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Authors

Vliet, Sara M
Ho, Trina C
Volz, David C

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Behavioral screening of the LOPAC^{1280} library in zebrafish embryos

Sara M. Vliet[a,b], **Trina C. Ho**[b], and **David C. Volz**[b,*]

[a]Environmental Toxicology Graduate Program, University of California, Riverside, CA, USA

[b]Department of Environmental Sciences, University of California, Riverside, CA, USA

Abstract

Spontaneous activity represents an early, primitive form of motor activity within zebrafish embryos, providing a potential readout for identification of neuroactive compounds. However, despite use as an endpoint in chemical screens around the world, the predictive power and limitations of assays relying on spontaneous activity remain unclear. Using an improved high-content screening assay that increased throughput from 384 to 3,072 wells per week, we screened a well-characterized library of 1,280 pharmacologically active compounds (LOPAC^{1280}) – 612 of which target neurotransmission – to identify which targets are detected using spontaneous activity as a readout. Results from this screen revealed that (1) 8% of the LOPAC^{1280} library was biologically active; (2) spontaneous activity was affected by compounds spanning a broad array of targets; (3) only 4% of compounds targeting neurotransmission impacted spontaneous activity; and (4) hypoactivity was observed for 100% of hits detected, including those that exhibit opposing mechanisms of action for the same target. Therefore, while this assay was able to rapidly identify potent neuroactive chemicals, these data suggest that spontaneous activity may lack the ability to discriminate modes of action for compounds interfering with neurotransmission, an issue that may be due to systemic uptake following waterborne exposure, persistent control variation, and/or interference with non-neurotransmission-related mechanisms.

Keywords

zebrafish; spontaneous activity; LOPAC^{1280}; high-content screening; behavior

1. Introduction

The use of mammalian models for drug discovery and toxicity testing is costly, time-intensive, and requires millions of dollars per chemical (Kuhlmann, 2000, 1999; Zon and Peterson, 2005). Moreover, ethical concerns regarding high animal use have accelerated the

*Corresponding author. Tel: +1 951 827 4450; fax: +1 951 827 3993. david.volz@ucr.edu (D.C. Volz).

Conflict of Interest Statement

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development of alternative testing methods to reduce, refine, and ultimately replace animal use for chemical testing (Arora et al., 2011; Bal-Price et al., 2012; Crofton et al., 2011; National Academies Press, 2007; Richmond, 2002; Zon and Peterson, 2005). As a result, high-throughput screening (HTS) and high-content screening (HCS) assays have been developed over the last decade to screen more chemicals at a lower cost within a shorter period of time (Möller and Slack, 2010; Persson and Hornberg, 2016; Zhu et al., 2014). The vast majority of HTS and HCS assays used for drug discovery and toxicity testing utilize cell-free and cell-based methods that model key biological events across a wide range of pharmacologically- or toxicologically-relevant pathways (Fernandes et al., 2009; Zanella et al., 2010; Zhu et al., 2014). However, since these assays do not adequately reflect the complex physiology of an intact organism, the use of smaller, alternative non-mammalian animal models (such as nematodes and fish embryos) have been proposed as complementary models, as these models are (1) suitable for microplate-based assays and (2) not protected by animal use regulations around the world (Coecke et al., 2007; Crofton et al., 2012).

Zebrafish offer one of the most promising alternative and cost-effective vertebrate models to support drug discovery and toxicity testing (MacRae and Peterson, 2015; Persson and Hornberg, 2016; Zon and Peterson, 2005), particularly for identification of neuroactive drugs and/or neurotoxic chemicals (Kokel et al., 2010; Lee and Freeman, 2014; Rihel and Schier, 2012). As such, a repertoire of zebrafish-based behavioral assays across multiple life-stages (including adulthood) have been developed over the last 10–15 years (Bang et al., 2002; Brockerhoff et al., 1995; Egan et al., 2009; Kokel et al., 2010). Although different forms of larval and adult zebrafish locomotion have been leveraged within behavioral assays, spontaneous activity (tail contraction) – a behavior that occurs from late-segmentation (~17–19 hours post-fertilization, hpf) through early-pharyngula (~27–29 hpf) – represents an early, primitive form of motor activity within zebrafish embryos, providing a potential readout for rapid identification of neuroactive chemicals (Kokel et al., 2010; Raftery et al., 2014; Reif et al., 2015; Truong et al., 2016). Although previous studies have explored the biological basis of this behavior (Knogler et al., 2014; Knogler and Drapeau, 2014; Saint-Amant and Drapeau, 2001, 2000), it remains unclear whether spontaneous activity is responsive only to certain compounds with specific mechanisms of action.

In 2014, we developed a high-content screening (HCS) assay that quantifies background (unstimulated) spontaneous activity within single zebrafish embryos after exposure to chemicals in 384-well plates (Raftery et al., 2014). Within this assay, 192 viable embryos were arrayed into a 384-well plate, resulting in one embryo per well and 16 initial embryos per treatment. Following static exposure from 5 to 25 hpf, automated image acquisition procedures and custom analysis protocols were then used to quantify spontaneous activity within live, non-malformed embryos using a 6-s video per well. Although survival and imaging success rates were >85% and the total assay duration was <30 hours, we observed a high degree of natural variability in the percent of control embryos exhibiting spontaneous activity despite efforts to control for developmental stage, temperature, and light conditions (Raftery et al., 2014). Moreover, initial attempts to use 384 embryos per plate were unsuccessful due to prolonged image acquisition times, resulting in across-plate variation in spontaneous activity (Raftery et al., 2014).

To address these challenges, the objectives of the present study were to (1) decrease assay control variability by increasing video duration per well from 6 to 18 s; (2) increase assay throughput from 384 (one plate) to 3,072 wells (eight plates) per week by imaging four wells simultaneously (rather than one well at a time); (3) assess the reproducibility of our improved assay using negative and positive control wells across replicate plates; and (4) using our improved assay, reveal which targets are detected using spontaneous activity as an integrative behavioral readout. To accomplish the final objective, we screened the commercially available LOPAC^{1280} (Library of Pharmacologically Active Compounds) library – a widely used library of 1,280 marketed drugs, failed development candidates, and well-characterized small molecules that (1) span a broad molecular weight range (36 to 1,485 g/mol), (2) represent multiple mechanisms of action, and (3) target a diverse set of biological receptors. In addition, nearly half of the LOPAC^{1280} library targets neurotransmission, providing an ideal, commercially available resource for identifying which targets are detected using spontaneous activity while, at the same time, increasing our understanding of targets that may be involved in regulating this behavior during early zebrafish embryogenesis (and presumably other vertebrates). For all assays, abamectin – an avermectin insecticide and potent anticonvulsant within zebrafish embryos (Raftery et al., 2014; Raftery and Volz, 2015) – was used as a positive control.

2. Materials and Methods

2.1 Animals

For this study, we used a transgenic (*fli1:egfp)* strain of zebrafish that stably express enhanced green fluorescent (eGFP) protein within vascular endothelial cells (Lawson and Weinstein, 2002), as this strain begins expressing eGFP at ~14 hpf. Adult *fli1:egfp* zebrafish were maintained on a recirculating system with UV sterilization and mechanical/biological filtration units (Aquaneering, San Diego, CA, USA), and were kept under a 14-h:10-h light:dark cycle at a water temperature of ~27–28°C, pH of ~7.2, and conductivity of ~900–950 μS. Water quality was constantly monitored for pH, temperature, and conductivity using a real-time water quality monitoring and control system. Ammonia, nitrate, nitrite, alkalinity, and hardness levels were manually monitored weekly by test strip (Lifeguard Aquatics, Cerritos, CA). Zebrafish were fed twice per day with dry diet (Gemma Micro 300, Skretting, Fontaine-lès-Vervins, France). Adult males and females were bred directly on-system using in-tank breeding traps suspended within 3-L tanks. For all experiments described, newly fertilized eggs were collected within 30 min of spawning, rinsed, and reared in a temperature-controlled incubator (28°C) under a 14-h:10-h light:dark cycle. All embryos were sorted and staged according to previously described methods (Kimmel et al., 1995). All adult breeders were handled and treated in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved animal use protocol (#20150035) at the University of California, Riverside.

2.2 Chemicals

Abamectin was purchased from ChemService, Inc. (West Chester, PA, USA), and a low-volume (25 μl per compound) library of 1,280 pharmacologically active compounds (LOPAC^{1280}) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of

abamectin (50 mM) were prepared by dissolving abamectin in high performance liquid chromatography (HPLC)-grade dimethyl sulfoxide (DMSO) and stored at room temperature within 2-ml amber glass vials containing polytetrafluoroethylene (PTFE)-lined caps. Stock solutions (25 μl of 10 mM stock per compound) of the LOPAC^{1280} library (16 96-well racks containing 80 compounds per rack) were prepared and provided in DMSO by Sigma-Aldrich and stored at −30°C upon arrival; with the exception of partition coefficient (LogP) values, all compound-specific information was provided by Sigma-Aldrich within a Microsoft Excel spreadsheet following acquisition of the LOPAC^{1280} library. For each individual plate, working solutions of all treatments were freshly prepared by diluting stock solutions 1:1000 into embryo media (EM) (10 mM NaCl, 0.17 mM KCL, 0.66 mM $CaCl_2$, 0.66 mM $MgSO_4$), resulting in 0.1% DMSO within all vehicle control and treatment groups.

2.3 Assay Setup

Newly fertilized embryos were collected immediately following spawning and incubated in groups of approximately 50 per plastic petri dish until 5 hpf. Embryo media, vehicle control (0.1% DMSO), positive control (abamectin), or test solution (50 μl/well) was loaded into a black 384-well microplate containing 0.17-mm glass-bottom wells (Matrical Bioscience, Spokane, WA, USA). For the LOPAC^{1280} screens, vehicle (0.1% DMSO) and positive (6.25 μM abamectin) control groups each occupied two columns (32 wells per group) flanking the left and right sides of the plate (0.1% DMSO: columns 1 and 24; 6.25 μM abamectin: columns 2 and 23), whereas each LOPAC^{1280} compound occupied one column (16 wells) on each plate. At 5 hpf, 384 viable *fli1:egfp* embryos were manually arrayed into each well of a 384-well plate over a 45-min time period, resulting in one embryo per well. The plate was then covered with a plate lid, wrapped with parafilm to minimize evaporation, and incubated at 28°C under a 14-h:10-h light:dark cycle until 24 hpf. At 24 hpf, the plate was placed in a second incubator at 25°C for 1 h to acclimate embryos to room temperature prior to imaging. At 25 hpf, the plate was then centrifuged for 2 min at 200 rpm to ensure all embryos were positioned at the well bottom.

2.4 Image Acquisition and Analysis

Using a time-lapsed image acquisition protocol optimized for our ImageXpress Micro (IXM) XLS Widefield High-Content Screening System equipped with MetaXpress 6.0.3.1658 (Molecular Devices, Sunnyvale, CA), four wells were simultaneously imaged every 0.3 s over an 18-s time period using a 2X objective and FITC filter cube, resulting in a total of 96 acquisitions per 384-well plate, 60 frames per acquisition, and 5,760 frames per 384-well plate. In addition, a 4X objective and FITC filter cube was used to acquire one frame per entire well for assessment of survival and quantification of total body area. During the entire ~30-min image acquisition period, internal temperature within the IXM system was maintained using previously described procedures (Raftery et al., 2014). Embryos were then euthanized by placing the plate at −30°C.

Using fully automated custom journal scripts, four-well frames were divided into individual quadrants and used to generate 18-s videos (.AVI files) representing individual wells. Videos (384 per plate) were manually checked to assess the presence or absence of spontaneous tail contractions and then analyzed within EthoVision XT 9.0 (Noldus Information Technology,

Leesburg, VA) using previously described procedures (Raftery et al., 2014). Using images captured with a 4X objective, survival and total body area was also quantified using previously described procedures (Raftery et al., 2014). Treatments resulting in a significant decrease in total body area, <85% survival, or gross malformations were not analyzed for spontaneous activity.

2.5 LOPAC^{1280} Library Screen

We relied on a two-tiered strategy to screen the LOPAC^{1280} library. For Tier I, embryos were exposed from 5–25 hpf to each compound at a single limit concentration of 10 μM. Compounds were identified as Tier-I hits if exposure resulted in a significant effect on survival, total body area, or spontaneous activity. Compounds resulting in autofluorescence, gross malformations (deformed axis, tail malformations, or underdeveloped head), or <85% survival were not analyzed for effects on total body area and spontaneous activity, and compounds resulting in a significant decrease in total body area were not analyzed for effects on spontaneous activity (Supplementary Figure S1).

2.6 Statistical Analysis

All statistical analyses were performed using SPSS Statistics 23.0 (IBM, Chicago, IL, USA). Total body area was analyzed using a general linear model (GLM) analysis of variance (ANOVA) ($\alpha = 0.05$), as these data did not meet the equal variance assumption for non-GLM ANOVAs. Pair-wise Tukey-based multiple comparisons of least-squares means were performed to identify significant treatment-related effects; treatments were only considered significant if total body area was statistically different from both vehicle control columns. Spontaneous activity data were analyzed using nonparametric tests, as these data were categorical and did not meet assumptions of normality. A Kruskal–Wallis test ($\alpha = 0.05$) was used to test for main effect of treatment, and Mann-Whitney pairwise comparisons were used to test for differences between and among vehicle control, positive control, and treatment columns. For the LOPAC^{1280} screens, plates were analyzed for differences between vehicle controls (columns 1 and 24) and positive controls (columns 2 and 23) as well as treatments (columns 3–22) relative to vehicle and positive controls ($\alpha = 0.05$); treatments were only considered significant if statistically different from both vehicle control columns and both positive control columns. If spontaneous activity between vehicle or positive control columns was statistically different based on the statistical tests described above, treatment columns were not analyzed and the plate was repeated.

3. Results

3.1 Assay Variability

To determine whether differences in 1-h acclimation temperatures affected spontaneous activity, we reared and acclimated a total of 96 embryos across two independent plates at 23, 28, or 33°C from 24–25 hpf (following incubation from 5–24 hpf at 28°C) under normal light conditions. For all temperatures, percent survival was >85% for each column and total body area was consistent within and across acclimation temperatures (Supplementary Figure S2). Although there was a slight increase in the percent of embryos displaying spontaneous

activity following acclimation at 33°C, no significant differences in spontaneous activity were observed among all three acclimation temperatures (Figure 1).

After confirming that spontaneous activity was not significantly impacted by acclimation temperature, we reared and acclimated a total of 1,152 embryos across three independent negative control (embryo media only) plates from 5–24 hpf at 28°C and 24–25 hpf at 25°C; each plate was loaded on separate days to account for potential day-to-day variation. At 25 hpf, each plate was analyzed for survival, total body area, and spontaneous activity. For all control plates, embryo survival was >85% and there were no significant within-plate nor plate-to-plate differences in total body area (Supplementary Figure S3). However, the percent of embryos with spontaneous activity ranged from 30–93% per column across all three control plates, with the majority of columns displaying 50–74% activity (Figure 2).

3.2 Assay Reproducibility

Two replicate concentration-response curves for abamectin (0.09–50 μM) – a positive control within our assay – were screened on a single plate to identify the lowest concentration resulting in complete elimination of spontaneous activity in the absence of effects on survival or total body area (Supplementary Figure S4). Based on these concentration-response curves (Figure 3A), three independent plates were then screened using 6.25 μM abamectin to confirm that effects on spontaneous activity were reproducible within and across plates (Figure 3B). For all three plates, embryo survival was >85% and there were no significant within-plate nor plate-to-plate differences in total body area (Supplementary Figure S5). Although vehicle controls were variable, there were no significant differences in spontaneous activity among vehicle control columns across all three plates. However, exposure to 6.25 μM abamectin resulted in complete elimination of spontaneous activity within and across all three plates (Figure 3B).

3.3 LOPAC^{1280} Library Screen

Based on a 5- to 25-hpf (20-h) exposure, approximately 8% (102 compounds) of the LOPAC^{1280} library was biologically active (relative to vehicle controls) due to significant effects on survival (51 compounds), total body area (5 compounds), or spontaneous activity (46 compounds) (Figure 4). For all three endpoints, Tier-I hits represented a wide range of compounds that were specific to both neurotransmission- and non-neurotransmission-related targets (Figure 5; Supplementary Data S1). Interestingly, all Tier-I spontaneous activity hits were driven by a significant decrease in spontaneous activity (hypoactivity), even for compounds that target the same receptor (e.g., dopamine receptor) but exhibit opposing modes of action (e.g., agonist vs. antagonist) (Supplementary Data S1).

For Tier II, all Tier-I hits were screened at a 10-μM limit concentration using a 23- to 25-hpf (2-h) exposure to eliminate false positives associated with adverse developmental effects prior to 23 hpf. Based on this secondary screen, there were no significant effects on survival or total body area, and approximately 15% (15 compounds) of the Tier-I hits were positive for significant impacts on spontaneous activity (Figure 4). Out of these 15 compounds, approximately 73% (11 compounds) resulted in significant impacts to spontaneous activity in both Tier I and II screens. Similar to Tier I, all 15 Tier-II spontaneous activity hits were

driven by hypoactive effects relative to vehicle controls, and spanned a wide range of neurotransmission- and non-neurotransmission-related targets with varying modes of action (Figure 5 and Supplementary Data S1).

3.4 Data Mining

Partition coefficient (LogP) values were retrieved for all 1,280 compounds from the NCBI's PubChem database (https://pubchem.ncbi.nlm.nih.gov) (Supplementary Data S1), and molecular weights for all compounds were correlated by endpoint to determine whether hydrophobicity and/or chemical size predicted the potential for a Tier-I or Tier-II hit within our assay. There was no association between LogP and the potential for a Tier-I hit, with LogP values ranging from 0 to 10 for 98% of Tier-I hits (Figure 6A). Similarly, there was no association between molecular weight and the potential for a Tier-I hit, where molecular weights for all Tier-I hits ranged from 98 to 875 g/mol. Interestingly, while Tier-II spontaneous activity hits were not associated with molecular weights (180–875 g/mol), these hits clustered within a narrow range of LogP values (1.8–5.2) relative to Tier-I hits (Figure 6B).

Using NCBI's PubChem Chemical Structural Clustering Tool, the entire LOPAC^{1280} library was clustered based on two-dimensional (2D) structural similarity using the Single Linkage algorithm (https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?p=clustering). Within this model, a Tanimoto Similarity score of 0.68 or higher denotes a statistically significant 2D structural similarity at the 95% confidence interval. Based on this analysis, the potential for a positive hit within Tier I – as well as impacts on survival, total body area, or spontaneous activity within both Tier I and II screens – were not associated with 2D compound structural similarity (Supplementary Figure S6).

4. Discussion

This study has revealed that (1) within the Tier-I screen, approximately 8% (102 compounds) of the LOPAC^{1280} library was biologically active (at a 10-μM limit concentration) based on significant effects on survival, body area, or spontaneous activity relative to vehicle controls; (2) within the Tier-I screen, only 4% (25 compounds) of 612 LOPAC^{1280} compounds that interfere with neurotransmission impacted spontaneous activity; (3) within both screens, spontaneous activity was adversely affected by LOPAC^{1280} compounds that spanned a broad array of non-neurotransmission and neurotransmission targets; and (4) within both screens, hypoactivity was observed for 100% of hits detected, including those that exhibit opposing mechanisms of action (e.g., agonist vs. antagonist) for the same target (e.g., dopamine receptor). Therefore, while our assay was able to identify potent neuroactive chemicals, these data suggest that spontaneous activity may lack the ability to discriminate modes of action (e.g., stimulants vs. sedatives) for compounds interfering with neurotransmission, an issue that may be a result of (1) systemic uptake following waterborne exposure; (2) persistent control variation despite negligible effects of temperature acclimation and assay improvements (increased video duration per well and assay throughput) relative to our previous study (Raftery et al., 2014); and/or (3) interference with non-neurotransmission-related mechanisms.

Within embryonic zebrafish, spontaneous activity is characterized by a series of trunk coils and represents the first sign of sensory-independent locomotion (Kimmel et al., 1995; Saint-Amant and Drapeau, 1998). Early spontaneous activity consists of single coils that are driven by periodic, non-chemically-mediated depolarizations, excitation spikes, and gap-junction-mediated electrical coupling within early spinal neurons (Saint-Amant and Drapeau, 2001, 2000). On the other hand, late spontaneous activity consists of side-to-side double coils that arise through the addition of chemically-mediated synapses to the existing electrical circuit and is regulated within the spinal cord as well as the hindbrain of embryonic zebrafish (Behra et al., 2002; Knogler et al., 2014; Raftery and Volz, 2015). As such, late spontaneous activity represents an intermediate form of behavior that precedes secondary motoneuron development and bridges early spontaneous activity with stimuli-induced responses observed during later stages of embryonic and larval development (Knogler et al., 2014).

As image acquisition within our assay occurred during the peak frequency of spontaneous tail contractions (25–26 hpf when reared at 28°C) (Yozzo et al., 2013), LOPAC^{1280} compounds that interfere with electrical coupling and/or chemically-mediated neurotransmission may have the potential to adversely affect late spontaneous activity following a 5–25 hpf exposure. Based on results from both screens, spontaneous activity was similarly impacted by LOPAC^{1280} compounds targeting neurotransmission- and non-neurotransmission-related processes, where the magnitude of effect following a 10-μM exposure was, in a number of cases, equivalent for both groups of compounds. After eliminating the potential for false positive hits associated with systemic toxicity (based on survival and total body area), LOPAC^{1280} hits for spontaneous activity were not limited to compounds targeting neurotransmission, raising the possibility that, at least for our Tier-I screen (exposure from 5–25 hpf), non-neurotransmission-related compounds that impact electrical coupling during early spontaneous activity may lead to downstream effects on late spontaneous activity. However, even following a 2-h exposure from 23–25 hpf (Tier-II screen), four out of 15 hits for spontaneous activity were not classified as targeting neurotransmission, suggesting that other variables or mechanisms of action such as impaired muscle function and/or energy metabolism may influence spontaneous activity within our assay.

Interestingly, our results demonstrate that spontaneous activity may lack the ability to discriminate opposing modes of action (e.g., stimulants vs. sedatives) for neurotransmission-interfering compounds. Indeed, hypoactivity was observed for all compounds affecting spontaneous activity – even for compounds with opposing mechanisms of action for the same target. For example, within our Tier-I screen, exposure to seven different dopaminergic drugs with varying mechanisms of action (e.g., agonist, antagonist, or inhibitor) all resulted in a significant decrease in spontaneous activity. In contrast, Irons et al. (2013) examined the effect of six different dopaminergic drugs – all of which were present within the LOPAC^{1280} library – on larval zebrafish locomotion, and demonstrated that exposure to dopaminergic agonists and antagonists induce hyperactivity and hypoactivity, respectively (Irons et al., 2013). Interestingly, dopaminergic drugs that resulted in changes to larval locomotion did not significantly alter embryonic spontaneous activity within our Tier-II screen. This discrepancy between life-stages may be due to differences in the presence and function of

dopamine receptors within embryonic vs. larval zebrafish, as the expression of dopamine receptor (D1–D4) genes is not initiated until ~24 hpf (Boehmler et al., 2007, 2004; Li et al., 2007). Therefore, given that other receptors and targets for neurotransmission-related LOPAC^{1280} compounds are likely absent or minimally functional at 25–26 hpf, our data suggest that compounds may be acting through other pathways and that, regardless of whether the receptor or target is present and functional, other variables such as compound uptake, distribution, and exposure concentration may be influencing behavior within our assay.

Similar to our findings within embryonic zebrafish, a recently published study demonstrates that, even within larval zebrafish harboring a more complex nervous system, the observed behavioral response is not always consistent with the expected behavioral response based on the known mechanism and mode of action within mammals (Kirla et al., 2016). Within this study, the authors relied on cocaine as a model compound, as cocaine acts on the monoaminergic neurotransmitter systems and is a stimulant within mammals. However, contrary to the expected outcome (hyperactivity), acute exposure to non-teratogenic concentrations of cocaine resulted in a concentration-dependent decrease in locomotion (hypoactivity) within both dark and light conditions following a waterborne exposure of cocaine (Kirla et al., 2016). Importantly, the authors also quantified cocaine uptake and distribution using matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI MSI), and observed significant accumulation within the brain, eyes, and trunk of larval zebrafish. Therefore, the authors concluded that cocaine-induced hypoactivity was as a result of systemic (rather than targeted) cocaine uptake across the skin of larval zebrafish, leading to an anesthetic effect on the peripheral nervous system that suppressed stimulatory targets present in the central nervous system (Kirla et al., 2016). Likewise, within our assay, there is a strong possibility that, for compounds expected to act as stimulants, waterborne exposure may have led to non-targeted, systemic uptake and distribution that overwhelmed the intended target and significantly biased behavior (spontaneous activity) toward an unidirectional hypoactive response.

Finally, the ability of our assay to identify and classify negative vs. positive hits was likely influenced by a complex interaction among exposure duration, compound potency (at a single limit concentration of 10 μM), and toxicokinetics (compound uptake over time) – variables that are dependent on assay design and physicochemical attributes. Clearly, exposure duration relative to the timing of key developmental landmarks is an essential consideration for assay design, as 87 of 102 Tier-I (5–25 hpf) hits for survival, total body area, or spontaneous activity were negative for all three endpoints within our Tier-II (23–25 hpf) screen. However, a more significant challenge is related to uncertainties about the influence of compound partitioning from water into zebrafish embryos following a 5–25 hpf exposure. Since we did not quantify internal doses, we were unable to determine if negative hits within our Tier-I screen were a result of (1) insufficient compound uptake over a 20-h exposure (for compounds that may otherwise have been potent) or (2) sufficient compound uptake over a 20-h exposure, but minimal potency at the limit concentration (10 μM) tested. Importantly, our data suggests that physicochemical attributes do not have the potential to predict Tier-I hits within our assay, as negative and positive hits spanned a broad range of LogP values and molecular weights. On the other hand, the vast majority of Tier-I hits (98%)

had LogP values favoring water-to-embryo partitioning (LogP>0) and Tier-II hits clustered within a narrower range of LogP values relative to Tier-I hits, suggesting that a short (2-h) exposure biased hits to hydrophobic compounds within an optimal LogP range (LogP = ~2–5). Therefore, these results are consistent with other studies showing that compound bioactivity is not associated with compound size (for compounds ≤3000 g/mol) but, rather, tends to be dependent on compound hydrophobicity (LogP>0) and partitioning from water into zebrafish embryos (whether chorionated or not) over a specific exposure duration (de Koning et al., 2015; Gustafson et al., 2012; Pelka et al., 2017; Sachidanandan et al., 2008). Finally, the lack of structural similarity suggests that, similar to LogPs and molecular weights, 2D chemical structures do not have the potential to predict hits within our assay; however, it is important to note that this lack of predictability may be driven by the inherent chemical diversity of the LOPAC^{1280} library.

In conclusion, results from this study suggest that, despite the seemingly simple biological basis of spontaneous activity (relative to more complex behaviors later in development), this primitive form of locomotion was affected by a wide range of pharmacologically and structurally diverse compounds. As a result, although the use of background (unstimulated) spontaneous activity was able to identify neuroactive compounds, this behavioral readout (as used in our assay) was unable to predict biological targets and discriminate chemical modes of action. This lack of specificity was likely due to a complex set of uncertainties associated with the underlying biology of embryonic zebrafish, persistent control variability (despite our efforts to control for developmental stage, temperature, and light conditions, and increase video duration per well), decisions about assay design, and toxicokinetics (i.e., the rate and magnitude of water-to-embryo partitioning). Despite limitations highlighted in this study, spontaneous activity may still hold utility as a readout within other behavioral assays, such as the photomotor response (PMR) assay, which relies on a high-intensity light stimulus to generate more robust “behavioral barcodes” to identify compounds that interfere with startle-response and habituation (Kokel et al., 2013, 2010). However, to our knowledge, the LOPAC^{1280} library has not been screened using the PMR assay, so it’s currently unclear whether the PMR assay will also suffer from similar uncertainties and limitations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Arora T, Mehta AK, Joshi V, Mehta KD, Rathor N, Mediratta PK, Sharma KK. Substitute of animals in drug research: an approach towards fulfillment of 4Rs. Indian J Pharm Sci. 2011; 73:1. [PubMed: 22131615]

Bal-Price AK, Coecke S, Costa L, Crofton KM, Fritsche E, Goldberg A, Grandjean P, Lein PJ, Li A, Lucchini R, Mundy WR, Padilla S, Persico AM, Seiler AEM, Kreysa J. Advancing the science of

developmental neurotoxicity (DNT): testing for better safety evaluation. ALTEX. 2012; 29:202–215. [PubMed: 22892558]

Bang PI, Yelick PC, Malicki JJ, Sewell WF. High-throughput behavioral screening method for detecting auditory response defects in zebrafish. J Neurosci Methods. 2002; 118:177–187. [PubMed: 12204308]

Behra M, Cousin X, Bertrand C, Vonesch JL, Biellmann D, Chatonnet A, Strähle U. Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. Nat Neurosci. 2002; 5:111–118. DOI: 10.1038/nn788 [PubMed: 11753420]

Boehmler W, Carr T, Thisse C, Thisse B, Canfield VA, Levenson R. D4 Dopamine receptor genes of zebrafish and effects of the antipsychotic clozapine on larval swimming behaviour. Genes Brain Behav. 2007; 6:155–166. DOI: 10.1111/j.1601-183X.2006.00243.x [PubMed: 16764679]

Boehmler W, Obrecht-Pflumio S, Canfield V, Thisse C, Thisse B, Levenson R. Evolution and expression of D2 and D3 dopamine receptor genes in zebrafish. Dev Dyn. 2004; 230:481–493. DOI: 10.1002/dvdy.20075 [PubMed: 15188433]

Brockerhoff SE, Hurley JB, Janssen-Bienhold U, Neuhauss SC, Driever W, Dowling JE. A behavioral screen for isolating zebrafish mutants with visual system defects. Proc Natl Acad Sci. 1995; 92:10545–10549. [PubMed: 7479837]

Coecke S, Goldberg AM, Allen S, Buzanska L, Calamandrei G, Crofton K, Hareng L, Hartung T, Knaut H, Honegger P, Jacobs M, Lein P, Li A, Mundy W, Owen D, Schneider S, Silbergeld E, Reum T, Trnovec T, Monnet-Tschudi F, Bal-Price A. Workgroup Report: Incorporating In Vitro Alternative Methods for Developmental Neurotoxicity into International Hazard and Risk Assessment Strategies. Environ Health Perspect. 2007; 115:924–931. DOI: 10.1289/ehp.9427 [PubMed: 17589601]

Crofton KM, Mundy WR, Lein PJ, Bal-Price A, Coecke S, Seiler AE, Knaut H, Buzanska L, Goldberg A. Developmental neurotoxicity testing: recommendations for developing alternative methods for the screening and prioritization of chemicals. Altex. 2011; 28:9–15. [PubMed: 21311847]

Crofton KM, Mundy WR, Shafer TJ. Developmental neurotoxicity testing: A path forward. Congenit Anom. 2012; 52:140–146. DOI: 10.1111/j.1741-4520.2012.00377.x

de Koning C, Beekhuijzen M, Tobor-Kap on M, de Vries-Buitenweg S, Schoutsen D, Leeijen N, van de Waart B, Emmen H. Visualizing Compound Distribution during Zebrafish Embryo Development: The Effects of Lipophilicity and DMSO: Compound Distribution in the zebrafish embryo. Birth Defects Res B Dev Reprod Toxicol. 2015; 104:253–272. DOI: 10.1002/bdrb.21166 [PubMed: 26663754]

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res. 2009; 205:38–44. DOI: 10.1016/j.bbr.2009.06.022 [PubMed: 19540270]

Fernandes TG, Diogo MM, Clark DS, Dordick JS, Cabral JMS. High-throughput cellular microarray platforms: applications in drug discovery, toxicology and stem cell research. Trends Biotechnol. 2009; 27:342–349. DOI: 10.1016/j.tibtech.2009.02.009 [PubMed: 19398140]

Gustafson AL, Stedman DB, Ball J, Hillegass JM, Flood A, Zhang CX, Panzica-Kelly J, Cao J, Coburn A, Enright BP, Tornesi MB, Hetheridge M, Augustine-Rauch KA. Inter-laboratory assessment of a harmonized zebrafish developmental toxicology assay – Progress report on phase I. Reprod Toxicol. 2012; 33:155–164. DOI: 10.1016/j.reprotox.2011.12.004 [PubMed: 22210281]

Irons TD, Kelly PE, Hunter DL, MacPhail RC, Padilla S. Acute administration of dopaminergic drugs has differential effects on locomotion in larval zebrafish. Pharmacol Biochem Behav. 2013; 103:792–813. DOI: 10.1016/j.pbb.2012.12.010 [PubMed: 23274813]

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995; 203:253–310. DOI: 10.1002/aja.1002030302 [PubMed: 8589427]

Kirla KT, Groh KJ, Steuer AE, Poetzsch M, Banote RK, Stadnicka-Michalak J, Eggen RIL, Schirmer K, Kraemer T. Zebrafish Larvae Are Insensitive to Stimulation by Cocaine: Importance of Exposure Route and Toxicokinetics. Toxicol Sci. 2016; 154:183–193. DOI: 10.1093/toxsci/kfw156 [PubMed: 27521082]

Knogler LD, Drapeau P. Sensory gating of an embryonic zebrafish interneuron during spontaneous motor behaviors. Front Neural Circuits. 2014; 8doi: 10.3389/fncir.2014.00121

Knogler LD, Ryan J, Saint-Amant L, Drapeau P. A Hybrid Electrical/Chemical Circuit in the Spinal Cord Generates a Transient Embryonic Motor Behavior. J Neurosci. 2014; 34:9644–9655. DOI: 10.1523/JNEUROSCI.1225-14.2014 [PubMed: 25031404]

Kokel D, Bryan J, Laggner C, White R, Cheung CYJ, Mateus R, Healey D, Kim S, Werdich AA, Haggarty SJ, MacRae CA, Shoichet B, Peterson RT. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nat Chem Biol. 2010; 6:231–237. DOI: 10.1038/nchembio.307 [PubMed: 20081854]

Kokel D, Dunn TW, Ahrens MB, Alshut R, Cheung CYJ, Saint-Amant L, Bruni G, Mateus R, van Ham TJ, Shiraki T, Fukada Y, Kojima D, Yeh JRJ, Mikut R, von Lintig J, Engert F, Peterson RT. Identification of Nonvisual Photomotor Response Cells in the Vertebrate Hindbrain. J Neurosci. 2013; 33:3834–3843. DOI: 10.1523/JNEUROSCI.3689-12.2013 [PubMed: 23447595]

Kuhlmann J. Responsibilities of clinical pharmacology in the early phase of drug development. Med Klin Munich Ger 1983. 2000; 95:31–40.

Kuhlmann J. Alternative strategies in drug development: clinical pharmacological aspects. Int J Clin Pharmacol Ther. 1999; 37:575–583. [PubMed: 10599949]

Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev Biol. 2002; 248:307–318. DOI: 10.1006/dbio.2002.0711 [PubMed: 12167406]

Lee J, Freeman JL. Zebrafish as a Model for Developmental Neurotoxicity Assessment: The Application of the Zebrafish in Defining the Effects of Arsenic, Methylmercury, or Lead on Early Neurodevelopment. Toxics. 2014; 2:464–495. DOI: 10.3390/toxics2030464

Li P, Shah S, Huang L, Carr AL, Gao Y, Thisse C, Thisse B, Li L. Cloning and spatial and temporal expression of the zebrafish dopamine D1 receptor. Dev Dyn. 2007; 236:1339–1346. DOI: 10.1002/dvdy.21130 [PubMed: 17393486]

MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. Nat Rev Drug Discov. 2015; 14:721–731. DOI: 10.1038/nrd4627 [PubMed: 26361349]

Möller C, Slack M. Impact of new technologies for cellular screening along the drug value chain. Drug Discov Today. 2010; 15:384–390. DOI: 10.1016/j.drudis.2010.02.010 [PubMed: 20206290]

National Academies Press. Toxicity Testing in the 21st Century: A Vision and a Strategy. National Academies Press; Washington, D.C: 2007.

Pelka KE, Henn K, Keck A, Sapel B, Braunbeck T. Size does matter – Determination of the critical molecular size for the uptake of chemicals across the chorion of zebrafish (Danio rerio) embryos. Aquat Toxicol. 2017; 185:1–10. DOI: 10.1016/j.aquatox.2016.12.015 [PubMed: 28142078]

Persson M, Hornberg JJ. Advances in Predictive Toxicology for Discovery Safety through High Content Screening. Chem Res Toxicol. 2016; 29:1998–2007. DOI: 10.1021/acs.chemrestox.6b00248 [PubMed: 27766849]

Raftery TD, Isales GM, Yozzo KL, Volz DC. High-content screening assay for identification of chemicals impacting spontaneous activity in zebrafish embryos. Environ Sci Technol. 2014; 48:804–810. DOI: 10.1021/es404322p [PubMed: 24328182]

Raftery TD, Volz DC. Abamectin induces rapid and reversible hypoactivity within early zebrafish embryos. Neurotoxicol Teratol. 2015; 49:10–18. DOI: 10.1016/j.ntt.2015.02.006 [PubMed: 25733401]

Reif DM, Truong L, Mandrell D, Marvel S, Zhang G, Tanguay RL. High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Arch Toxicol. 2015; :1–12. DOI: 10.1007/s00204-015-1554-1

Richmond J. Refinement, Reduction, and Replacement of Animal Use for Regulatory Testing: Future Improvements and Implementation Within the Regulatory Framework. ILAR J. 2002; 43:S63–S68. DOI: 10.1093/ilar.43.Suppl_1.S63 [PubMed: 12388854]

Rihel J, Schier AF. Behavioral screening for neuroactive drugs in zebrafish. Dev Neurobiol. 2012; 72:373–385. DOI: 10.1002/dneu.20910 [PubMed: 21567979]

Sachidanandan C, Yeh JRJ, Peterson QP, Peterson RT. Identification of a Novel Retinoid by Small Molecule Screening with Zebrafish Embryos. PLoS ONE. 2008; 3:e1947.doi: 10.1371/journal.pone.0001947 [PubMed: 18398471]

Saint-Amant L, Drapeau P. Synchronization of an Embryonic Network of Identified Spinal Interneurons Solely by Electrical Coupling. Neuron. 2001; 31:1035–1046. DOI: 10.1016/S0896-6273(01)00416-0 [PubMed: 11580902]

Saint-Amant L, Drapeau P. Motoneuron Activity Patterns Related to the Earliest Behavior of the Zebrafish Embryo. J Neurosci. 2000; 20:3964–3972. [PubMed: 10818131]

Saint-Amant L, Drapeau P. Time course of the development of motor behaviors in the zebrafish embryo. J Neurobiol. 1998; 37:622–632. DOI: 10.1002/(SICI)1097-4695(199812)37:4<622::AID-NEU10>3.0.CO;2-S [PubMed: 9858263]

Truong L, Gonnerman G, Simonich MT, Tanguay RL. Assessment of the developmental and neurotoxicity of the mosquito control larvicide, pyriproxyfen, using embryonic zebrafish. Environ Pollut. 2016; 218:1089–1093. DOI: 10.1016/j.envpol.2016.08.061 [PubMed: 27593350]

Yozzo KL, McGee SP, Volz DC. Adverse outcome pathways during zebrafish embryogenesis: A case study with paraoxon. Aquat Toxicol. 2013; 126:346–354. DOI: 10.1016/j.aquatox.2012.09.008 [PubMed: 23046524]

Zanella F, Lorens JB, Link W. High content screening: seeing is believing. Trends Biotechnol. 2010; 28:237–245. DOI: 10.1016/j.tibtech.2010.02.005 [PubMed: 20346526]

Zhu H, Zhang J, Kim MT, Boison A, Sedykh A, Moran K. Big Data in Chemical Toxicity Research: The Use of High-Throughput Screening Assays To Identify Potential Toxicants. Chem Res Toxicol. 2014; 27:1643–1651. DOI: 10.1021/tx500145h [PubMed: 25195622]

Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. Nat Rev Drug Discov. 2005; 4:35–44. DOI: 10.1038/nrd1606 [PubMed: 15688071]

Highlights

- Assay throughput was increased from 384 to 3,072 wells per week.
- 8% of the LOPAC^{1280} library was biologically active within zebrafish embryos.
- Spontaneous activity was affected by compounds spanning a broad array of targets.
- Only 4% of compounds targeting neurotransmission impacted spontaneous activity.
- Hypoactivity was observed for 100% of hits detected.

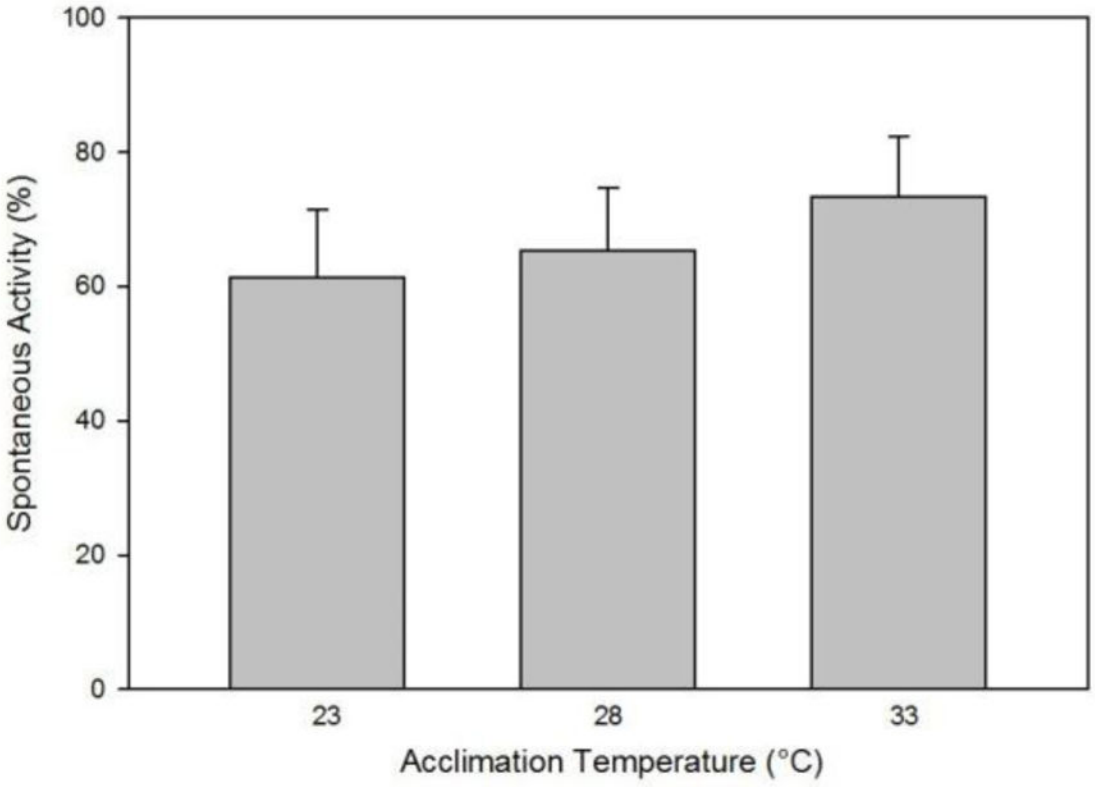

Figure 1.

Spontaneous activity is not significantly impacted by acclimation temperature from 24–25 hpf. Spontaneous activity (%) after a 1-h acclimation from 24–25 hpf at 23, 28, or 33°C under normal light conditions. Data are presented as mean ± standard deviation across two independent plates. N=96 initial embryos (six columns) per acclimation temperature.

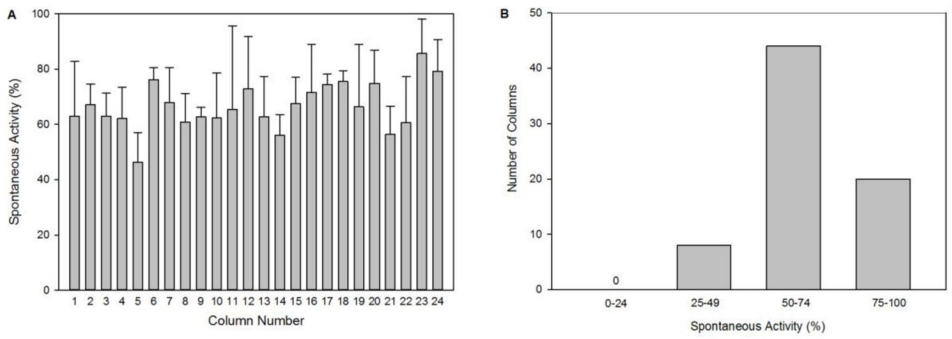

Figure 2.

Spontaneous activity is variable among control embryos within and across plates. Spontaneous activity (%) by column (A) and the number of columns within spontaneous activity (%) bins (B). Data represents spontaneous activity across three independent control plates (N= 48 initial embryos per column number). Spontaneous activity data in Panel A are presented as mean ± standard deviation.

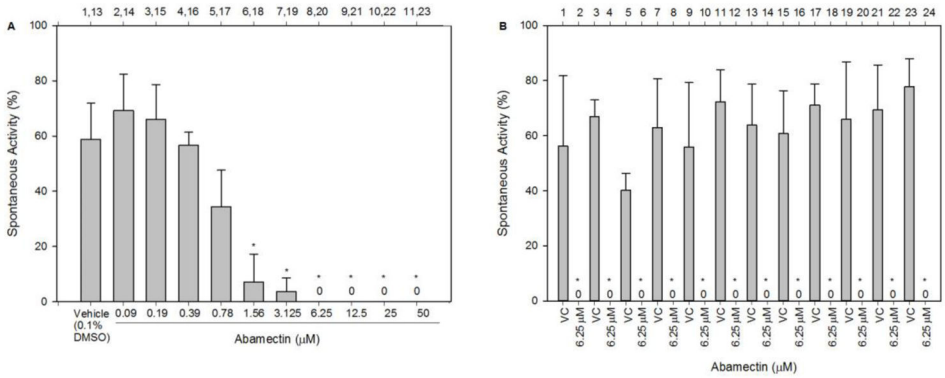

Figure 3.

Abamectin induces a concentration-dependent and reproducible decrease in spontaneous activity. Spontaneous activity (%) following exposure to abamectin in concentration-response format (A) or across three replicate plates (B). Spontaneous activity (%) in Panel A is presented as mean ± standard deviation based on two concentration-response curves (N=32 embryos per treatment group) within a single plate and asterisk denotes significant difference from vehicle controls ($p<0.05$). Spontaneous activity (%) in Panel B is presented as mean ± standard deviation across three replicate plates (N=48 initial embryos per treatment group). VC denotes vehicle control (0.1% DMSO). Asterisk denotes significant difference from all vehicle control columns on plate ($p<0.05$). Column numbers corresponding to each treatment group are provided along the x-axis above each panel.

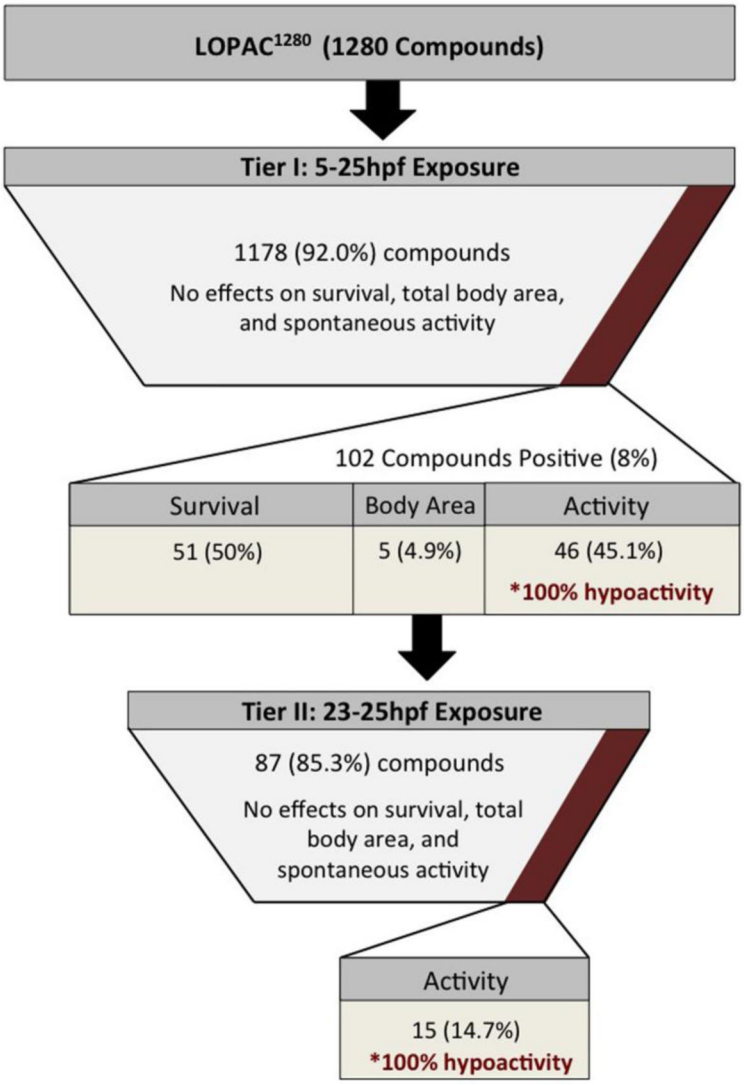

Figure 4.

Summary of results from two-tiered behavioral screening of the Library of Pharmacologically Active Compounds (LOPAC^{1280}).

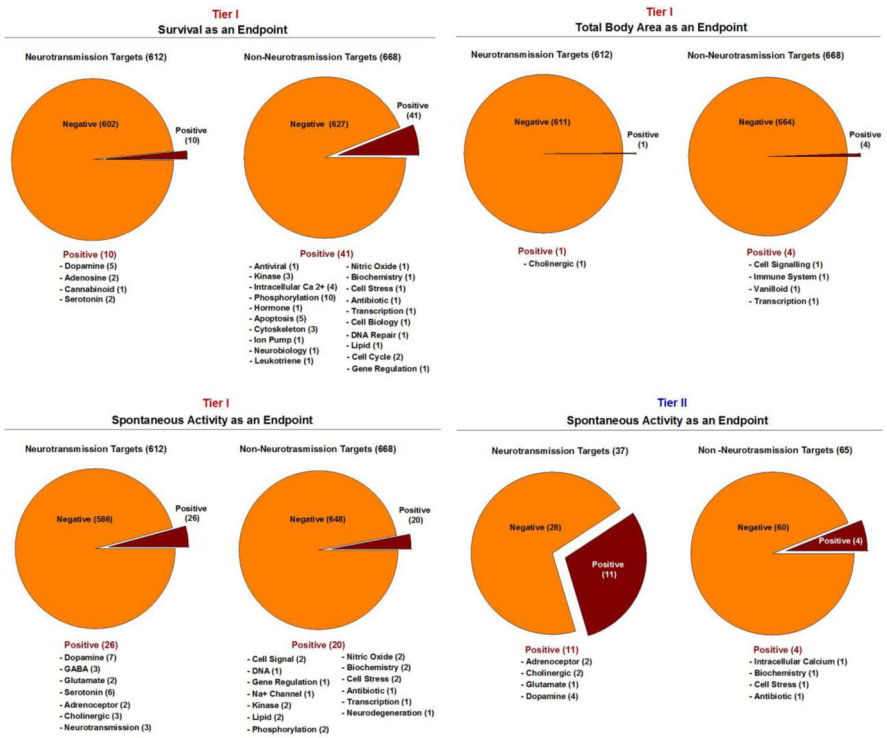

Figure 5.

LOPAC^{1280} Tier I and II hits are not associated with unique Sigma-Aldrich-defined classes of biological targets. LOPAC^{1280} Tier I and II hits are presented as a function of 1) endpoint (survival, total body area, or spontaneous activity); 2) whether the compound targets neurotransmission; and 3) Sigma-Aldrich-defined biological class. Numbers within parentheses represent the total number of positive compounds within each group.

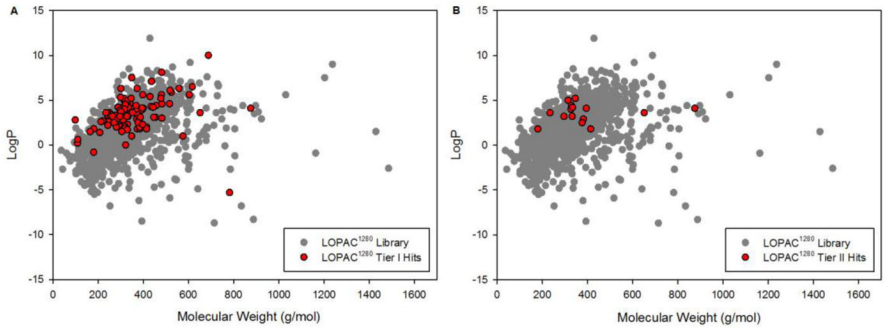

Figure 6.

LOPAC^{1280} hits are not associated with LogP and molecular weight. LogP values and molecular weights for all compounds were correlated to determine whether hydrophobicity and chemical size predicted the potential for a Tier I (A) or Tier II (B) hit within our assay.