Anxiolytic Drug Screening - Tools and Strategies for Reliable Predictions

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INTRODUCTION

Anxiety disorders are the most common mental health conditions worldwide. However, many current treatments show limited efficacy or cause side effects. This limitation drives the search for new and safer anxiolytic drugs. Zebrafish (*Danio rerio*) larvae provide a rapid and cost-effective *in vivo* model for the early-stages drug screening. Their suitability for toxicity testing, behavioural analysis, and molecular profiling makes them ideal for evaluating psychoactive compounds. Nevertheless, each assay has its limitations.

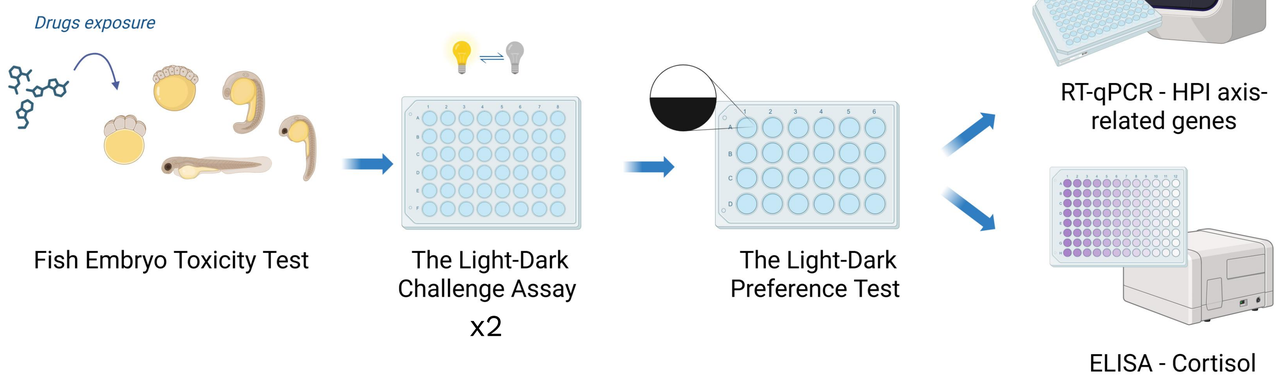
METHODS

Table 1. Screening Platform – concentrations of substances used in each experiment

Test / Substance	Fish Embryo Toxicity Test	Dark-Light Challenge Assay (48-well plate)	Dark-Light Challenge Assay (24-well plate)	Light-Dark Preference Test	ELISA	RT-qPCR
	Concentration [µM]					
Diazepam	-	10	10 ✓	10 ✓	10 ×	10 ✓×
Amitriptyline	0.033, 0.165, 0.33, 1.65, 3.3, 10, 20, 30	0.033, 0.165, 0.33, 1.65, 3.3, 10, 20, 30	3.3, 10 ✓	3.3, 10 ×	10 ✓	10 ✓×
Fluoxetine	0.3, 1.5, 3, 10.5, 21, 60	0.3, 1.5, 3, 10.5, 21, 60	3, 10.5 ✓	3, 10.5 ×	-	-
TP003	0.1, 1, 5, 10, 20, 50	0.03, 0.06, 0.1, 0.25, 0.5, 1	-	-	-	-
DOI	0.1, 1, 5, 10, 50, 100	0.1, 1, 5, 10, 50, 100	10, 50 ✓	10, 50 ✓	50 ×	50 ✓×
5-MeO-DMT	0.23, 0.46, 0.92, 2.3, 4.6, 13.8	0.23, 0.46, 0.92, 2.3, 4.5, 14	4.5, 14 ✓	4.5, 14 ×	-	-
Psilocybin	1, 2.5, 5, 10, 25, 50	1, 2.5, 5, 10, 25, 50	1, 5 ×	1, 5 ×	-	-

✓ - expected effect, × - no effect, ✓× - variable outcomes

Experimental design



*HPI axis- related genes: *hsd11b2, crha, crhbp, pomca, and ucn3l*

RESULTS

Fish Embryo Toxicity Test

All tested compounds were non-toxic at selected concentrations, except TP003 at doses >5 µM, which adversely affected embryonic development.

Observed malformations included head malformation (HM), tail/somite malformation (TM) and yolk sac edema (YSE).

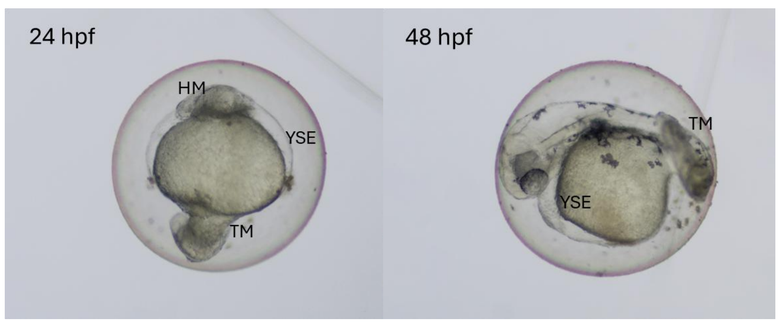


Fig. 1 Zebrafish in different stages of development after exposure to 10 µM TP003.

Dark-Light Challenge Assay (48-well plate)

Based on screening six concentrations using the Light–Dark Challenge Assay (L-DCA), two doses showing the strongest anxiolytic-like effects were selected for further analysis.

Key observations include:
- Larval exploratory space significantly affects anxiety-like phenotype clarity,
- 48-well plates are suitable for initial screening,
- Full L-DCA procedure is recommended in 24-well plates for more accurate results.

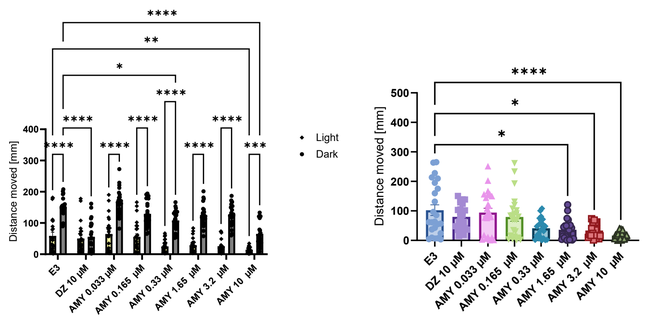


Fig. 2 Average distances moved in 1 min time bins under light and dark conditions (left) and continuous light phase (right; AMY – amitriptyline, DZ – diazepam). Data presented as mean ± SEM, n=24 animals per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (Two- and One- way ANOVA).

Dark-Light Challenge Assay (24-well plate)

The analysis revealed anxiolytic properties of fluoxetine, amitriptyline, and DOI, relaxing effects of 5-MeO-DMT, and no observable effect of psilocybin.

The Light–Dark Challenge Assay proved particularly informative, offering:
- assessment of relaxing effects under continuous light,
- detection of anxiolytic-like effects during alternating light–dark phases,
- compatibility with 24-well plates, allowing for additional parameters (e.g., thigmotaxis).

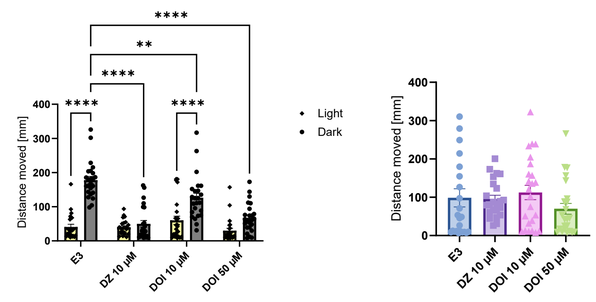


Fig. 3 Average distances moved in 1 min time bins under light and dark conditions (left) and continuous light phase (right; DOI – 2,5-Dimethoxy-4-iodoamphetamine, DZ – diazepam). Data presented as mean ± SEM, n=24 animals per group.**p<0.01, ****p<0.0001 (Two- and One- way ANOVA).

Light-Dark Preference Test

In the Light–Dark Preference test, anxiolytic properties were confirmed only for diazepam and DOI.

This assay appears more restrictive as a standalone screening tool due to:
- Reliance on choice-based behaviour rather than escape response,
- Reduced sensitivity to moderate anxiolytic effects,
- Limited interpretability in the presence of sedation.

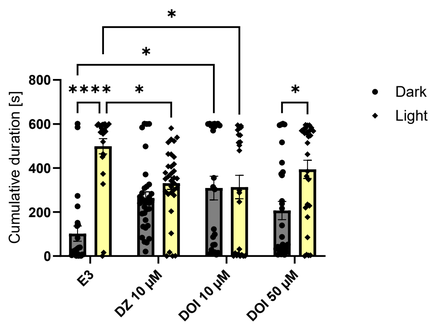


Fig. 4 Time spent in the dark or light zones (DOI – 2,5-Dimethoxy-4-iodoamphetamine,DZ – diazepam). Data presented as mean ± SEM, n=30. *p<0.05, **p<0.01, ****p<0.0001 (Two-way ANOVA).

ELISA
Cortisol

Whole-body cortisol levels increased following the L-DCA procedure, confirming its stress-inducing effect in zebrafish larvae. Among the anxiolytic candidates, only amitriptyline significantly reduced cortisol levels.

While cortisol measurement is useful for drugs acting through the stress axis, not all anxiolytics reduce anxiety by modulating cortisol levels; some act via other mechanisms.

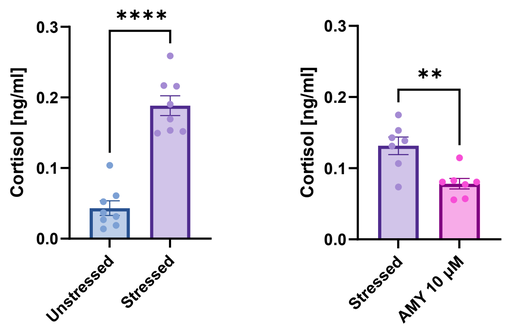


Fig. 5 Average whole-body cortisol levels.: Cortisol level change between unstressed and stressed groups (left); Cortisol level change between stressed (no treatment) and stressed after AMY – amitriptyline (right). Data presented as mean ± SEM, n=7-8. **p<0.01, ****p<0.0001 (t-student test for parametric data).

RT-qPCR
HPI axis-related genes

Gene expression analysis yielded inconsistent results; few genes responded to stress or treatment. Most genes remained unchanged under both conditions.

We conclude that gene expression analysis has several limitations:
- Requires strict methodological consistency (incubation, treatment, sampling).
- Highly sensitive to sampling time due to dynamic transcription.
- Interpretation complicated by regulatory complexity.
- Technically challenging in larvae due to small size and limited brain access.

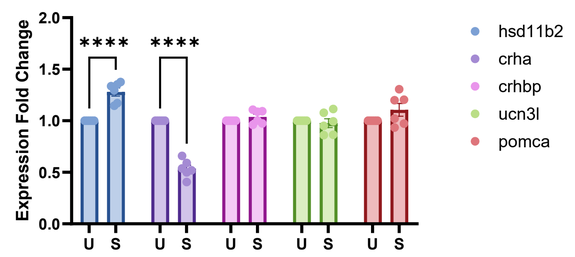


Fig. 6 Expression fold change of genes involved in the hypothalamic–pituitary–interrenal (HPI) axis between unstressed (U) and stressed (S) groups. Data presented as mean ± SEM, n=6. ****p<0.0001 (Two-way ANOVA)

CONCLUSIONS

- Developmental toxicity might not indicate the effects experienced in adulthood.
- Behavioural outcomes can vary between tests.
- Gene expression does not always correlate with phenotype.
- Cortisol levels are useful markers, but mainly when a drug acts via the hypothalamic–pituitary–interrenal (HPI) axis.
- Using a multi-endpoint approach enhances the reliability of evaluating the potential anxiolytic effects.