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Article

Administration of *Stephania tetrandra* to *Drosophila melanogaster* to model obsessive compulsive disorder

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SUMMARY

Obsessive compulsive disorder (OCD) is a neuropsychiatric condition that affects 2-3% of the world population, with more research needed to better understand its mechanisms. Theories positing the involvement of neurotransmitters like serotonin, dopamine, and glutamate in OCD have gained traction, however no specific cause has been identified. There are currently no research models of OCD in invertebrates, and existing vertebrate models fail to accurately model the disorder. Often, these existing models exhibit abnormal behaviors not associated with OCD, or do not respond appropriately to OCD treatments. The current study aimed to create a model of OCD in *Drosophila melanogaster* through administration of *Stephania tetrandra* extract. *S. tetrandra* extract is a plant extract with antiserotonergic and antidopaminergic properties similar to those of second-generation antipsychotics, a class of drugs that can induce obsessive compulsive symptoms (OCS). We hypothesized that flies administered the extract would display more OCS than control flies. We determined the highest non-lethal dose of S. tetrandra in *Drosophila* and administered it in two assays for OCS: a locomotion assay that measured movement as a metric of anxiety, and a grooming assay that quantified repetitive grooming behavior. These two assays were selected because OCD is characterized by repetitive behaviors and anxiety. Administration of the *S. tetranda* extract significantly increased both anxiety and repetitive behavior, suggesting that flies administered the extract had increased OCS and that S. tetranda treatment could serve as a potential model of OCD in *Drosophila*.

INTRODUCTION

Obsessive compulsive disorder (OCD) is a prevalent neuropsychiatric condition that affects 2–3% of the world population and is characterized by the presence of two key features: obsessions and compulsions (1). According to the Diagnostics and Statistical Manual of Mental Disorders (DSM-V), the standard manual used by mental health professionals for diagnoses, obsessions are recurring intrusive thoughts or images, and compulsions are repeated behaviors performed to alleviate the discomfort caused by obsessions (2). OCD impairs the functioning of those who suffer from it due to compulsive behaviors and irrational anxiety that interrupt daily tasks (2).

The exact cause of OCD is still unknown; however, there is abundant evidence supporting the involvement of dopamine and serotonin (3). In fact, OCD patients often respond to selective serotonin reuptake inhibitors (SSRIs), which block the reabsorption of excess serotonin, increasing the amount of the neurotransmitter at the synapse and amplifying serotonin's effects (4, 5). Some patients respond best when SSRIs are supplemented with a dopamine D2 receptor antagonist (6). D2 antagonists are commonly used to treat schizophrenia by blocking D2 receptors and decreasing dopaminergic neurotransmission (6). However, at low doses, D2 antagonists have the same mechanism of action as antidepressants and actually increase dopaminergic neurotransmission in the brain's prefrontal cortex (6). The efficacy of treating OCD by augmenting SSRIs with a D2 antagonist suggests that dopamine also plays a role in OCD's pathophysiology (6).

Recent studies have highlighted other possible causes of OCD, such as the involvement of glutamate neurons and differences in brain structure (7). In short, while there are many possible causes of OCD, no exact cause has been pinpointed (3). It is likely the disorder is caused by a multitude of factors that vary from patient to patient, with some overlapping biological phenomenon connecting all cases (3). Thus, further investigation needs to be done into the mechanisms of OCD. One common method for studying and understanding disorders in humans is creating and using animal models (8). Unfortunately, OCD models are imperfect in their ability to recapitulate the disorder when evaluated based on three kinds of animal model criteria: construct validity, face validity, and predictive validity (9). Construct validity evaluates whether the mechanisms of the animal model involved in producing symptoms are similar or the same to those indicated to be involved in the human version of the condition, and this validity is important when considering if a model is truly an accurate reflection of a condition (8). Face validity is whether the animal model exhibits the same symptoms or behaviors as those seen in the condition and is the most obvious indicator of whether a model is accurate (8). Lastly, predictive validity measures whether the animal model responds to treatment and outside factors in the same way humans who have the condition do (8). There are currently seven genetic models of OCD in mice; however, many exhibit additional behaviors and abnormalities not associated with OCD and do not respond to known OCD treatments (9). Behavioral manipulation-based models of OCD — a subset of models that attempt to conceptualize the disorder by reinforcing behavioral patterns associated with OCD — have been able to accurately replicate some compulsive behaviors in mice but have only been able to exhibit stereotypic behaviors not specific to OCD (9). Pharmacologically induced models have been found to induce behaviors not associated with OCD and do not respond appropriately to drugs known to treat OCD (9). Thus, none of the existing models of OCD accurately model the disorder in both mechanism and behavior (9).

Drosophila melanogaster is a commonly used model

organism in neurological and neuropharmacological research (10). Many mammalian neurological processes are preserved in *D. melanogaster*, and they have been used to model autism spectrum disorder and Fragile X syndrome, where they exhibit repetitive behaviors (10). Thus, a model of OCD in *D. melanogaster* would increase accessibility to the study of OCD, leading to a better understanding of the disorder. Despite its viability and ease of study, there are currently no models of OCD in *D. melanogaster*, or in any invertebrate (9). Second-generation antipsychotics (SGAs) can induce and worsen Obsessive Compulsive Symptoms (OCS) in patients with schizophrenia, suggesting SGAs could be used to create drug-induced OCD models (11). Stepholidine (SPD) and tetrahydroprotoberberines are naturally occurring alkaloids found in the root extract of plants in the genus *Stephania* (12). SPD increases dopaminergic neurotransmission at D1 receptors and decreases dopaminergic neurotransmission at D2 receptors (12). levo-tetrahydroberberrubine (*l*-THBr), a derivative of tetrahydroprotoberberine, is a 5-HT_{1A} agonist that interacts with dopamine receptors (13). The alkaloids in *S. tetrandra* bind to both serotonin and dopamine receptors, behaving similarly to the OCS-inducing SGA-class drug clozapine (12, 13). This similar biochemistry suggests that SPD could also cause OCS. Unlike SGAs, however, *S. tetrandra* extract has the advantage of being a cost-effective and accessible substance, making it ideally suited for use in a model organism.

The purpose of this study was to test the efficacy of *S. tetrandra* extract as an OCS-inducing compound in *D. melanogaster*. We hypothesized that administering high doses of *S. tetrandra* to Oregon-R, wild type *D. melanogaster* would cause an increase of OCS in the flies compared to flies not administered the extract. Repetitive behavior and anxiety were assessed using assays to quantify the frequency of grooming and locomotion, respectively. Flies administered *S. tetrandra* extract groomed more often and moved less often. Thus, the hypothesis that *S. tetrandra* extract would increase OCS in *D. melanogaster* is supported. These results support the face validity of a potential *D. melanogaster* model of OCD created using the administration of *S. tetrandra* extract.

RESULTS

To determine the appropriate dosage of the *S. tetrandra* extract for *D. melanogaster*, we performed a toxicity assay. The toxicity assay tested five concentrations of the extract all increasing by tenfold, except the greatest concentration which was halved due to anticipated supply issues. At the conclusion of four days, we recorded the number of flies still living after being fed each of the five concentrations. All dosages administered were nonlethal, with all flies still living after four days (**Table 1**). We decided to experiment using the dosage of 36.88 mg/mL of food, as this was the full concentration that would have been used in the toxicity assay if not for supply issues.

Repetitive behaviors are a major symptom of OCD. To determine whether *S. tetrandra* induces repetitive behavior in *D. melanogaster*, we performed a grooming assay. In this assay, flies are dusted with a dye that they remove by grooming. Either zero or 30 minutes after the addition of the dye, the flies are removed from the dye and added to an ethanol solution. After allowing the flies to incubate in the ethanol solution for five hours, the flies are removed and

Group Number	Concentration (mg/mL)	Flies Living	Flies Dead	Percent Survival
1	0.003688	6	0	100
2	0.03688	6	0	100
3	0.3688	6	0	100
4	3.688	6	0	100
5	18.440	6	0	100

Table 1. *S. tetrandra* extract is non-toxic in *D. melanogaster* up to 18.440 mg/mL. A toxicity assay was conducted to determine the highest non-lethal dose of *S. tetrandra* extract in *Drosophila*. The quarter batch concentrations (mg of extract per mL of food) are listed along with the number of flies living after four days, the number of flies dead after four days, and the percent survival for each group. The concentrations increased by tenfold in each trial except for the final iteration, where the amount was halved relative to anticipated due to a lack of supplies.

absorbance values at 297 nm are measured as a metric for the quantity of dye remaining on each fly. High absorbance values indicate low levels of grooming, whereas low absorbance indicates high grooming (i.e., more dye removal). We collected absorbance values from flies administered the control diet either zero or 30 minutes after dusting and compared to confirm that control flies groomed during this period, resulting in reduced dye content. Absorbances were significantly lower in control flies given 30 minutes to groom compared to flies given zero minutes (1.589 ± 0.017 vs. 2.597 ± 0.866, $p < 0.0001$), suggesting that the flies were grooming and the assay could capture this behavior (**Figure 1**). We then compared absorbances between flies on the control diet and flies on the diet supplemented with *S. tetrandra* each given 30 minutes to groom to determine whether the extract increased grooming. Flies administered *S. tetrandra* had lower absorbance readings compared to controls (0.890 ± 0.007 vs. 1.588 ± 0.017, $p < 0.0001$), supporting the hypothesis that the extract would increase repetitive behavior.

We conducted a locomotion assay to measure anxiety levels in control versus *S. tetrandra* flies. Anxiety in *D. melanogaster* is negatively associated with locomotion, so high anxiety can be observed as reduced movement. We recorded the amount of time flies spent moving over the course of one minute while inside a petri dish. Flies administered *S. tetrandra* spent less time in motion compared to control flies (4.149 sec vs. 26.729 sec, respectively. $p < 0.001$) (**Figure 2**). These data suggest that the extract increases anxiety, a key hallmark of OCD.

DISCUSSION

In the current study, we aimed to determine the validity of *S. tetrandra* extract treatment in *D. melanogaster* as a model for OCD. Flies administered *S. tetrandra* extract yielded significantly lower absorbances of dye than the flies not given the extract, meaning that the flies given the extract groomed more during the given time period. In the locomotion assay, flies treated with *S. tetrandra* spent significantly less time in locomotion than untreated flies. A lack of locomotion in *D. melanogaster* is indicative of anxiety in the flies, and anxiety is a common symptom of OCD (3, 14). The increased grooming behavior is a consistent indicator of OCD associated with low levels of serotonin and dopamine (9). Together, increased anxiety and repetitive behavior in the *S. tetrandra*-treated

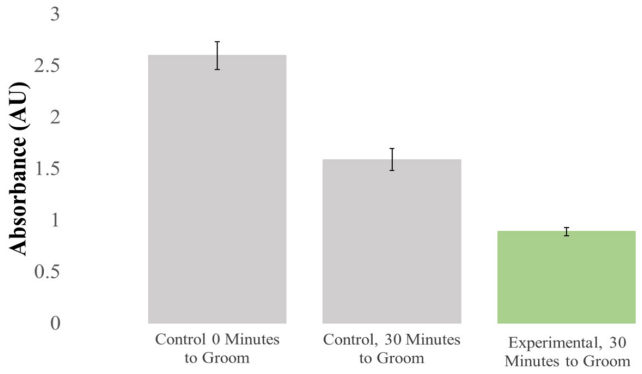

Figure 1. Grooming activity was increased in flies given *S. tetrandra* extract. Grooming activity was measured in flies on control diets or supplemented with *S. tetrandra* by collecting absorbances at 297 nm from fly samples dusted with dye and allowed zero or 30 minutes to groom. Control flies with zero minutes to groom had significantly less dye absorbance than controls given 30 minutes to groom (*p < 0.0001), supporting that the flies were grooming. Flies given the S. tetrandra extract had a lower dye absorbance than flies not given the extract (*p < 0.0001). Control diet– gray; experimental diet – green.

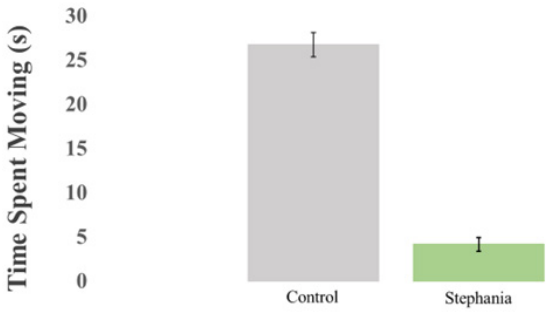

Figure 2. Locomotion times were reduced in flies given *S. tetrandra* extract. The amount of time spent in motion for one minute was measured in 10 flies on control diet and 10 flies on control supplemented with *S. tetrandra* extract. Experimental flies spent notably less time in movement (*p < 0.0001). Control diet – gray; experimental diet – green.

flies support the hypothesis that flies administered the extract would display more OCS than flies not administered the extract. Taking into consideration the link between excessive grooming and the connection between OCD, serotonin, and dopamine, we theorize the increase in repetitive behaviors exhibited by the experimental group was due to the inhibition of serotonin and dopamine receptors by SPD in the *S. tetrandra* extract (12). However, further experimentation is needed to uncover the molecular mechanisms underlying *S. tetrandra*'s effects.

Decreased dopaminergic transmission in *D. melanogaster* has been correlated with lower locomotion, while increased dopaminergic transmission has been associated with hyperactivity (15). It could be speculated that the decreased levels of locomotion seen in the experimental group are due to a motor deficit created by the D2 antagonistic properties of *S. tetrandra* extract. However, the central complex, the locomotion center of the fly brain, is composed primarily of D1 receptors (16). Therefore, given *S. tetrandra*'s partial agonistic effect of D1 receptors, it is more likely that the central complex would instead see an increase in dopaminergic transmission. This difference in D1 versus D2 responses to *S. tetrandra* and the makeup of the central complex suggest that the decreased locomotion seen in the experimental group is driven by anti-locomotive anxiety symptoms rather than motor deficits (15, 16). It is also worth noting that D1 agonists have been associated with increasing stereotypical grooming behavior in *D. melanogaster*, so the D1 agonistic properties of *S. tetrandra* may have influenced the results of the grooming assay (15).

The presence of these two OCS in the flies administered *S. tetrandra* extract contributes to the face and construct validity of this model – two forms of animal model validity that previous OCD models have not met (9). The model created in this experiment meets face validity criteria because the flies exhibited behaviors similar to behaviors seen in humans with the disorder. Additionally, the creation of this model using an extract with anti-serotonergic and anti-dopaminergic properties contributes to its construct validity because it is already known serotonin and dopamine play a role in OCD (3). However, further research is required to confirm whether the extract is truly inducing anti-serotonergic and anti-dopaminergic effects. While no significant difference in results would be expected, this study should also be replicated using male flies to eliminate the limitations of only testing with one sex. The neurochemical changes induced by *S. tetrandra* extract will be compared to neurochemical abnormalities in human OCD patients to answer this question.

The results of this study demonstrate the potential of using SGAs or SGA-like substances – such as *S. tetrandra* extract – to create drug-induced OCD models. These findings suggest that *D. melanogaster* may be capable of modeling OCD and are the first to introduce a feasible invertebrate OCD model. As knowledge of OCD continues to grow, the scientific community needs to devote more time to understanding the causes and underlying mechanisms of the disorder to better treat the condition. Once accurate animal models are established, their use can help propel research forward, aiding in the development of new psychiatric medications and alternative treatment options.

MATERIALS AND METHODS

Fly Maintenance

All fly stocks used in the experiment were female Oregon-R strain, obtained from the Carolina Biological Supply Co. and raised using yeast-cornmeal-agar fly food. Only female flies were used to maintain sex as an experimental constant. The control group was administered an unmodified version of this food, while the experimental group was administered fly food containing *S. tetrandra* extract at a concentration of 36.88 mg/mL of food from larval stage to adulthood. Stocks were stored in an incubator at 22°C, 65% humidity, and a day-night cycle of 12 hours for optimal growth. Flies were transferred to new vials regularly to ensure that the vials did not become overpopulated. All flies used in experimentation were two to five days old.

Toxicity Assay

We conducted a toxicity assay to identify the highest nonlethal dose of *S. tetrandra.* This assay was performed by testing the toxicity of five different concentrations of the extract and selecting the highest nonlethal dosage (**Figure**

3). After creating the yeast-cornmeal-agar fly food, the batch was divided into four equal parts, each poured in a different beaker. An additional quarter batch was made because five different concentrations of the extract were being tested in the assay. Each beaker was labeled with its assigned concentration of *S. tetrandra* root extract.

Gradually increasing amounts of *S. tetrandra* root extract were added to each batch, with the concentration of extract increasing by tenfold (**Table 1**). Final concentrations were 0.003688, 0.03688, 0.3688, 3.688, and 18.44 mg/mL of food. The variations in concentrations were widely spaced because there is currently no data on the toxicity of *S. tetrandra* root extract in *D. melanogaster.* The fifth and highest concentration was reduced to half of what would be the highest tenfold concentration due to supply issues at the time; however, the second part of the assay would test the toxicity of the full, highest tenfold concentration on a larger group of flies.

To find the starting dosage for the toxicity assay, the following Human Equivalent Dose (HED) formula was used to scale dosages between species (17):

$$\text{HED dose}\,\frac{\text{mg}}{\text{kg}} = \text{Animal dose}\,\frac{\text{mg}}{\text{kg}} \times \left(\frac{\text{Weight}_{\text{animal}}[\text{kg}]}{\text{Weight}_{\text{human}}[\text{kg}]}\right)^{(1-0.67)}$$

This equation was solved for the animal dosage of *S. tetrandra* in mg/kg, based on the ideal starting human equivalent dose of 100 mg/kg and assuming an average *D. melanogaster* mass of 1 x 10-6 kg (18). This formula indicated a dosage of 0.369 mg per *D. melanogaster.* The dosage was converted into a concentration under the assumption that there are approximately 10 flies per vial and each vial contains 10 mL of food.

To perform the toxicity assay, we administered each batch of food to groups of six flies. One fly was placed in each vial of food, with six vials in all for each concentration.

The maximum tolerated dose (MTD) selected was the highest concentration of *S. tetrandra* extract that did not kill more than one third of the flies in its tested group as of 4 days post-administration. The procedure for selecting the MTD was based on the 3 + 3 dose escalation method, which is a common method in pharmaceutical research to determine the highest possible dosage of a new substance for an organism (19).

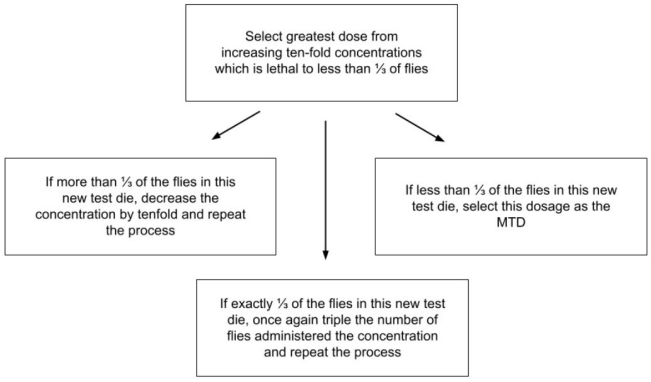

Figure 3. Process of 3+3 dose escalation method. To choose the highest possible non-lethal dosage of *S. tetrandra* extract, the 3+3 dose escalation method was referenced. Based on the number of flies that died for 4-days after administration of each concentration group, the MTD was determined. However, due to a lack of supplies, the procedure for this method was modified; after finding the highest dose that killed less than ⅓ of flies, the dose was doubled and used as the MTD.

Due to a limited supply of the extract, half of the highest tenfold concentration (18.44 mg/mL of food) was used to perform the toxicity assay. We determined a priori that if this halved concentration was selected as the MTD, the full tenfold concentration (36.88 mg/mL of food) would be used for experimentation, maximizing the likelihood of inducing OCS. The full concentration was deemed acceptable, as the extract was proven to be largely non-toxic. The concentration of 36.88 mg/mL of food was used all throughout experimentation.

Locomotion Assay

Ten flies on a control diet and 10 flies on a diet supplemented with *S. tetrandra* extract were anesthetized using a cold pad. Female *D. melanogaster* spend less time walking than male *D. melanogaster* (20). Sex was preserved as a constant by only using female *D. melanogaster.* The anesthetized flies were moved to a 15 cm petri dish. An iPhone camera was positioned above the apparatus with its flashlight turned on to illuminate the flies. Flies were given two minutes to awake from being anesthetized, after which the movements of the flies were filmed for one minute. Videos were analyzed manually at 0.5x speed to increase the accuracy of measurements. The locomotion of each fly in both the control and experimental trials was timed, with the timer running whenever the fly moved. At the conclusion of the video, the total time displayed on the timer was divided by two to account for the video being viewed at half speed. The resulting value represented the total time the fly spent in motion. No technical replicates were performed.

Grooming Assay

Ten flies from each group were anesthetized using a cold pad. Female and male *D. melanogaster* display differences in grooming behavior, so only female *D. melanogaster* were used to keep sex as a constant (21). Each anesthetized fly was brushed into a well containing 0.015 grams of Brilliant Yellow Dye and coated by flipping the well plate three times. Each group tested consisted of forty flies with no technical replicates. The flies in the control group that were given zero minutes to groom were immediately pinched by their wings with tweezers and dropped into prepared tubes containing 1 mL of 100% ethanol. The well plate containing the control group that was given 30 minutes to groom and the well plate containing the experimental group were both placed in an incubator for 30 minutes.

After 30 minutes, the well plates were placed in an ice bath to anesthetize the flies before each was placed into an ethanol tube. With the flies still inside, the ethanol tubes were flipped three times to mix the dye and ethanol. The tubes were then incubated for five hours at room temperature to allow for the full solubilization of the dye.

After the incubation period, 50 µL of solution were micropipetted from each of the tubes into a new 96-well plate. To prevent an overflow reading from the microplate reader, 200 µL of 100% ethanol were added to the wells to dilute each sample. The microplate equipped with BioTek Gen5 Microplate Reader software read the absorbances of the samples at 297 nm.

Statistical Tests

For all the statistical tests conducted in this experiment, the threshold for statistical significance was a p-value less than 0.05. Grooming assay data were independent, randomly sampled, and have equal variances, so the data were analyzed using two sample t-tests. The two-sample t-tests determined if there were significant differences between the average absorbances of groups of flies, thereby indicating if one group exhibited more grooming than the other. To confirm the flies were indeed grooming themselves, a two-sample t-test was performed comparing absorbances from control flies given zero minutes to groom versus control flies given 30 minutes to groom. To identify if the extract caused one group of flies to groom more than the other, a two-sample t-test was performed to compare the absorbances from control or experimental flies each given 30 minutes to groom. A statistically significant difference would show that the *S. tetrandra* extract did induce OCS in the flies.

For analysis of the locomotion assay data, a Mann-Whitney test was chosen because the data were independent, randomly sampled, and not normally distributed. The Mann-Whitney test determined if there was a significant difference between the locomotion times of the experimental and control flies, which would indicate that the *S. tetrandra* extract increased anxiety in the experimental flies.

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