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Zebrafish Behavioral Profiling Links Drugs to Biological Targets and Rest/Wake Regulation

Jason Rihel[*,1,#], **David A. Prober**[*,1,†], **Anthony Arvanites**[4], **Kelvin Lam**[4], **Steven Zimmerman**[1], **Sumin Jang**[1], **Stephen J. Haggarty**[5,6,7], **David Kokel**[8], **Lee L. Rubin**[4], **Randall T. Peterson**[5,8,9], and **Alexander F. Schier**[1,2,3,4,5,#]

[1]Department of Molecular and Cellular Biology; Harvard University, Cambridge, MA 02138

[2]Division of Sleep Medicine, Harvard Medical School, Boston, MA 02115

[3]Center for Brain Science, Harvard University, Cambridge, MA 02138, USA

[4]Harvard Stem Cell Institute; Harvard University, Cambridge, MA 02138, USA

[5]The Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

[6]Stanley Center for Psychiatric Research, Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA 02142, USA

[7]Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02142, USA

[8]Developmental Biology Laboratory, Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129, USA

[9]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

Abstract

A major obstacle for the discovery of psychoactive drugs is the inability to predict how small molecules will alter complex behaviors. We report the development and application of a high-throughput, quantitative screen for drugs that alter the behavior of larval zebrafish. We found that the multi-dimensional nature of observed phenotypes enabled the hierarchical clustering of molecules according to shared behaviors. Behavioral profiling revealed conserved functions of psychotropic molecules and predicted the mechanisms of action of poorly characterized compounds. In addition, behavioral profiling implicated new factors such as ether-a-go-go-related gene (ERG) potassium channels and immunomodulators in the control of rest and locomotor activity. These results demonstrate the power of high-throughput behavioral profiling in zebrafish to discover and characterize psychotropic drugs and to dissect the pharmacology of complex behaviors.

Most current drug discovery efforts focus on simple in vitro screening assays. Although such screens can be successful, they cannot recreate the complex network interactions of whole organisms. These limitations are particularly acute for psychotropic drugs because brain activity cannot be modeled in vitro (1,2 and supplemental text 1). Motivated by recent small molecule screens that probed zebrafish developmental processes (3–6), we developed a whole organism, high-throughput screen for small molecules that alter larval zebrafish locomotor behavior. We used an automated rest/wake behavioral assay (7,8) to monitor the activity of

#to whom correspondence should be addressed, schier@fas.harvard.edu, rihel@fas.harvard.edu, Tel: 617.496.4835, Fax: 617.495.9300.
†current address: Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA
*equal contribution

larvae exposed to small molecules at 10–30 μM for three days (Fig. 1A and supplemental text 2). Multiple behavioral parameters were measured, including the number and duration of rest bouts, rest latency, and waking activity (i.e. activity not including time spent at rest) (Fig. 1B and (8)). We screened 5648 compounds representing 3968 unique structures and 1680 duplicates and recorded more than 60,000 behavioral profiles. 547 compounds representing 463 unique structures significantly altered behavior relative to controls, using a stringent statistical cutoff (8).

Because the alterations in behavior were multi-dimensional and quantitative, we assigned a behavioral fingerprint to each compound and applied clustering algorithms to organize molecules according to their fingerprints (Fig. 2A and S1–S3). This analysis organized the dataset broadly into arousing and sedating compounds and identified multiple clusters corresponding to specific phenotypes (Fig. 2B–F, Fig. 3A–C, Fig. 4B–C, Fig. S1–S4). Clustering allowed us to address three questions: 1) Do structural, functional, and behavioral profiles overlap? 2) Does the dataset predict links between known and unknown small molecules and their mechanisms of action? 3) Does the dataset identify unexpected candidate pathways that regulate rest/wake states?

Cluster analysis revealed several lines of evidence that molecules with correlated behavioral phenotypes often shared annotated targets or therapeutic indications (Fig. 2B–F, Fig. S1–3). First, drug pairs were more likely to be correlated if the compounds shared at least one annotated target (median correlation when sharing one target, 0.561 vs. 0.297 when sharing zero targets; Fig. S5A–B). Second, analysis of 50 different structural and therapeutic classes revealed that drugs belonging to the same class produced highly correlated behaviors in nearly all cases (Figs. S5C-6 and supplemental text 3). For example, several structurally diverse selective serotonin reuptake inhibitors (SSRIs) similarly reduced waking, and sodium channel agonist insecticides induced large increases in waking activity (Figs. S5C and S6). Third, behavioral profiling uncovered the polypharmacology of drugs with multiple targets. For example, the profile of the dopamine reuptake inhibitor and muscarinic acetylcholine receptor antagonist 3α-bis-(4-fluorophenyl) methoxytropane correlated only with drugs that also shared both properties, such as the anti-Parkinson's drug trihexyphenidyl (Figure S7 and supplemental text 4). Fourth, modulators of the major neurotransmitter pathways often induced similar locomotor and rest/wake effects in zebrafish larvae as in mammals (Figs. S8–S15 and supplemental text 5). For example, α2-adrenergic receptor agonists (e.g. clonidine) were sedating whereas β-adrenergic agonists (e.g. clenbuterol) were arousing, as in mammals (Fig. S8). These analyses indicate that compounds with shared biological targets yield similar and conserved phenotypes in our high-throughput behavioral profiling.

Detailed analyses revealed that the clustering of well-known and poorly characterized drugs could predict targets for compounds whose mode of action has been unclear (Fig. 3). For example, the pesticide amitraz co-clustered with α2-adrenergic agonists (Fig. 3A), supporting reports that amitraz causes clonidine-like side effects in mammals and binds to α2-adrenoreceptors (9). Similarly, sinapic acid methyl ether co-clustered with N-methyl-D-aspartic acid (NMDA) receptor antagonists (Fig. 3B), suggesting that the mild anxiolytic effect of sinapic acid in mice is due to NMDA receptor antagonism rather than γ-aminobutyric acid (GABA) receptor activation, as proposed (10). Indeed, several sinapic acid analogs are known to block NMDA induced excitotoxicity in vitro (11–12). Finally, MRS-1220, an adenosine A3 receptor antagonist (13) clustered with monoamine oxidase (MAO) inhibitor antidepressants (Fig. 3C). To directly test whether MRS-1220 inhibits MAO, we performed an in vitro activity assay and found an IC_{50} of ~1 μM (Fig. 3D). Thus, behavioral profiling in zebrafish larvae can predict and identify targets of poorly characterized compounds.

In addition to revealing a conserved neuropharmacology between zebrafish and mammalian rest/wake states (supplemental text 5 and Figs. S8–S15), behavioral profiling identified additional pathways involved in rest/wake behaviors. 1) L-type calcium channel inhibitors of the verapamil class increased rest with minimal effects on waking activity (Fig. S16). This is likely a direct effect on rest regulation, because average waking activity and associated muscle activity were unaffected. 2) Cluster analysis identified two structurally related podocarpatrien-3-ones that specifically increased rest latency (Fig. 4A). These and other compounds also revealed that total rest, rest latency, and waking activity can be disassociated, indicating that these processes can be regulated by distinct mechanisms (supplemental text 6). 3) Although inflammatory cytokine signaling has long been known to promote sleep during infection, a role for the immune system in normal vertebrate sleep/wake behavior has not been described (14). Behavioral profiling revealed that a diverse set of anti-inflammatory compounds increased waking activity during the day with much less effect at night (Fig. 4B, Fig. S17). These anti-inflammatory compounds included the steroidal glucocorticoids, the non-steroidal anti-inflammatory drugs (NSAIDs), phosphodiesterase (PDE) inhibitors, and other compounds with anti-inflammatory properties, including the immunosuppressant cyclosporine and the mood stabilizer valproic acid. Taken together, these data suggest that inflammatory signaling pathways not only induce sleep during infection (14) but also play a role in setting normal daytime activity levels. 4) Ether-a-go-go-gene related (ERG) potassium channel blockers selectively increased waking activity at night without affecting total rest (Fig. 4C, S18). This phenotype was induced by compounds with divergent therapeutic indications (e.g. the anti-malarial halofantrine, the antipsychotic haloperidol, the anti-histamine terfenadine); however, these drugs also inhibit the ERG channel and can cause the heart rhythm disorder long QT syndrome (15, 16). Rank-sorting all the screened compounds by their fingerprint's mean correlation to the ERG-blocking cluster resulted in a significant enrichment of known ERG blockers in the top ranks (Fig. 4D). Moreover, the specific ERG inhibitor dofetilide increased nighttime activity, whereas structurally related non-ERG blocking compounds, including the antihistamines fexofenadine and cetirizine, did not (Fig. S18B). Finally, this phenotype was not caused by general misregulation of potassium channels because psora-4, a drug that blocks the related shaker potassium channel, Kv1.3, induced a distinct phenotype (Fig S18A). These results suggest that ERG potassium channels play a role in regulating wakefulness at night that is distinct from the role of shaker channels in regulating sleep in flies and mice (17, 18).

In summary, behavioral profiling reveals relationships between drugs and their targets, demonstrates a conserved vertebrate neuropharmacology, and identifies regulators of rest/wake states. Our findings have two major implications for the fields of neurobiology, pharmacology, and systems biology. First, behavioral profiling has the potential to complement traditional drug discovery methodologies by combining the physiological relevance of in vivo assays with high-throughput, low-cost screening (supplemental text 7). Future screens can be expanded to include many more uncharacterized compounds and to assay additional phenotypes, including those associated with human psychiatric disorders. In this way, behavioral profiling can characterize large classes of compounds and reveal differences in effectiveness, potential side effects, and combinatorial properties that might not be detected in vitro. Second, behavioral profiling allows for the systematic dissection of the pharmacology of complex behaviors. Our screen profiled the effects of dozens of neurotransmitter pathways and identified small molecules that regulate discrete aspects of rest/wake states. Future experiments can test drug combinations to identify synergistic or antagonistic effects among psychotropic compounds and build interaction maps. High throughput behavioral profiling thus opens the possibility to apply the logic and approaches of systems biology to neuropharmacology and behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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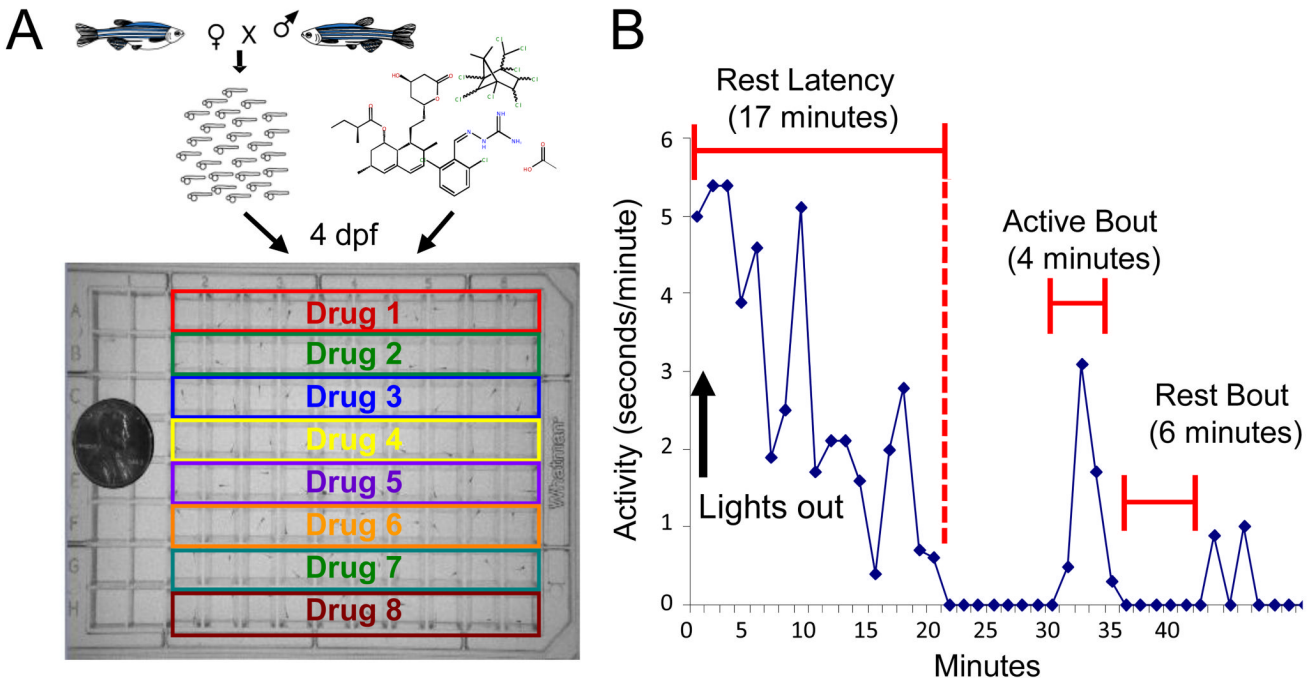

Figure 1. Larval zebrafish locomotor activity assay

(A) At four days post fertilization (dpf), an individual zebrafish larva is pipetted into each well of a 96-well plate with small molecules. Automated analysis software tracks the movement of each larva for 3 days. Each compound is tested on 10 larvae. (B) Locomotor activity of a representative larva. The rest and wake dynamics were recorded, including the number and duration of rest bouts (i.e. a continuous minute of inactivity, (7)), the timing of the first rest bout following a light transition (rest latency), the average waking activity (average activity excluding rest bouts), and the average total activity. Together, these measurements generate a behavioral fingerprint for each compound.

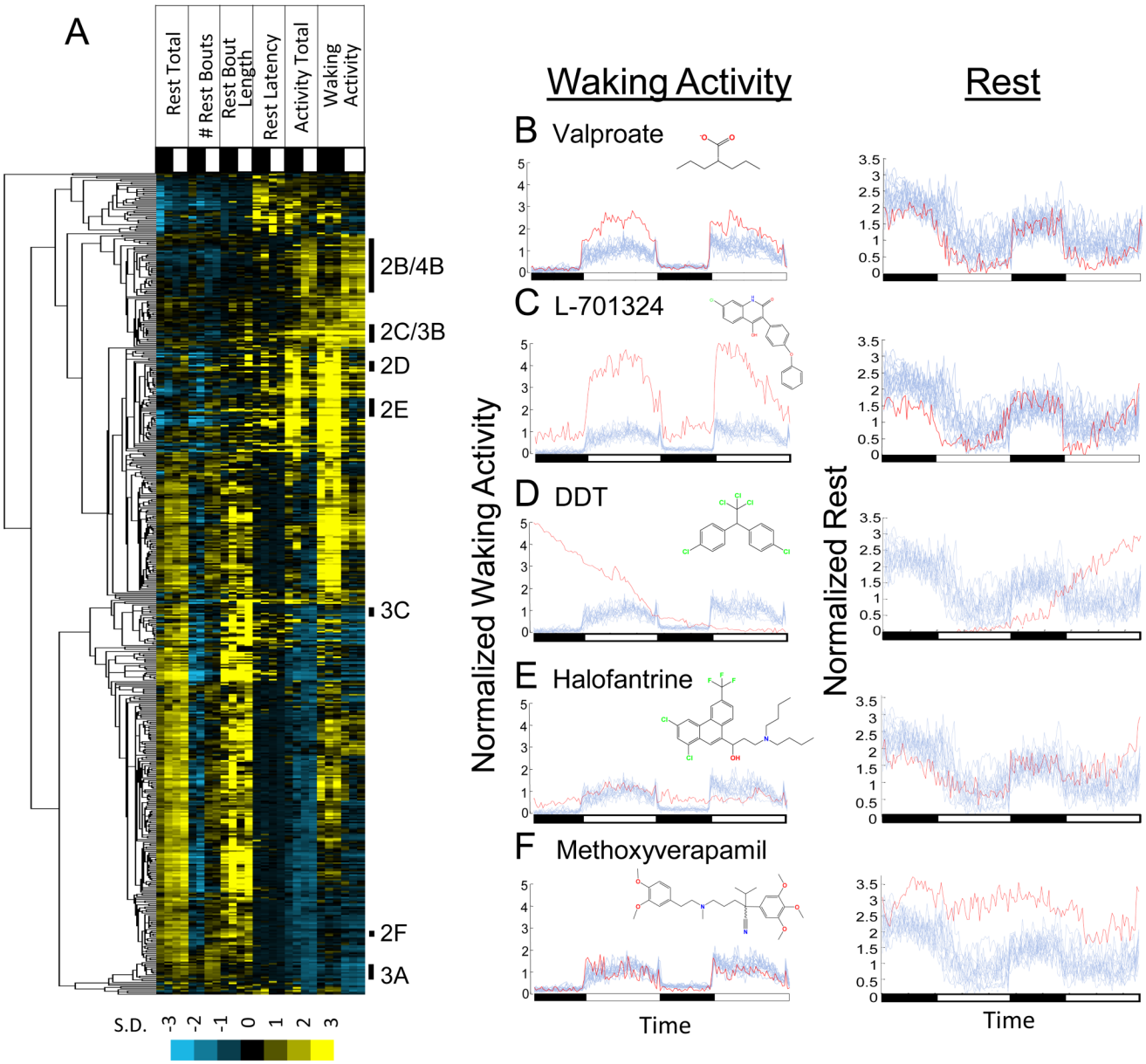

Figure 2. Hierarchical clustering reveals the diversity of drug-induced behaviors

(A) Behavioral profiles are hierarchically clustered to link compounds to behaviors. Each square of the clustergram represents the average relative value (in standard deviations; yellow = higher than controls, blue = lower than controls) for a single behavioral measurement. Dark bars indicate specific clusters analyzed in subsequent figures. (B–F) Normalized waking activity and rest graphs are plotted for behavior-altering compounds (red trace; average of 10 larvae) and representative controls (10 blue traces; average of 10 larvae each). Compounds that altered behavior include the mood stabilizer and anti-epileptic drug sodium valproate (B), the psychotomimetic NMDA antagonist L-701324 (C), the sodium channel agonist pesticide DDT (D), the anti-malarial halofantrine (E), and the calcium channel blocker methoxyverapamil (F).

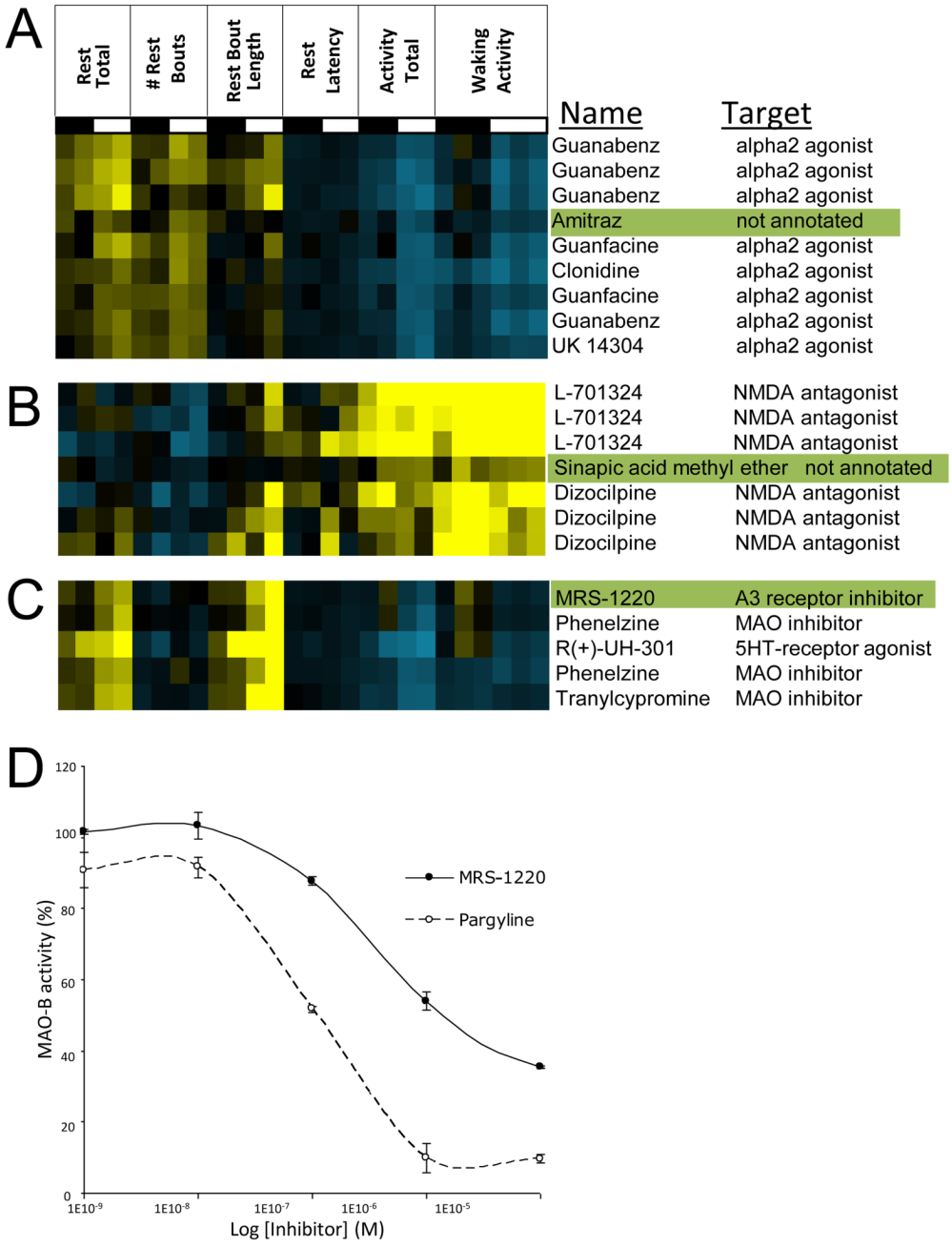

Figure 3. Predicting primary and secondary biological targets for poorly characterized compounds

(A) The pesticide amitraz co-clusters with α2-adrenergic agonists. (B) Sinapic acid methyl ether co-clusters with NMDA antagonists. (C) MRS-1220 co-clusters with MAO inhibitors. (D) MRS-1220 inhibits MAO-B activity in an enzymatic assay with an IC_{50} of ~1μM. Pargyline is a known MAO-B inhibitor (19). The clusters include repeats from different chemical libraries.

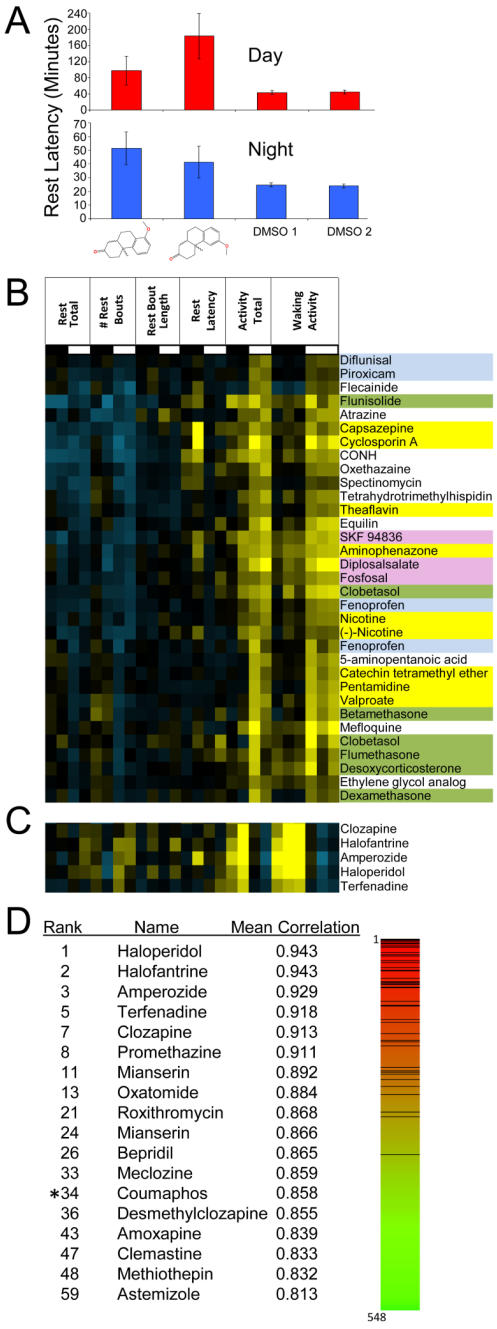

Figure 4. Unexpected regulators of zebrafish rest/wake states

(A) Podocarpatrien-3-one analogs increase rest latency, the time from light transition to the first rest bout, relative to controls. Error bars represent +/− SEM (B) Many wake-promoting anti-inflammatory and immunomodulating compounds co-cluster (blue—NSAIDs; green—glucocorticoids; pink—PDE inhibitors; yellow—miscellaneous; white—no anti-inflammatory annotation). See Figure S17 for an extended list. (C) A cluster of ERG-blocking compounds specifically increases waking activity at night. (D) Rank sorting the data set by correlation to the ERG blocking cluster results in a significant enrichment of ERG blockers in the top ranks [p<10–13 by the Kolmogorov-Smirnov statistic (see methods)]. Black lines indicate known ERG blockers; red indicates high correlation, green indicates low correlation to the ERG

cluster. This analysis also detected potential indirect regulators of ERG function, for example the organophosphate coumaphos (marked with an asterisk), which causes long QT through an unknown mechanism (20).