of compounds discovered by phenotype—based methods is the difficulty in determining their mechanisms of action. It has been proposed that systems—level analyses of content—rich phenotypic data could be used to identify mechanistic similarities between compounds and predict their mechanisms of action4. Repositories of high—throughput screening data such as PubChem and ChemBank are beginning to make such analyses possible, but difficulties remain, including the challenges of comparing phenotypes across disparate assay types, libraries, and experimental conditions. Theoretically, the behavioral effects of small molecules could provide sufficient content to enable compound characterization and prediction of their mechanisms of action. However, because behaviors can be complex and difficult to quantify, systems—level comparison of behavioral phenotypes would require conversion of the behaviors into simple, quantitative measures that are more amenable to such approaches.

Given the unmet need for novel psychotropic drugs, we sought to develop a small molecule discovery process that combined the scale of modern HT screening with the biological complexity of behavioral phenotyping in living animals. Here, we report development of a fully automated platform for analyzing the behavioral effects of small molecules on embryonic zebrafish. Using this platform, we have identified hundreds of behavior—modifying compounds. We further demonstrate that complex behavioral changes can be

distilled into simple behavioral `barcodes' to classify psychotropic drugs and determine their mechanisms of action.

## RESULTS

### The photomotor response

We discovered that a high intensity light stimulus elicits a stereotypic series of motor behaviors in embryonic zebrafish that we call the photomotor response (PMR) (Fig. 1a, b and Supplementary Movies 1–3). The PMR can be divided into 4 broad phases: a pre—stimulus background phase, a latency phase, an excitation phase, and a refractory phase (Fig. 1c). During the pre—stimulus phase, zebrafish embryos are mostly inactive, exhibiting low basal activity characterized by spontaneous and infrequent body flexions within their chorions. Presentation of a light stimulus elicits a robust motor excitation phase (lasting 5—7 s) characterized by vigorous shaking. This excitation phase is preceded by a latency phase (lasting 1—2 s), and is followed by a refractory phase during which basal activity is suppressed and animals do not respond to a second light pulse (Fig. 1a). We found that the PMR consistently occurred in almost all untreated animals and that the PMR assay could be scaled to 96—well plate format (Fig. 1d).

To quantify the effects of small molecules on the PMR, we selected fourteen quantitative features that can be automatically extracted from the behavioral recordings (Fig. 1c). The fourteen features are derived from the motion of embryos during seven periods of time, including one period during the pre—stimulus background phase, one period during the latency phase, three periods during the excitation phase, and two periods during the refractory phase (Fig. 1c). For each of the seven time periods, the first and third quartiles of the motion index were measured. These features were compared to the control population and depicted as a sequence of fourteen pseudo z—scores (which we call a `barcode') representing the effect of a compound on motor activity (Fig. 1c). We found that barcodes facilitated the analysis of large—scale datasets by providing a concise quantitative summary of the behaviors observed in each well (Fig. 1e).

### Effects of small molecules on the PMR

We found that known psychoactive drugs altered the PMR in distinct and reproducible ways (Fig. 2). For example, isoproterenol (**1**), a psychostimulant, increased motor activity throughout the PMR, whereas diazepam (**2**), an anxiolytic, decreased activity (Fig. 2a—i). The dopamine agonist apomorphine (**3**) lengthened the PMR latency period, whereas the cardenolide digitoxigenin (**4**) caused a vigorous and prolonged motor response to the stimulus (Fig. 2j—o). Animals exposed to 6—nitroquipazine (**5**), a serotonin reuptake inhibitor, exhibited a robust but brief motor response to the first stimulus, but unlike untreated animals also responded to the second stimulus (Fig. 2p—r). All of these small molecule—induced behavioral effects were statistically significant and reproducible across different days and different animals (Fig. 2 right column; Supplementary Table 1). We found that many neuroactive compounds showed activity between 10 uM and 100 uM in this assay, whereas they lost activity at lower concentrations (Supplementary Fig. 1) and sometimes induced toxicities at higher concentrations (Supplementary Fig. 1, digitoxigenin).

These data show that small molecules with different mechanisms of action (e.g. adrenergic, dopaminergic, serotonergic) produce distinct changes in the PMR assay, suggesting the involvement of multiple neurotransmitter pathways in the various components of the PMR. Thus, despite the novelty of this behavior, we reasoned that the PMR might have predictive value for neuroactive drug discovery.

## High—throughput behavior—based chemical screening

To determine if HT behavioral phenotyping could be used to identify novel neuroactive drugs and their mechanisms of action, we used the PMR and embryonic touch response (ETR) assays to assess the behavioral phenotypes of animals exposed to 14,000 different small molecules. To evaluate the behavioral effects of such a large number of compounds, we built a high—throughput automated screening platform capable of screening approximately 5,000 animals per microscope—day (Supplementary Table 2, Movie 4). Together, we collected behavioral information from over 25,000 wells, representing the behaviors of over a quarter million animals. The similarity of barcodes obtained from independent wells treated with a common chemical is substantially and significantly higher than background well—to—well similarity ($p < 1\times10^{-70}$; Supplementary Fig. 2), suggesting good assay reproducibility. We identified 1,627 wells (a 7% hit rate) in which at least one behavioral feature was statistically distinct from control wells (which had a 2.5% false positive rate). Overall, 56% of hits (655 unique molecules) caused various patterns of increased activity, whereas the remaining 44% of hits (327 unique molecules) were generally sedating (Fig. 3a). Although many of the molecules that reduced activity in the PMR assay were also annotated as causing a toxic (Tox) phenotype, other molecules, including known anesthetics and analgesics, were annotated as healthy sedatives (Sed) in the ETR screen (Fig. 3a) based on observation of sedation without severe cardiovascular depression or other obvious toxicities.

## Systems—level analysis of behavior—modifying molecules

Hierarchical clustering was used to organize compounds into clusters of phenotypically related molecules. For all featured clusters, we formally compared the featured barcodes with those observed in control wells and confirmed that these groups represent statistically distinct and coherent sets of behavior—modifying molecules (Supplementary Table 1). We found that clusters of barcodes representing similar phenotypes (phenoclusters) were often enriched with functionally related compounds (Fig. 3b, c, d, e). For example, one phenocluster, characterized by elevated activity in all PMR phases, is largely composed of beta—adrenergic receptor (ADBR) agonists, including isoproterenol, clenbuterol (**6**), and epinephrine (**7**) (Fig. 3c). As expected, beta—blockers have the opposite effect, and generally decrease motor activity (Supplementary Fig. 3). A second phenocluster, characterized by a prolonged latency period, is highly enriched for dopamine agonists, including dihydroergocristine (**8**), dihydrexidine (**9**), and compound PD128907 (**10**) (Fig. 3d). A third cluster, characterized by an abbreviated PMR excitation period, is enriched for adenosine receptor antagonists (Fig. 3e). In addition to sharing common mechanisms of action, many of these compounds also share common chemical scaffolds (Fig. 3f, g, h, i). Thus, behavioral barcodes can be used to sort molecules into biologically relevant functional classes.

Because functionally related molecules cause similar phenotypes, behavioral barcodes may be used to sort molecules with different cellular targets into common pathways. For example, we identified a phenocluster characterized by prolongation of the PMR excitation period that included aconitine (**11**), veratridine (**12**), strophanthidinic acid (**13**) and digitoxigenin (Fig. 3b). Aconitine and veratridine are inhibitors of voltage gated sodium ($Na^+$) channel inactivation, whereas strophanthidinic acid and digitoxigenin are inhibitors of the $Na^+/K^+$ ATPase5,6. How these molecules with different cellular targets generate the same behavioral phenotype is unknown. However, it is interesting to note that both types of molecules are predicted to increase intracellular $Na^+$ concentration. It is possible that these related mechanisms could underlie their phenotypic similarity.

## Target identification for novel hits

We hypothesized that behavioral barcodes could be used to predict mechanisms of action for novel behavior—modifying compounds. To test this possibility, we looked for clusters containing both well—characterized and completely uncharacterized molecules. For example, we identified fifteen molecules that all caused a distinctive slow—to—relax (STR) phenotype in the ETR assay (Fig. 4a). The STR phenotype involves prolonged tail flexion and a slow return to the relaxed state (Supplementary Movie 5, 6). Several of these compounds, including eserine (**14**) are known to inhibit acetylcholinesterase (AChE), whereas two other compounds, STR—1 (**15**) and STR—2 (**16**), are structurally unrelated molecules with no known activity in animals (Fig. 4a, b). The co—clustering of STR—1 and STR—2 with known AChE inhibitors suggested that these novel compounds might inhibit AChE. To test this possibility, we determined the activity of these molecules in AChE activity assays. We found that STR—1 inhibits AChE activity *in vitro* ($IC_{50}$= 500 nM), confirming that it is a novel AChE inhibitor (Fig. 4c and Supplementary Fig. 4a). Interestingly, a high concentration of STR—2 (10 μM) did not inhibit purified AChE *in vitro* (Fig. 4c and Supplementary Fig. 4a). However, STR—2 did inhibit AChE *in vivo* ($IC_{50}$= 500 nM) (Fig. 4c). Together, these observations demonstrate that *in vivo* behavioral phenotyping in zebrafish can identify novel neuroactive compounds and their mechanisms of action, including molecules that require bioactivation and would likely have been missed using *in vitro* screening assays.

In addition to clustering barcodes based on phenotypic similarity, it is also possible to cluster them using small molecule structural information. For example, we identified several structurally—related coumarins that all caused a similar PMR “magnitude stimulant” (MAG) phenotype characterized by increased PMR excitation magnitude and duration (Fig. 4d—g and Supplementary Fig. 5). To identify possible targets of the MAG compounds, we calculated their similarity against a broad panel of ligand—target sets reported as expectation (E) values7,8. The lowest E—values, reflecting the highest confidence predictions, were obtained for MAG—1 (**17**) against monoamine oxidase B (MAO—B, E—value $1.0 \times 10^{-12}$), and to a lesser extent MAO—A (E—value $9.8 \times 10^{-9}$). Other coumarins have been previously described as inhibitors of MAO—A and B9,10. However, no ligands with substitution patterns like the MAG compounds have been reported, nor have any phenotypes been described for these molecules *in vivo*. To determine if MAG compounds inhibit MAO, we tested several of them in MAO activity assays. We found that the MAG

compounds inhibit MAO *in vitro* (Supplementary Fig. 5) and that MAG—1 is particularly potent ($IC_{50}$= 1 nM), inhibiting MAO with 100 times greater potency than the known MAO inhibitor pargyline (**18**) (Fig. 4h). By contrast, high concentrations (10 μM) of the structurally related coumarin, warfarin (**19**), do not produce a MAG phenotype nor inhibit MAO activity. Together, these observations suggest that MAG compounds are novel MAO inhibitors.

### Behavior—based chemical modifier screens

Beyond identifying molecules that affect normal behaviors, it may be possible to use HT behavioral screening to identify compounds that normalize abnormal phenotypes caused by genetic mutations or pharmacological treatments. To investigate this possibility, we analyzed the suppression of behavioral phenotypes induced by molecules acting on cholinergic and adrenergic signaling pathways. Nerve agents cause seizures, paralysis and death by disrupting cholinergic signaling11. We found that the nerve agent mimetic azinphos methyl (**20**) (AzMet) also caused paralysis in zebrafish, as evidenced by reduced levels of PMR excitation in animals treated with AzMet compared to untreated controls (Fig. 5a, b). Importantly, two nerve agent antidotes used in humans, atropine (**21**) and pralidoxime (**22**) (2—PAM), counteracted the behavioral effects of AzMet in zebrafish, rescuing PMR excitation to almost normal levels (Fig. 5a, b). Similarly, the beta—adrenergic receptor antagonist bopindolol **(23**) was able to counteract the behavioral effects of beta—receptor agonist clenbuterol, restoring normal PMR profiles to agonist—treated animals (Fig. 5c, d). These examples suggest that it may be possible to establish HT behavioral assays in zebrafish to identify chemical modifiers of these, and possibly other, behavioral phenotypes.

## DISCUSSION

To date, most pharmacological studies of behavior have been relatively small scale, involving a few small molecules at most. Here, we describe the behavioral phenotypes induced by thousands of small molecules, all tested under the same carefully controlled conditions. This large—scale data set provides opportunities for 1) systematic neuroactive drug discovery in the context of the intact nervous system and 2) improving our understanding how chemicals affect the brain and behavior.

Large—scale behavioral screening provides an opportunity to discover novel neuroactive compounds for research and therapy. For example, the STR and MAG compounds described here are novel AChE inhibitors (STR—1, STR—2) or MAO inhibitors (MAG compounds) that could have therapeutic activity for treating memory loss and depression, respectively12,13. It is worth noting that for each of these novel compounds, comparison with existing neuroactive drugs was central to predicting their mechanisms of action. In the case of the STR compounds, we observed that they caused behavioral phenotypes similar to those caused by known AChE inhibitors. In the case of the MAG compounds, the SEA algorithm relied upon structural similarities between the MAG compounds and existing MAOIs to predict mechanisms. Not surprisingly, mechanism prediction is easiest when phenotypic or structural similarity exists between novel and known compounds. However,

not all behavior—modifying compounds will have structural or phenotypic similarity to well characterized molecules. In these cases, other target identification strategies will be necessary.

Future screens may be devised to identify compounds that suppress pharmacologically induced behavioral defects. These screens could be performed using the PMR assay or other behavioral assays. The feasibility of such an approach is supported by our observation that the PMR assay can detect the suppression of pharmacologically induced behavioral defects ranging from organophosphate—induced immobility to stimulant—induced hyperactivity (Fig. 5). Beyond pharmacologically induced phenotypes, it may be possible to generate genetic models of nervous system disorders and screen for small molecule modifiers of the associated behavioral defects. Recently developed technologies for targeted gene mutation in zebrafish14–16 are likely to aid such efforts.

Behavioral assays like the PMR may also be useful for screening chemicals for neurotoxicology or other undesirable behavioral effects. Testing drug candidates for neurotoxicity remains a significant challenge, and new regulations requiring the testing of thousands of compounds are expected to require millions of rodents and cost billions of dollars17. Given these costs and the ethical implications of increased animal use, inexpensive, high—throughput means of testing for neurotoxicity are needed, especially those involving non—mammalian species. Of course, questions remain about the degree of conservation of nervous system drug effects in zebrafish and humans. Although we found that many drugs with psychotropic effects in humans also cause reproducible behavioral effects on the PMR, many other psychotropic drugs failed to produce any detectable change in the PMR screen. We do not yet know the degree to which these “false negatives” are due to screening dose, failure of absorption, or imperfect conservation between zebrafish and humans. Clearly, answering these questions will be an important step toward translating behavioral pharmacology findings from zebrafish to humans.

In addition to its potential applications for drug discovery, large—scale behavior—based chemical phenotyping provides a new perspective on the relationship between small molecules and behaviors. In the experiments described here, the principal behavior under study had not previously been characterized. Despite this, the systematic approach we employed has begun to provide some hints about how the PMR is regulated. The fact that adrenergic, dopaminergic, serotonergic, and other drug classes modify the PMR in distinct ways suggests that these neurotransmitter pathways all contribute to PMR regulation. In addition, the fact that specific molecules modulate individual features of the PMR (such as the response latency, the excitation magnitude, excitation duration, etc.) suggests that this poorly understood behavior is comprised of specific functional components under distinct mechanisms of control. This information provides testable hypotheses about the functional architecture of the behavior.

In summary, we have sought to combine the *in vivo* relevance of traditional, behavior—based phenotyping with the scale and automation of modern drug discovery. Compared to *in vitro* and cell based assays, systematic phenotyping in living vertebrate animals, provides a more holistic understanding of neuropharmacology, including those effects that are

modulated by compound metabolism or caused by complex interactions with multiple biological pathways. Thus, behavioral barcoding in zebrafish could accelerate the pace of neuroactive drug discovery and improve our understanding of how chemicals affect the brain and behavior.

## METHODS

### Aquaculture

A large number of fertilized eggs (up to 5,000 embryos per day) were collected from group matings of Ekkwill or TuAB zebrafish. Embryos were raised in HEPES (10 mM) buffered E3 media in a dark incubator at 28 °C until 30 hpf. Groups of 8—10 embryos (28 hpf) were distributed into the wells of flat bottom black 96 well plates filled with E3 media (360 μl). Embryos were then incubated in a dark incubator 25 °C for chemical treatment and subsequent experiments.

### Chemical libraries

The following 6 libraries were screened: 1) The DiverSet library (10,000 compounds) from Chembridge Corporation (San Diego). 2) The Spectrum library (2,000 compounds) from Microsource Discovery Systems, Inc. (Gaylordsville, CT). 3) The Prestwick Library (1,120 compounds) from Prestwick Chemical (Illkirch, France. The Neurotransmitter (700 compounds; cat# 2810), Ion Channel (72 compounds; cat # 2805) and Orphan Ligand (84 compounds; cat # 2825) libraries were from Biomol International.

### Automated PMR assay and measurement

In a typical assay, 1000 frames of digital video were recorded at 33fps using a Hamamatsu ORCA—ER camera mounted on a Nikon TE200 microscope with a 1× objective. Instrument control and data measurement were performed using custom scripts for Metamorph Software (Molecular Devices). Each video was saved for review. The assay, including video recording and data processing, takes ~40s per well, enabling us routinely to screen approximately 500 wells (using ~ 5,000 animals) per microscope—day.

To analyze digital video recordings, custom software scripts were used to automatically draw six evenly spaced line segments across each well such that each embryo is likely to be crossed by one of the lines. The software then tracks the average intensity of the pixels for each segment over time. As the embryos move, the light intensity at some of the pixels changes. A motion index is formed by taking the absolute value of the difference in average pixel intensity for adjacent time points and then summing over the six segments. This motion index correlates with the overall amount of motion in the well, both in terms of contraction frequency and number of animals in motion.

### The embryonic touch response (ETR) assay

Following the PMR assay, each individual animal in every well was gently touched with a fine plastic probe, and motor responses were observed using a dissecting microscope. ETR phenotypes were categorized as normal, toxic (Tox), sedative (Sed), or `Other'. A normal ETR is characterized by brief motor activity in response to the stimulus. Toxicity was

evaluated based on visual evaluation of developmental retardation, morphological abnormalities (brain, eye, ear, nose, somite, notochord, trunk, tail, fin), cardiovascular defects (heart morphology, heart rate, circulation), touch response and overall appearance. Wells in which animals did not respond to the touch stimulus, but appeared to be healthy with strong heart beats and circulation were annotated as `sedative' to distinguish them from toxic compounds that may also have suppressed the normal touch response. Chemicals were annotated as `Other' if they induced some other remarkable behavioral phenotype that was not commonly seen including uncommonly strong or prolonged motor responses to the stimulus.

### Chemoinformatics

We used the Similarity Ensemble Approach (SEA) algorithms8,18 to predict candidate molecular targets for every hit compound, using Daylight topological fingerprints19, as previously described8. A filtered version of the Wombat 2006.2 database20 (Sunset Molecular, New Mexico) provided a reference set of target—annotated compounds. For the clustering by chemical similarity, pairwise Tanimoto similarities were calculated between all compounds using ECFP_4 fingerprints in Pipeline Pilot v6.1.1.0 (Accelrys, San Diego California).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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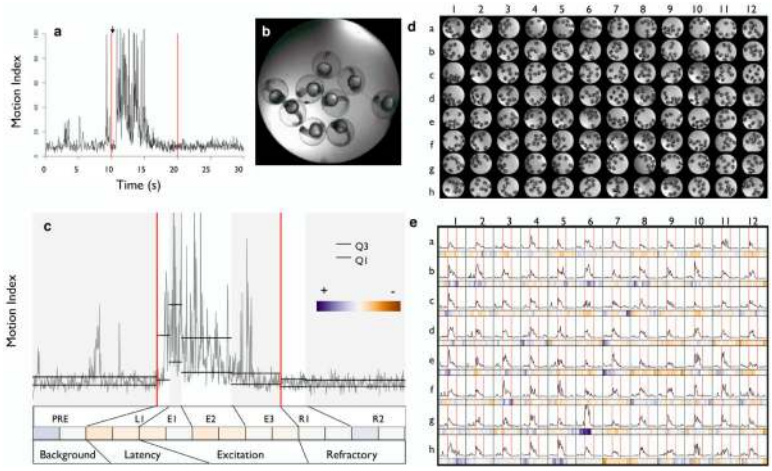


**Figure 1.**

The PMR and high—throughput behavioral barcoding. (**a**) Representative plot of the aggregate motor activity (in arbitrary units) over time (in seconds) from all animals in a single control well during the PMR assay. Prior to the first light stimulus (red bar at 10s), brief asynchronous movements in individual animals are recorded as narrow spikes with short (< 1 s) duration. A latency phase, lasting for approximately 1 s (marked with an arrow), exists between the first light pulse and the onset of the excitation phase. The PMR excitation phase is clearly distinguished from background as a large sustained increase in motor activity. Following the excitation phase, animals enter a refractory phase in which a second light pulse (red bar at 20 s) fails to elicit a response. (**b**) Nine zebrafish embryos in a single well of a 96—well plate. (**c**) Composite photograph of a 96—well microtiter plate prior to behavioral analysis. Each well contains 8—10 unhatched zebrafish embryos. (**d**) Fourteen features extracted from each PMR plot are converted into a `behavioral barcode.' Horizontal parallel lines in each of 7 time periods show the values of the motion index at the $75^{th}$ percentile (Q3), and $25^{th}$ percentile (Q1). Colors in the heat map represent their deviation from the average control phenotype. Purple represents increased activity and orange represents decreased activity. (**e**) PMR profiles and the respective behavioral barcodes of 96 untreated control wells.

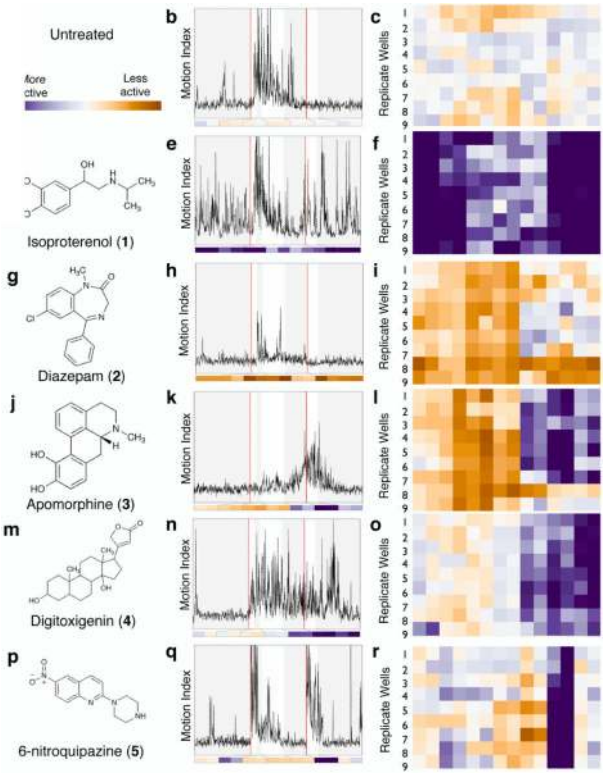


**Figure 2.**

Neuroactive chemicals cause specific patterns of behavior. (**a**—**c)** The photomotor response (PMR) in untreated control animals. Colors indicate motor activity levels as compared to the control population, with purple representing increased activity and orange representing decreased activity. (**d**—**f**) Isoproterenol, a psychostimulant, increases zebrafish motor activity (p = 6.53e-08). (**g**—**i**) Diazepam, an anxiolytic, reduces motor activity (p = 2.33e-07). (**j**—**l**) Apomorphine, a dopamine agonist, increases PMR latency period duration (p = 1.88e-06). (**m**—**o**) Digitoxigenin, a cardiotonic steroid, prolongs duration of the PMR excitation phase (p = 6.03e-07). (**p**—**r**) 6—Nitroquipazine, an SSRI, modulates the PMR refractory period (p = 6.73e-07). (**c, f, i, l, o, r**) Nine replicate behavioral barcodes from independent wells treated with the indicated compound.

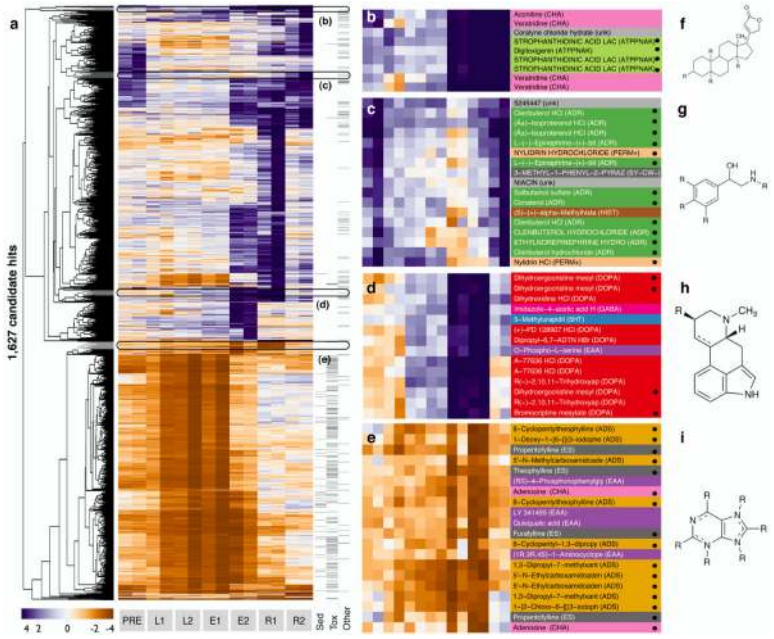


**Figure 3.**

Hierarchical clustering reveals that compounds cluster with functionally similar molecules. (**a**) A dendrogram of behavioral barcodes for all 1,627 hits clustered by phenotypic similarity. Candidate hits (*y*—axis) are clustered based on behavioral features (*x*—axis) from the PMR assay. Grey bars denote the presence of the indicated phenotypes in the ETR assay. The four clusters circled on the dendrogram are shown at higher resolution in panels **b, c, d** and **e**. Chemical names are colored based on annotated activity codes provided in parentheses. Compounds without functional annotation are labeled unknown (unk). (**b**) A phenocluster exhibiting excitation period prolongation is enriched for compounds that alter intracellular sodium levels (p = 4.71e-07). Inhibitors of $Na^+$ channel inactivation (CHA) and the $Na^+/K^+$ pump (ATPPNAK) are colored pink and light green respectively. (**c**) A cluster exhibiting generalized stimulation is enriched for β-adrenergic receptor agonists (p = 9.94e-14)(ADR, dark green). (**d**) A cluster exhibiting prolonged latency is enriched for dopamine receptor agonists (p < 2.2e-16) (DOPA, red). (**e**) A cluster exhibiting attenuated excitation is enriched for adenosine receptor antagonists (p = < 2.2e-16) (ADS, orange). Indicated compounds (•) are structural analogs of the scaffold shown to the right of each cluster (**f, g, h, i**). $PERM^+$, membrane permeability inhibitor; HIST, histaminergic; GABA, GABAergic; 5HT, serotonergic; EAA, glutamatergic; ES, esterase inhibitor.

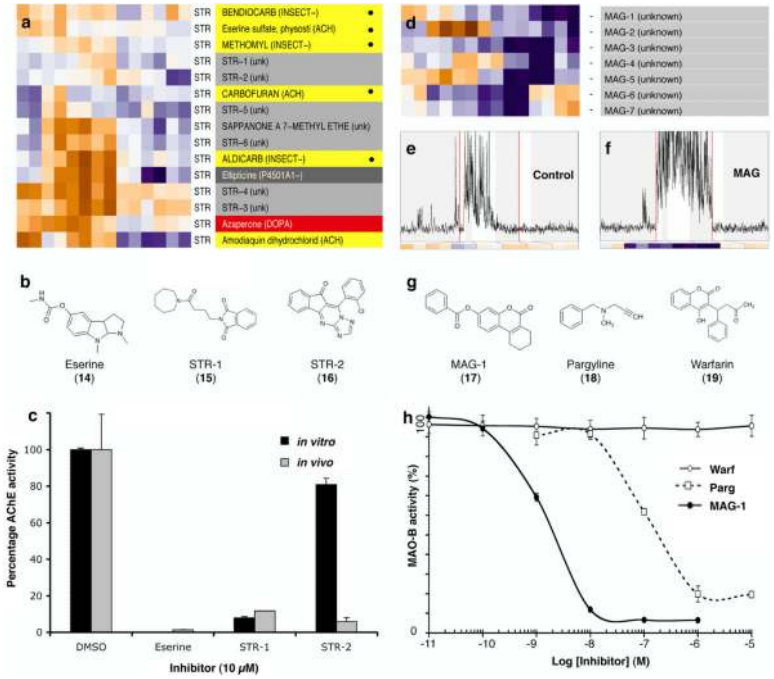


**Figure 4.**

Behavior—based discovery of novel neuroactive small—molecules. (**a**) Behavioral barcodes of chemicals causing the STR phenotype (p = 0.00034). Known AChE inhibitors are colored yellow. (**b**) Structures of eserine, a known AChE inhibitor, and two previously uncharacterized compounds that also cause the STR phenotype, STR—1 and STR—2. (**c**) AChE activity *in vitro* (black bars) or *in vivo* (gray bars). Data represent mean values ± s.d.. INSECT, insecticide; ACH, AChE inhibitor; P4501A1, cytochrome P450. (**d**) (**e**) Behavioral barcodes of structurally related MAG compounds. (**e, f**) Representative PMR plots of a control well compared to the effect of a MAG compound. Note the stimulus—dependent increase in excitation magnitude and duration. (**g**) Chemical structures of warfarin, a negative control, the known MAO inhibitor pargyline, and MAG—1. (**h**) MAO—B activity after treatment with the indicated compounds. Data points and error bars represent mean values ± s.d..

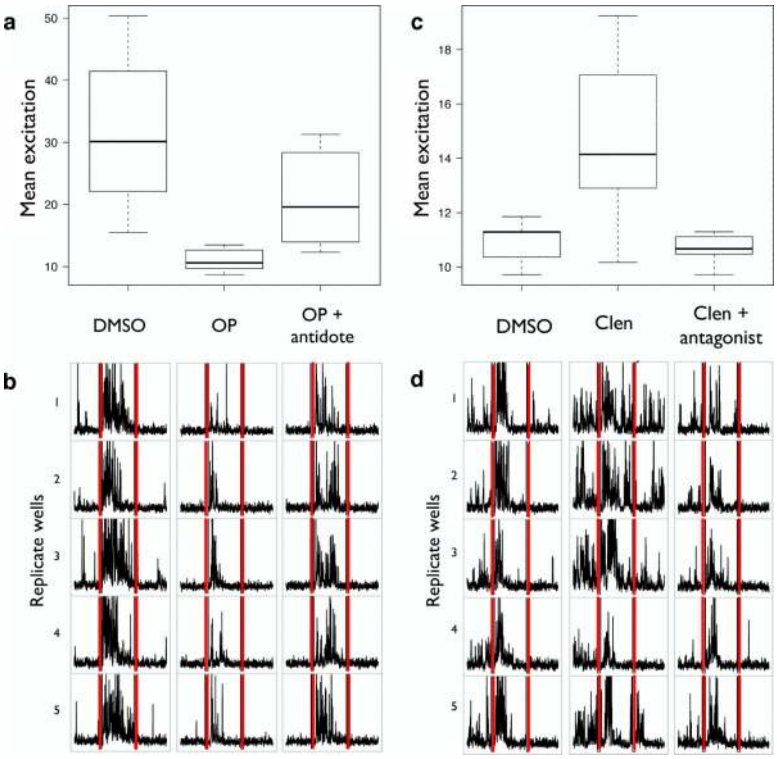


**Figure 5.**

Chemical suppression of behavioral phenotypes. (**a**) Boxplots showing the mean excitation values for the 5 replicate wells shown in (**b**) and treated with DMSO alone, AzMet, an OP nerve poison, or AzMet co—treated with 2—PAM and atropine. **(c)** Boxplots showing the mean excitation values for the 5 replicate wells shown in (**d**) and treated with DMSO, clenbuterol (Clen), or clenbuterol and the beta—receptor antagonist bopindolol. For each treatment the box represents the middle half of the distribution of the data points stretching from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The bold line across the box represents the median. The lengths of the lines above and below the box are defined by the maximum and minimum datapoint values.