Chapter 15

Assessing the Maximum Predictive Validity for Neuropharmacological Anxiety Screening Assays Using Zebrafish

Amanda Linker, Adam Stewart, Siddharth Gaikwad, Jonathan M. Cachat, Marco F. Elegante, Allan V. Kalueff, and Jason E. Warnick

Abstract

The development of reliable pharmacological screening assays is an important task. However, it is based upon the ability of animal models, such as the zebrafish, to demonstrate predictive validity for a specific set of drug classes. A popular assay used for this purpose is the novel tank diving paradigm, where zebrafish behavior can easily be modulated by anxiolytic or anxiogenic drug exposure. However, predictive validity may fail to provide crucial information about the model, such as comparisons of drug efficacy and the effects of drugs on varying behavioral phenotypes. This deficit is accounted for by a novel measure termed the Maximum Predictive Value (MPV), which provides an estimate of how sensitive a particular model is when assessing its potential pharmacologically. Here we provide a protocol detailing how to employ this measure to validate behavioral endpoints in the novel tank test for use in pharmacological studies in zebrafish. Similar approaches can be used to examine drug efficacy in other zebrafish-based behavioral tests.

Key words: Maximum predictive value, zebrafish, pharmacological screening, model, novel tank.

1. Introduction

In behavioral neuroscience, the use of animal models rests on the assumption that appropriate assays have been chosen to assess the desired phenotype, disease, or drug. While considerable attention has been given to the development and assessment of animal-based biobehavioral assays and simulations of neuropsychiatric disorders (1–11), little scrutiny was given to improving

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pharmacological screening assays. In determining which drug screens to employ, researchers often turn to predictive validity to assess the models' effectiveness (8–11). Predictive validity is the selectivity whereby an animal model responds to a specific class of drugs. Since these assays are primarily utilized as industrial-based tools, attainment of predictive validity is a critical necessity (10). For instance, if a screening-assay yields false negatives, researchers may unknowingly dismiss chemicals that may have therapeutic potential. Likewise, when screening assay exhibits false positives, a researcher may waste valuable resources on a substance with no potential for future development (10).

Although predictive validity is an important measure, it is dichotomous in nature (i.e., a model either does or does not possess it) (12). This represents a major shortcoming for pharmacological research, since predictive validity fails to provide the ability to differentiate the level of efficacy between drugs in a model (12). Furthermore, if multiple drug screening assays are found to possess predictive validity, this evaluative standard does not have the ability to compare the level of drug effects between these models (12). This fails to provide important information necessary for model development or selection, like statistical power, which could influence important decisions such as the number of animals to be utilized, and necessary drug dosage (12).

In an effort to move beyond the evaluative standard of predictive validity, the measure known as maximum predictive value (MPV) was developed (12, 13). This measure converts a drug's effect in a model to a standardized mean difference and allows researchers to look across multiple scores to find the largest, which provides a general estimate of how sensitive a particular model is when assessing its potential in pharmacological testing (12). This measure is a good complement to psychopharmacology research as it accounts for several factors common to this field. First, this statistic utilizes the measures of group mean differences, which is the typical data reported in behavioral research. Second, the MPV score provides a common metric that allows the comparison of multiple models. That is, this measure provides the ability to directly compare diverse behavioral measures like the number of open arm entries in the elevated-plus maze and the amount of time spent in social contact in the social interaction test. Lastly, the measure moves beyond a simple measure of statistical significance on which predictive validity is often determined.

While statistical significance testing is an important research tool, it has major limitations that can influence the interpretation of predictive validity. For example, statistical significance can be influenced by the number of subjects used in an experiment. Thus, when a drug fails to produce a statistically significant effect, it might not reflect the model's predictive validity but inadequate sample sizes. Similarly, the experiment needs to

possess enough statistical power to produce a statistically significant effect. Therefore, a failure to produce a statistically significant effect might be due to a drug dose that is too low instead of poor predictive validity.

Again, the MPV measure allows researchers to look across multiple scores to find the largest, which provides an estimate of how sensitive a particular model is when assessing its potential in pharmacological testing (12). Due to the differences in protocols between laboratories (e.g., strain differences, drug dosage differences, vehicle differences, etc), looking across multiple studies to find the largest score keeps the findings in the context of the original study. This measure allows researchers to make critical decisions about choice of organism, drug dose, and experimental protocol (12, 13).

Assessing the MPV for a variety of pharmacological agents can reveal response patterns that would be missed by simply evaluating predictive validity (12, 13). These analyses will allow us to quantitatively assess the validity of specific behavioral endpoints, collectively revealing our model's overall validity. Moreover, the modulation of several behavioral endpoints can be used to derive a specific MPV score, such as through testing a variety of anxiolytic and anxiogenic drugs, with varying doses and durations. Additionally, the data generated using this approach serves to identify which endpoints, associated with a particular behavioral assay, correlate with the highest positive MPV value (e.g., thereby indicating the drugs' ability to function as an anxiolytic or anxiogenic).

One of the most popular zebrafish behavioral paradigms is the novel tank diving test, extensively used for modeling the anxiolytic and anxiogenic properties of pharmacological agents and already comprehensively covered in this volume (*see* Chapter 1 of this book for details). Utilizing the exploratory behavior and robust endpoints exhibited by zebrafish, this assay allows for the quantification of various indices to assess a drug's overall functionality at a given dose. Here we provide a protocol that utilizes the MPV measure to assess a zebrafish model of anxiety based on the novel tank diving test, to determine which behavioral endpoints are valid constructs to test pharmacological compounds.

2. Methods and Materials

2.1. Animals and Housing

Adult zebrafish (≈50:50 male:female ratio) can be obtained from commercial distributors and tested in a standard novel tank test (refer to Chapter 1 by Cachat et al. in this book for details). Room and water temperatures are maintained at 25–27°C, with illumination provided by ceiling-mounted fluorescent light tubes

on a 12-h cycle (on at 8.00, off at 20.00). All fish are experimentally naïve at the time of testing.

Apparatus: The novel tank used for this protocol is a 1.5-L trapezoidal tank (15.2 height × 27.9 top × 22.5 bottom × 7.1 width cm; Aquatic Habitats, Apopka, FL) maximally filled with aquarium-treated water. Novel tanks are to be rested on a level, stable surface and divided into two equal virtual horizontal portions, marked by a dividing line on the outside walls of the tank. The setup may also include a camera or webcam (e.g., 2.0-Megapixel, Gigaware, UK) for further video-aided analysis of recorded trials.

2.2. Maximal Predictive Validity

The behavioral data obtained from a particular experiment shows how many standard deviations apart the two groups (e.g., experimental and control cohorts) are. Data for the MPV is taken from manual and computer-based observations. In the current protocol, positive MPV values indicate a drug's anxiolytic effect (reduction of anxiety-like behaviors) whereas negative values demonstrate anxiogenic effects (enhancement of an anxious state).

3. Procedure

3.1. Acclimation and Pre-treatment

Move the fish from their holding room to the experimental room for acclimation 1 h prior to testing. After acclimation, pre-treat the animals via individual immersion into a 3–4 L beaker containing the drug dissolved in ~3 L water. Drug concentration and treatment duration are determined through examination of previous literature.

3.2. Novel Tank Testing

Following pharmacological pre-treatment, zebrafish are individually placed in the novel tank. Once relocated to novel tanks, behavior should be recorded over a 6-min period manually by two trained observers and by a computer. The following endpoints are recorded: number of transitions (entries) to the upper portion of the tank, time spent in the upper portion of the tank (s), number of erratic movements, number of freezing bouts, freezing duration (s), and latency to reach the upper portion of the tank (s) (14–15). Erratic movements were defined as sharp changes in direction or velocity and repeated rapid darting behaviors. Freezing was defined as a total absence of movement, except for the gills and eyes, for 2 s or longer. Significant decreases in exploratory behavior (longer latency to reach the top, fewer entries to the top, longer freezing) or elevated erratic movements and freezing represent behavioral phenotypes indicative of high stress and anxiety (for details, *see* Chapter 1 in this book).

3.3. Measuring the Maximum Predictive Value of a Model

To determine the maximum predictive value (MPV), calculate the ratio of the mean difference between two groups and their pooled standard deviations as follows:

$$\text{Maximum Predictive Value} = \frac{\text{Mean}_{\text{treatment}}}{\frac{\text{Pooled Standard Deviations}}{\sqrt{2}}}.$$

$$\text{Pooled Standard Deviations} = \sqrt{\frac{(n_{\text{control}} - 1)\,\text{Variance}_{\text{control}} + (n_{\text{treatment}} - 1)\,\text{Variance}_{\text{treatment}}}{n_{\text{control}} + n_{\text{treatment}}}}.$$

Given the mathematical simplicity of this measure, our lab typically calculates MPV scores with a spreadsheet software program (e.g., Microsoft Office Excel).

4. Anticipated Results

The administration of anxiogenic and anxiolytic compounds can be expected to produce MPV values that correlate with the functionality of a drug. For example, our group has found that treatment with the anxiolytics, diazepam and fluoxetine, possess scores paralleling known drug effects. For example, in our experiments with diazepam, three of four trials resulted in significant positive MPV values for both # *of Entries to Upper Half* and *Duration In Upper Half*, with respective values of 2.268 and 2.005 for one trial, and 2.859 and 3.192 for the second, both providing interpretation as behavioral anxiolytic endpoints (**Table 15.1**). Furthermore, we have also found that fluoxetine produces a dramatic increase in MPV scores for *Duration in Upper Half*, *Average Entry Duration,* and *Latency to 1st Transition* in comparison to acute and chronic administration studies (**Table 15.1**).

However, the experimenter should also expect data of considerable complexity that warrants careful interpretation. For example, the acute administration of alarm pheromone (7 mL) can produce both anxiogenic and anxiolytic results. Our group found that zebrafish in this group demonstrate a greater # *of Erratic Movements* (MPV –1.958) and *Freezing Bouts* (MPV –1.673), as well as longer *Freezing Duration* (MPV –1.005), and an increased *Latency to the 1st Transition* (MPV –3.472). These behaviors indicate higher anxiety levels. Interestingly, the zebrafish in this group also had a higher # *of Entries to Upper Half* (MPV 3.559) and

Table 15.1
Maximum predictive validity (MPV) analyses for selected anxiolytic compounds

Experimental conditions	Endpoint	MPV score	Experimental conditions	Endpoint	MPV score
Diazepam	# of Entries to Upper Half	–0.05	Diazepam	# of Entries to Upper Half	**2.27**
Dose			*Dose*		
0.0284 mg/L	Time in Upper Half	–0.41	0.149 mg/L	Time in Upper Half	**2.01**
N			*N*		
12 control	# of Erratic Movements	0.30	12 control	# of Erratic Movements	0.16
12 experimental	Average Entry Duration	–	12 experimental	Average Entry Duration	–
	# of Freezing Bouts	–0.58		# of Freezing Bouts	–0.90
	Freezing Duration	–0.58		Freezing Duration	–0.88
	Latency to 1st Transition	–		Latency to 1st Transition	–
Diazepam	# of Entries to Upper Half	**2.86**	Diazepam	# of Entries to Upper Half	0.86
Study I			Study II		
Dose			*Dose*		
3.6 mg/0.05 l	Time in Upper Half	**3.19**	3.6 mg/0.05 l	Time in Upper Half	0.65
5 min exposure			5 min exposure		
N			*N*		
10 control	# of Erratic Movements	0.32	10 control	# of Erratic Movements	–0.66
10 experimental	Average Entry Duration	–	11 experimental	Average Entry Duration	–
	# of Freezing Bouts	–0.95		# of Freezing Bouts	–1.43
	Freezing Duration	–0.81		Freezing Duration	–1.09
	Latency to 1st Transition	–		Latency to 1st Transition	–

Table 15.1 (continued)

Experimental conditions	Endpoint	MPV score	Experimental conditions	Endpoint	MPV score
Fluoxetine (Acute)	# of Entries to Upper Half	0.28	Fluoxetine (Chronic) 3 weeks	# of Entries to Upper Half	–0.54
Dose			*Dose*		
100 μg/L	Time in Upper Half	0.10	100 μg/L	Time in Upper Half	**–9.13**
N			*N*		
16 control	# of Erratic Movements	–0.11	5 control	# of Erratic Movements	0.84
14 experimental	Average Entry Duration	–0.49	4 experimental	Average Entry Duration	**–3.04**
	# of Freezing Bouts	0.80		# of Freezing Bouts	1.37
	Freezing Duration	0.83		Freezing Duration	1.08
	Latency to 1st Transition	0.23		Latency to 1st Transition	**3.28**

Bold numbers indicate statistically significant results ($P < 0.05$).

Table 15.2
Maximum predictive validity (MPV) analyses for selected anxiogenic manipulations

Experimental conditions	Endpoint	MPV score	Experimental conditions	Endpoint	MPV score
Alarm Pheromone	# of Entries to Upper Half	0.06	Caffeine	# of Entries to Upper Half	1.00
Dose			*Dose*		
200 mL undiluted	Time in Upper Half	0.81	100 mg/L 15 min exposure	Time in Upper Half	0.95
N			*N*		
10 control	# of Erratic Movements	–1.34	21 experimental	# of Erratic Movements	–0.85
10 experimental	Average Entry Duration	0.31	21 experimental	Average Entry Duration	0.77
	# of Freezing Bouts	**–14.43**		# of Freezing Bouts	–0.30
	Freezing Duration	0.04		Freezing Duration	–0.27
	Latency to 1st Transition	0.06		Latency to 1st Transition	–

spent a great amount of *Time in the Upper Half* (MPV 2.381). Furthermore, we have found that subjects receiving 200 mL of undiluted alarm pheromone had positive MPV scores in all behavior parameters according to the pilot data. However, in another study at this dose, zebrafish had an MPV of –14.425 in # *of Freezing Bouts*, indicating an anxiogenic effect (**Table 15.2**). Likewise, the acute administration of caffeine also appears to induce anxiogenic symptoms in our zebrafish. For example, subjects treated with 100 mg/L of caffeine displayed an increase in # *of Erratic Movements* and *Freezing Bouts* and experienced longer episodes of freezing behavior (**Table 15.2**).

5. Summary

Using the MPV measure can be a beneficial tool for the development and characterization of new animal models for behavioral pharmacology research. In this protocol, the MPV measure allowed our laboratory to analyze multiple behavioral measures to assess drug efficacy and treatment reliability. This also allows for the assessment of validity while also enabling fine-grained analysis not addressed by the dichotomous measure of predictive validity (*see* above) (12). For example, a promising measure resulting from our alarm pheromone trials is the *Freezing Duration* measure, which produced an expected anxiogenic response. This suggests a potential importance of employing this specific behavioral endpoint when analyzing anxiogenic compounds.

The strength of the MPV as an analytical tool is most profound when observing our diazepam results. Diazepam would be expected to produce effects associated with eliminating the fear, and to evoke more entries to, and longer time in, top. These behaviors would likely be anxiety-provoking to zebrafish in their native environment due to the risk of predators near the water's surface. In our studies, the MPV value calculated for two of the three trials give positive values associated with # *of Entries to Upper Half* and *Duration in Upper Half* as valid behavioral endpoints in assessing diazepam as an anxiolytic. It is important to note that all trials except for the lowest dosage yielded positive values for these two endpoints. This represents consistency and reliability for these measures in regards to accurately representing diazepam as an anxiolytic compound. Analyzing MPV values for specific endpoints across different trials can help elucidate information such as the most effective dose, as seen by the increasing MPV values when increasing the dosage from 0.149 mg/L to the 3.6 mg/L. Collectively, this provided further evidence that the MPV measure can allow a researcher to make precise decisions about drug

doses for specific compounds that goes beyond the measure of predictive validity (12, 13).

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