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RESEARCH ARTICLE

Decynium-22 affects behavior in the zebrafish light/dark test

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Abstract

Decynium-22 (D-22) is an inhibitor of the uptake$_2$ system of monoamine clearance, resulting in increased levels of dopamine and norepinephrine (and in some cases serotonin) in the nervous system and elsewhere. Uptake$_2$ is mediated by low-affinity, high-capacity transporters that are inhibited by glucocorticoids, suggesting a mechanism of fast glucocorticoid-monoamine interaction in the brain and a possible target for antidepressants. D-22 dose-dependently increased anxiety-like behavior in adult zebrafish exposed to the light/dark test, monotonically increasing scototaxis (dark preference), but affecting risk assessment with an inverted-U-shaped response. These results suggest that the uptake$_2$ system has a role in defensive behavior in zebrafish, presenting a novel mechanism by which stress and glucocorticoids could produce fast neurobehavioral adjustments in vertebrates.

Related Objects: Preprint - https://doi.org/10.1101/2021.01.14.426728; Protocol - https://doi.org/10.17504/protocols.io.srfed3n; Dataset - https://doi.org/10.5281/zenodo.5121722
Key words: Uptake2; Monoamines; Stress; Defensive behavior; Zebrafish

1. Introduction

Clearance of the monoamine neurotransmitters dopamine (DA), norepinephrine (NE), and serotonin (5-HT) released in the synaptic cleft is executed by two distinct mechanisms, uptake$_1$ and uptake$_2$ [1–5]. Uptake$_1$ is mediated by high-affinity, low-capacity transporters which include the NE transporter (SLC6A2, NET), the DA transporter (SLC6A3, DAT), and the serotonin transporter (SCL6A4, SERT)[6,7]. This SLC6A family has been implicated in the pathophysiology of mental disorders, including alterations of anxiety [8–11], and are the target for major classes of anxiolytic drugs, including tricyclic antidepressants, selective 5-HT reuptake inhibitors, and 5-HT-NE reuptake inhibitors [12]. Uptake$_2$ is mediated by low-affinity, high-capacity transporters which include organic cation transporters (OCT1-3; SLC22A1-3) and the plasma membrane monoamine transporter (PMAT; SLC29A4) [4,5,13–15]. Evidence suggests that uptake$_2$ plays significant roles in the regulation of monoaminergic neurotransmission and maintenance of synaptic homeostasis [2,3,7], with numerous studies suggesting that uptake$_2$ plays significant roles in various psychological disorders, such as anxiety and depression [16–23].

Uptake$_2$ has been described as an "extraneuronal transport system" [2,3], due to its low-affinity, high-capacity, "promiscuous" characteristic, and evidence for that includes the perisynaptic location of transporters [1] and the fact that the uptake$_2$ inhibitor decynium-22 does not necessarily increase basal serotonin levels, but may instead produce effects in situations in which 5-HT brain concentrations are high [24]. Uptake$_2$ is an interesting system not only because it is best suited for extraneuronal uptake, but also because it is blocked by glucocorticoids [13,25]. Glucocorticoids have been shown to inhibit OCT3 [26], and, with low affinity, PMAT [27]. As a result, uptake$_2$ represents an intersection in the pathophysiology of stress and anxiety, a mechanism by which circulating glucocorticoids (GCs) can rapidly increase monoamine levels in the brain [9]. Uptake$_2$ has been shown to participate in anxiety-like behavior: SLC22A3 knockout mice show decreased anxiety-like behavior in the open field test and in the elevated plus-maze [19, but see 28]. Knockdown of SLC22A3 expression in the brains of mice decreases immobility time in the forced swim test [17], a screen for antidepressant-like effects [29]. Finally, while decynium-22 (D-22), an uptake$_2$ inhibitor [4], had no behavioral effect by itself, co-treatment with fluvoxamine produced synergistic effects on 5-HT clearance and immobility in the forced swimming test [23].

Zebrafish (*Danio rerio* Hamilton 1822) have been proposed as model organisms in the study of behavioral functions and its dis-

orders [30–33]. The advantages of using this species in behavioral studies stem from its use in developmental biology (i.e., small size, fast generation times, high reproduction rates) and the availability of tools to image and manipulate its nervous system [33]. Zebrafish demonstrate a robust endocrine response to acute stressors [34–37]; importantly, simple acute stressors such as net chasing induce robust behavioral responses which are blocked by 5-HT reuptake inhibitors [38,39] and DAergic and NErgic drugs [36,37].

A few behavioral assays for anxiety-like behavior have been described for adult zebrafish, with the novel tank test (NTT) and the light/dark test (LDT) being the most widely used at the moment [40]. In particular, the LDT involves an approach-avoidance motivational conflict [41] that results in scototaxis (preference for dark environments vs brightly-lit or white environments) that is accompanied by risk assessment (brief entries in the white compartment), erratic swimming, freezing, and thigmotaxis while in the white compartment [40–42]. These variables are particularly sensitive to anxioselective compounds [40,43], and drug effects on scototaxis are negatively correlated with drug effects on 5-HT turnover [43]. The LDT has been used to investigate the role of specific 5-HTergic mechanisms [44], but little is known about the role of uptake$_2$ in behavior in this test.

Currently, it is unknown whether zebrafish possess a functional uptake$_2$ system. Fourteen *slc22* genes have been identified in zebrafish, and OCT3 appears absent [45,46]; *oct1* shows moderate expression in the brain, suggesting a role in neurotransmitter homeostasis [45], and therefore is likely to be of behavioral relevance. Little information on zebrafish PMAT is available [47,48]; Sivasubbu et al. [49] reported a gene that is "Similar to solute carrier family 29, member 4" (deposited on ZFIN as ZDB-GENE-070112-1932 and Ensembl as ENSDARG00000059690), but expression patterns and function has not yet been described. Nonetheless, the interplay between serotonin, dopamine, and cortisol in behavioral responses to threatening and stressful stimuli in zebrafish [see 50 for a review] suggests a participation of uptake$_2$. Here, I show that D-22 dose-dependently increases anxiety-like behavior in the zebrafish LDT. These results suggest that uptake$_2$ is present in this species, and that it functions as a mediator of stress and defensive behavior.

This manuscript is a complete report of all the studies performed to test the hypothesis of a dose-dependent effect of D-22 on anxiety-like behavior. I report all data exclusions, all manipulations, and all measures in the study.

2. Materials and methods

2.1. Animals and housing

A total of 100 animals were used. Animals were bought from a commercial vendor (AcquaPeixes, Goiânia/GO, Brazil) and arrived in the laboratory with an approximate age of 3 months (standard length = 13.2 ± 1.4 mm), and were quarantined for two weeks; the experiment began when animals had an approximate age of 4 months (standard length = 23.0 ± 3.2 mm). Housing standards were as described by Pimentel et al. [51]: "Animals were kept in mixed-sex tanks during acclimation, with an approximate ratio of 50-50 males to females (confirmed by body morphology). Adult zebrafish from the wildtype strain (longfin phenotype) were used in the experiments. Outbred populations were used for increased genetic variability, thus decreasing the effects of random genetic drift which could lead to the development of uniquely heritable traits [52,53]. Thus, the animals used in the experiments were expected to better represent the natural populations in the wild. The breeder was licensed for aquaculture under Ibama's (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) Resolution 95/1993. Animals were group-housed in 40 L tanks, with a maximum density of 25 fish per tank, for at least 2 weeks before experiments begun. Tanks were filled with non-chlorinated water at room temperature (28 °C) and a pH of 7.0-8.0. Lighting was provided by fluorescent lamps in a cycle of 14-10 hours (LD), according to standards of care for zebrafish [54]. Water quality parameters were as follows: pH 7.0-8.0; hardness 100-150 mg/L $CaCO_3$; dissolved oxygen 7.5-8.0 mg/L; ammonia and nitrite < 0.001 ppm. All manipulations minimized their potential suffering of animals, and followed Brazilian legislation [55]. Animals were used for only one experiment and in a single behavioural test, to reduce interference from apparatus exposure. Experiments were approved by UEPA's IACUC under protocol 06/18."

2.2. Sample size calculation and exclusion criteria

Sample sizes were calculated based on a power analysis, using the effects of fluoxetine on the light/dark test [44] as estimates of effect sizes. Using an effect size of 0.7, a significance level of 0.005, and a power of 90%, a sample size of 11 animals per group was calculated. Final sample sizes were 20 animals/group. Animals were excluded if they displayed signals of overt ataxia (swimming on a side, swimming upside-down, vertical swimming) during the exposure period [56]. Outliers were detected using an *a priori* rule based on median absolute deviation (MAD) of time on white (the main endpoint of the LDT), with values above or below 3 MADs being removed [57].

2.3. Drug treatments

Zebrafish were randomly drawn from the holding tank immediately before injection and assigned to four independent groups (n = 20/group). Animals were injected with vehicle (Cortland's salt solution) or D-22 (CAS #977-96-8, Cat#: 323764, Sigma-Aldrich/Merck, Brazil; 0.01, 0.1, 1, or 10 mg/kg). The injection volume was 1 µL/0.1 g b.w. following procedures described by Kinkel et al. [58]. The order with which groups were tested was randomized via generation of random numbers using the randomization tool in http://www.randomization.com/. The experimenter and data analyst was blinded to treatment by using coded vials for drug doses and by using coding to reflect treatments in the resulting datasets; after analysis, data was unblinded. Codes were created and kept by a research assistant.

2.4. Light/dark test

The light/dark preference (scototaxis) test was performed as described elsewhere [32, 59]. Animals were individually tested, and groups were independent from each other. 30 min after drug injection, an individual was individually transferred to the central compartment of a half-black, half-white tank (15 cm height × 10 cm width × 45 cm length; Figure 1A) and left for 3 min, during which the animal acclimated to the tank. After this acclimation period, the doors which delimit this compartment were removed, allowing the animal to freely explore the apparatus. Spatiotemporal variables (below) were recorded for the entire 15 min trial. While the whole experimental tank was illuminated from above by a homogeneous light source, due to differences the reflectivity of the apparatus walls and floor average illumination (measured just above the water line) above each compartment was different: 225 ± 64.2 (mean ± S.D.) lux above the black compartment, and 307 ± 96.7 lux above the white compartment. A digital video camera (Samsung ES68, Carl Zeiss lens) was installed above the apparatus to record the behavioral activity of the zebrafish. The following variables were recorded:

- *Time in the white compartment:* the time spent in the white half

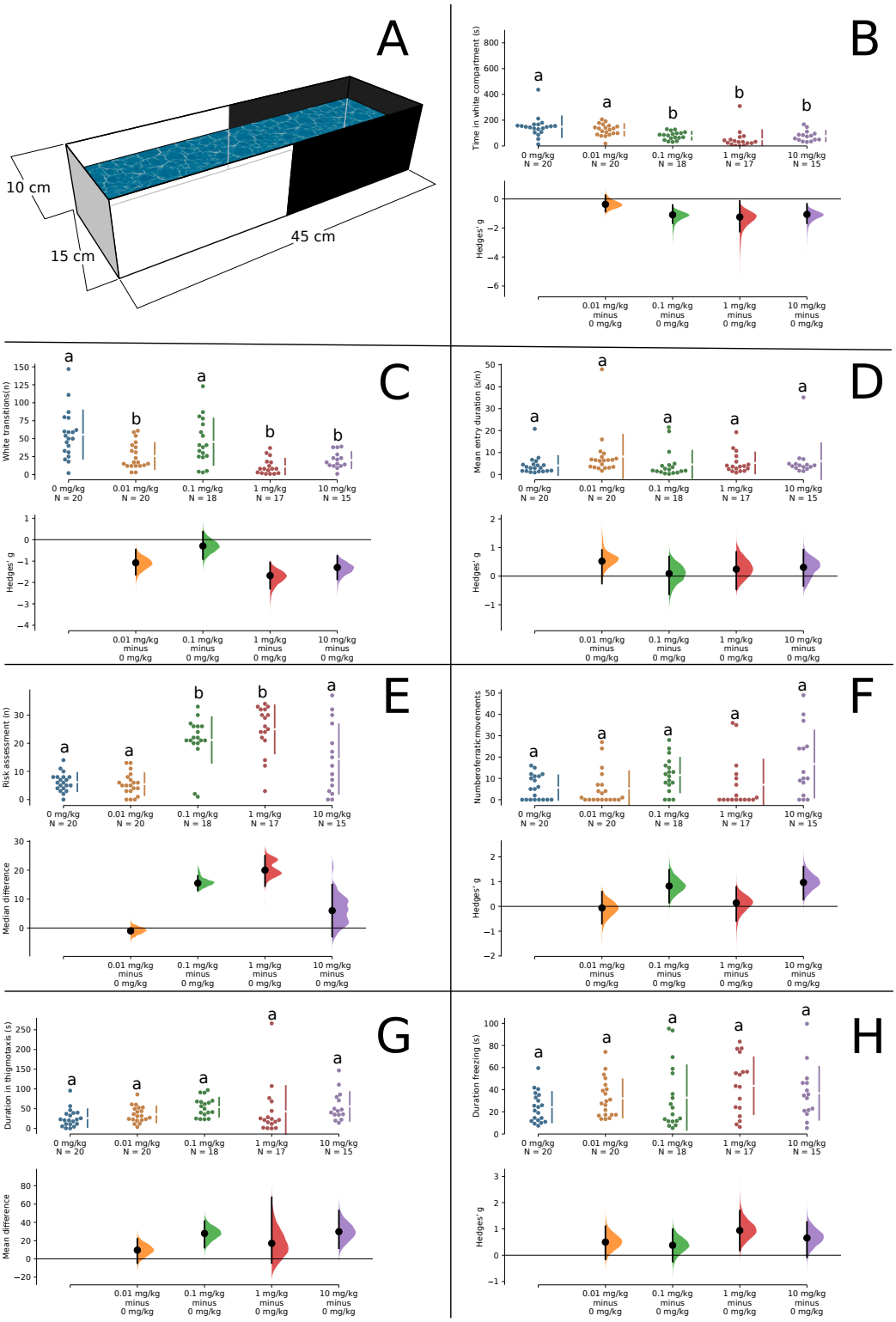

Figure 1. (A) Test apparatus. (B) Scototaxis (time spent in the white compartment). (C) Transitions to the white compartment. (D) Duration of entries in the white compartment. (E) Risk assessment. (F) Erratic swimming. (G) Thigmotaxis. (H) Freezing duration. The Hedges' *g* for 4 comparisons against the shared control 0 mg/kg are shown in the above Cumming estimation plots. The raw data is plotted on the upper axes. On the lower axes, mean differences are plotted as bootstrap sampling distributions. Each mean difference is depicted as a dot. Each 95% confidence interval is indicated by the ends of the vertical error bars. 5000 bootstrap samples were taken; the confidence interval is bias-corrected and accelerated. Letters indicate results from post-hoc tests; different letters indicate statistically significant differences ($p < 0.05$).

of the tank (*s*);

- *White transitions:* the number of entries in the white compartment made by the animal throughout the session;
- *Entry duration:* the average duration of an entry (time on white / transitions);
- *Number of erratic movements:* defined as the number of zig-zag, fast, and unpredictable swimming behavior of short duration;
- *Duration in freezing:* the duration of freezing events (*s*), defined as complete cessation of movements with the exception of eye and operculum movements;
- *Duration in thigmotaxis:* the duration of thigmotaxic events (*s*), defined as swimming in a distance of 2 cm or less from the

white compartment's walls;
- *Frequency of risk assessment:* defined as a fast (<1 s) entry in the white compartment followed by re-entry in the black compartment, or as a partial entry in the white compartment (i.e., the pectoral fin does not cross the midline).

Two independent observers, blinded to treatment, manually measured the behavioral variables using X-Plo-Rat 2005 (https://github.com/lanec-unifesspa/x-plo-rat). Inter-observer reliability was at least > 0.95.

2.5. Data analysis

Drug effects were assessed using asymptotic general independence tests, using the R package 'coin' [60]. Independence tests are conditional, resampling-based procedures which replace the unknown null distribution by a conditional null distribution (i.e., the distribution of a given test statistic given the actually observed data), and are therefore not limited by distributional assumptions and by the assumption that random samples of a population (instead of randomization of a nonrandom sampling) took place [60–62]. Post-hoc analysis was made using pairwise permutation tests with correction for the false discovery rate. Data were presented as Cumming estimation plots, with Hedges' *g* used to estimate effect sizes, using the R package DABESTR [63]. Cumming estimates were made using 5000 bootstrap resamples, and confidence intervals were bias-corrected and accelerated. Bootstrapping was used to derive sampling-error curves due to the robustness of this method in relation to deviances from normality and unequal variances [64]. Estimation statistics were chosen for graphical representation because they estimate effect sizes and their uncertainties, emphasizing quantitative reasoning beyond dichotomous thinking (effect/no effect) [63,65].

3. Results

5 animals were removed from analysis in the highest dose group due to overt ataxia, and 2 animals were removed from the 1 mg/kg group for the same reason. 1 animal from the 1 mg/kg group was detected as outlier and removed from further analysis. A dose-dependent decrease in time on white was found (maxT = -3.773, p = 0.0007; Figure 1B); significant effects were found for 0.1-10 mg/kg. Likewise, dose-dependent decreases were found for transitions to white (maxT = 4.0277, p = 0.0003; Figure 1C); significant effects were found for all doses, except 0.1 mg/kg. No significant effects were found for entry duration (maxT = 1.8191, p = 0.2779; Figure 1D). An inverted-U-shaped response was found for risk assessment (maxT = 4.6248, p = 0.019; Figure 1E), with 0.1, and 1 mg/kg increasing risk assessment, and 0.01 mg/kg having no effect; the effect of 10 mg/kg was smaller than the other effects. A main effect of dose was found in erratic swimming (maxT = 3.106, p = 0.0091; Figure 1F), but *post-hoc* comparisons failed to detect differences. No effects were found for thigmotaxis (maxT = 2.1474, p = 0.1396; Figure 1G) or freezing (maxT = 2.0629, p = 0.1688; Figure 1H). Table 1 presents false discovery rate-adjusted p-values for multiple comparisons.

4. Discussion

The present experiment showed evidence that D-22, an uptake$_2$ inhibitor, dose-dependently increased anxiety-like behavior in the LDT in unstressed zebrafish. Dose-dependent effects were found for time on white (scototaxis), transitions to white, and risk assessment, with the latter suggesting better effects at intermediate doses (0.1 and 1 mg/kg). No effects were observed in other variables (freezing and erratic swimming, thigmotaxis). D-22 decreased time on white (suggesting an increase in preference for dark), an index of anxiogenic-like effects [40,41,43,66], at doses of 0.1 mg/kg and higher, while decreasing transitions to white and increasing risk assessment. In general, effect sizes for time on white and transitions to white were small, while effect sizes for risk assessment were average.

The LDT has been proposed as a screening test for anxiolytic-like and anxiogenic-like effects of treatments in adult zebrafish [66]. The test shows good predictive validity, being sensitive to agents that act at different targets [40]. The main endpoint of this test, light/dark preference, is sensitive to anxiolytic-like and anxiogenic-like effects, and represents an "avoidance" dimension of behavior in the LDT, while risk assessment clusters in a different group and represents a more "cognitive" aspect of anxiety-like behavior [43]. Moreover, exposure to the LDT induces a cortisol response in unstressed animals [40], suggesting that the conflict that is induced in the test is mildly stressful.

Behavioral effects of D-22 have been described in rodents; while by itself D-22 (0.01-0.32 mg/kg) was not able to change immobility time in the tail suspension test in mice, a screen for antidepressant-like effects, it produced a synergistic effect with fluvoxamine [23]. Species- and strain-specific effects can be responsible for this lack of effect of D-22, as this drug (0.001-0.01 mg/kg) reduced immobility time in the forced swim test (another screen for antidepressant-like effects) in Wistar-Kyoto, but not Long Evans, rats [67]. Although these effects are usually attributed to effects on serotonin clearance [1], it is not possible to discard an effect on norepineprhine.

Importantly, effects of manipulations of the uptake$_2$ system in rodents produce either opposite [19] or no effect [28] in transgenic mice. These differences could be attributed to a role of monoamines in adulthood (e.g., fast modulation of mood and behavior) vs. their roles during development [68–70]. Similar effects are observed with acute drug treatment vs. transgenics in the case of serotonin transporters [e.g. 71], suggesting that monoamines participate in the development of brain regions that are involved in defensive/emotional behavior, and that lacking monoamine transporters disrupts these developmental trajectories in ways that acute drug treatment does not. Indeed, it has been shown that serotonin participates in the development of neural circuits associated with emotion in a sensitive developmental window [71], and since knocking out uptake$_2$ transporters from birth should affect the levels of monoamines at periods which are critical for the development of neurocircuits associated with anxiety-like behavior, the effects of this manipulation are expected to be different than acute treatment with D-22 in adult animals.

D-22 blocks the uptake$_2$ monoamine transport system [1]. D-22 does not readily discriminate between OCT and PMAT systems [72], and therefore it is currently impossible to pharmacologically uncouple both transporters. Due to its low-affinity, high-capacity character, transporters in the uptake$_2$ system (OCT and PMAT) are "promiscuous", participating in the elimination of most monoamines from synaptic and extrasynaptic sites [73]. Importantly, uptake$_2$ may represent a link between acute stress and monoaminergic neurotransmission [9], as these transporters are blocked by glucocorticoids [25]. While currently it is not known whether the effects reported in this experiment are due to serotonin, norepinephrine, dopamine, or histamine, there is some evidence for anxiety-like behavior in zebrafish being increased by serotonin [74] and catecholamines [37].

Overall, these results suggest that uptake$_2$ is present in zebrafish, and that it functions as a mediator of stress and defensive behavior. These results point to novel avenues of investigation in the stress-monoamine interaction in anxiety, stress, and defensive behavior. Further studies are needed to better understand the mechanisms by which D-22 produces its behavioral effects.

Table 1. False discovery rate-adjusted p-values for multiple comparisons

Endpoint	Dose vs	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg
Time on white	0 mg/kg	0.2648	0.0050	0.0003	0.0056
0.01 mg/kg		0.0048	0.0634	0.0056	
0.1 mg/kg			0.0018	0.8041	
1 mg/kg				0.0056	
Transitions to white	0 mg/kg	0.0053	0.3522	0.0007	0.0035
0.01 mg/kg		0.0324	0.0078	0.3303	
0.1 mg/kg			0.0025	0.0137	
1 mg/kg				0.0137	
Entry duration	0 mg/kg	0.7876	0.8143	0.7888	0.7875
0.01 mg/kg		0.7876	0.7876	0.7876	
0.1 mg/kg			0.8143	0.7888	
1 mg/kg				0.8143	
Risk assessment	0 mg/kg	0.5085	< 0.0001	< 0.0001	0.0159
0.01 mg/kg		< 0.0001	< 0.0001	0.0124	
0.1 mg/kg			0.2216	0.0865	
1 mg/kg				0.0184	
Erratic swimming	0 mg/kg	0.9562	0.0594	0.9562	0.0594
0.01 mg/kg		0.0594	0.9243	0.0594	
0.1 mg/kg			0.0817	0.3241	
1 mg/kg				0.0594	
Thigmotaxis	0 mg/kg	0.2386	0.0152	0.8082	0.0385
0.01 mg/kg		0.0449	0.5570	0.0794	
0.1 mg/kg			0.0449	0.8553	
1 mg/kg				0.0770	
Freezing	0 mg/kg	0.2865	0.3952	0.0609	0.2865
0.01 mg/kg		0.9243	0.2865	0.6259	
0.1 mg/kg			0.3952	0.7481	
1 mg/kg				0.5310	

Significance statement

Uptake$_2$ is a low-affinity, high-capacity transport system that contributes to the clearance of extraneuronal monoamines (mainly norepinephrine, serotonin, and dopamine) and is sensitive to glucocorticoids, therefore representing a putative mechanism of glucocorticoid-monoamine interaction. Since both monoamines and glucocorticoids have been implicated as mediators of stress-induced behavioral adjustments, this interaction can be of relevance to understanding the mechanisms through which stress influences neurochemical and behavioral responses. Here I report that, in zebrafish, the uptake$_2$ inhibitor decynium-22 increases dark preference and risk assessment in the light/dark test, an assay for anxiety-like behavior. Thus, uptake$_2$ appears to act as a modulator of defensive behavior, and its inhibition by, e.g., glucocorticoids could represent a mechanism through which stress produces fast neurobehavioral adjustments in vertebrates.

Declarations

Funding

This research was conducted without funding.

Conflict of Interest

The author declares no conflict of interest.

Author Contributions

Caio Maximino: Conceptualization, Formal analysis, Methodology, Investigation, Data curation, Project Administration, Resources, Software, Validation, Visualization, Writing – original draft.

Data Availability

Data and analysis scripts for this work can be found at a GitHub repository (https://github.com/lanec-unifesspa/decynium22) and on Zenodo [75].

Editorial Notes

History

- Received: 2021-03-04
- Revisions Requested: 2021-04-12
- Revisions Received: 2021-07-07
- Accepted: 2021-07-17
- Published: 2021-08-02

Editorial Checks

- Plagiarism: Plagiarism detection software found no evidence of plagiarism.
- References: Zotero did not identify any references in the Re-

tractionWatch database.

Peer Review

This paper followed a standard single-blind review process.

For the benefit of readers, reviewers are asked to write a public summary of their review to highlight the key strengths and weaknesses of the paper. Signing of reviews is optional.

Reviewer 1 (Anonymous)

This paper showed that decynium-22 (D-22; an uptake$_2$ inhibitor) produced a dose-dependent increase in scototaxis and risk taking behaviour without altering other anxiety-related behaviours such as thigmotaxis or freezing. This experiment shows for the first time that zebrafish do indeed have an uptake$_2$ mechanism, but more research is needed to understand which neurochemicals play a role in this mechanism (dopamine, serotonin, norepinephrine or histamine).

Reviewer 2 (Anonymous)

Overall, the paper presents an interesting finding about the interaction of D-22 with neuronal receptors in zebrafish. Convincing evidence is presented that zebrafish have a similar mechanism of stress-anxiety response mediated by uptake$_2$ receptors compared to other vertebrates. However, since the mechanism of D-22 is not detailed and the findings contradict other studies looking at the uptake$_2$ system, it is unknown the true effect of the drug. The behavioral studies were blinded for both researcher and behavioral evaluator which is a strength of the study. In addition, the authors use a well established behavioral test as well as strong interpretation measures for the data. Finally, this paper adds important knowledge of the existence of the uptake$_2$ system in zebrafish as well as its possible role in defense behaviors.

Reviewer 3 - Statistical Review (Shawn Zheng Kai Tan, European Bioinformatics Institute (EMBL-EBI), United Kingdom.)

In their manuscript, the author uses, in my opinion, non-standard, though rather progressive statistics and visualisations to measure the effects of various dosages of decynium-22 on behavioural measures in zebrafish light/dark test. The data and statistics in this paper are fully disclosed and available on their github, something that should be applauded. While the author has employed admirable statistical methodology that addresses some issues in the field, the major issue I have is the lack of justification/explanation of them. Even if these statistics were more common in the zebrafish field (which I am not sure they are), the wider behavioural neuroscience readership of the journal would likely find it useful to further understand these choices. Therefore, having the right papers cited, together with basic explanation of why the statistical methods were chosen would greatly increase trust in the statistical methods, and help the reader understand why such methods were used.

Reviewer 4 - References Review (Anonymous)

I examined the literature review and checked cited references for appropriateness. I found no evidence of citations to predatory journals or papers under editorial notices and references generally provided good support to the claims that they were being cited for. However, I did find several instances where the introduction of the uptake$_1$ and uptake$_2$ systems could have provided a more complete and nuanced description of the system. For example, while papers showing glucocorticoid inhibition of uptake$_2$ were cited, there are also papers that suggest only low affinity inhibition of PMAT by corticosterone. I also suggested several citations to facilitate a more complete description of uptake$_1$/uptake$_2$ and in a couple of places I suggested alternative citations that provided a more up-to-date or comprehensive discussion of issues. I am not an author on any of the citations I recommended.

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