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PLDT (planarian light/dark test): an invertebrate assay to quantify defensive responding and study anxiety-like effects

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Abstract

Background—Planarians, like rodents, instinctively spend more time in dark versus light environments when given a choice. This behavioral phenomenon is called negative phototaxis, which may reflect defensive responding related to an anxiety-like phenotype.

New Method—We propose a planarian light/dark test, designated PLDT, to predict anxiogenic- or anxiolytic-like effects. Experimentally, we placed a planarian at the midline of a Petri dish, containing test compound or water, that was split evenly into light and dark compartments and determined time spent in the light over 10 min.

Results—A clinically-approved benzodiazepine agonist (clorazepate; 10 μ M) increased time spent in the light whereas an inverse benzodiazepine agonist (FG-7142; 1, 10 μ M) produced the opposite response. Fluoxetine (1 μ M) or ethanol (1 %), as well as the ‘bath salt’ cathinone *S*-mephedrone (300 μ M), enhanced time spent in the light. Planarians exposed to predator (frog) odor spent more time in the dark.

Comparison with Existing Methods—The light/dark box (LDB) test in rodents is used to screen putative medications for possible anxiolytic and anxiogenic effects. Our results showing that time spent in the light by planarians is enhanced by common anxiety-relieving drugs (*e.g.* benzodiazepine agonist, ethanol, fluoxetine) and decreased by anxiogenic substances (*e.g.* predator odor, benzodiazepine inverse agonist) reveal directionally similar effects in the established (LDB) and new (PLDT) assays.

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Conclusion—Our data identify the PLDT as a cost-effective, invertebrate assay for quantifying the effects of practically any water-soluble substance on defensive responding and for studying and teaching anxiety-like responses in a living organism.

Keywords

planarian; anxiety; invertebrate; benzodiazepine; clorazepate; fluoxetine; mephedrone; cathinone; carboline

1. Introduction

Planarians are flatworms of the Turbellaria class that are the simplest living animals with bilateral symmetry and a central nervous system with cephalization (Pagán, 2014). They express and utilize neurotransmitters, including dopamine, serotonin, GABA, glutamate and acetylcholine (Nishimura et al., 2008), and, when exposed to specific drugs and stimuli, display mammalian-like responses such as a change in motility, enhanced stereotypical activity, behavioral sensitization, drug seeking and withdrawal (Palladini et al., 1996). Although a remarkable regenerative capacity is the hallmark feature of planarians, another defining phenomenon is a tendency, akin to rodents, to spend greater time in light versus dark environments (*i.e.*, negative phototaxis or light avoidance in which planarians travel away from a light stimulus) (Davidson et al., 2011). The organ system responsible for detecting light in planarians (the so-called “visual system”) has been the subject of many studies (Dong et al., 2012). Planarians are indeed one of the most primitive animals to develop two forward facing eyecups, each composed of photoreceptors and pigment cells in a rhabdomeric structure registering the presence and direction of light.

Anxiety is a phenotype that has not been modeled extensively in planarians but might be predicted by light/dark inclination assays. Planarians, like rodents, tend to spend more time in dark versus light environments when given a choice. Moreover, at present, the light/dark box (LDB) test in rodents is one of the most widely used assays for screening putative medications for potential anxiolytic and anxiogenic activities (Ennaceur et al., 2013). In the LDB test the percentage of time a rodent spends in the light compartment following injection of a drug is used to predict of a drug’s anxiolytic or anxiogenic effects, with time spent in the light enhanced following injection with an anxiolytic drug and reduced following injection with an anxiogenic substance (Ennaceur et al., 2013).

In the present study, we propose a comparable test in planarians – one designated as the planarian light/dark dish test (PLDT). To validate the PLDT test, we screened 3 different classes of drugs: (1) benzodiazepine receptor compounds, which are clorazepate, a benzodiazepine agonist approved to treat anxiety and depression in humans, and FG-7142, a benzodiazepine inverse agonist that produces anxiogenic effects in mammals (Venault and Chapouthier, 2007); (2) synthetic cathinones, which are bupropion, approved to treat depression and nicotine dependence, and *S*-mephedrone (*S*MEPH), a ‘bath salt’ designer cathinone that shares structural similarity with bupropion and displays anxiolytic and antidepressant efficacy in rats (Philogene-Khalid et al., 2017); (3) fluoxetine (Prozac), a

selective serotonin reuptake inhibitor (SSRI) approved to treat depression and some anxiety disorders (*e.g.* panic disorder); and (4) ethanol.

2. Materials and Methods

2.1. Subjects and drugs

Planarians (*Dugesia dorotocephala*) were purchased from Carolina Biological Supply (Burlington, North Carolina, USA). Clorazepate dipotassium salt, fluoxetine hydrochloride, bupropion hydrochloride, FG-7142 (β -Carboline-3-carboxylic acid N-methylamide), and ethanol were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Drugs were dissolved in spring water (*i.e.*, water in which planarians are maintained). *S*-mephedrone (*S*-MEPH), a synthetic cathinone, was synthesized by Fox Chase Chemical Diversity (Doylestown PA, USA). Frog juice was purchased from Bog Baits (Beaver Dam, WI, USA).

2.2. PLDT tests (Light/dark experiments)

We assessed baseline responding in spring water at different times of the day to quantify the magnitude of time spent in the light versus dark compartments and to choose a suitable time of day for conducting behavioral experiments. Each planarian was removed from its home jar and placed at the midline of a Petri dish (5.5 cm diameter) containing spring water. A sleeve of black construction paper was positioned to cover one half of the dish on the top, bottom, and vertical sides to create distinct dark and ‘ambient’ light environments. Each planarian was provided free access to roam both sides of the dish, and time spent in the ambient light compartment was recorded over 10 min.

Using a similar paradigm, we investigated how known anxiolytic and anxiogenic stimuli affect time spent in the light compartment. Experiments were conducted between 10am and 4pm. Each planarian was removed from its home jar and placed into a secondary jar (identical to their home jar in shape) containing test compound or spring water for 30 min. Each secondary jar only contained a single planarian. Following the 30-min pretreatment in the secondary jar, each planarian was removed and placed at the midline of a Petri dish (5.5 cm diameter and split into light and dark compartments as described above) containing the same concentration of test compound or spring water. Each planarian was provided free access to roam both sides of the dish, and time spent in the ambient light compartment was recorded over 10 min. Concentrations selected for PLDT tests were based on earlier work (Tallarida et al., 2014) and did not reduce planarian motility [clorazepate (10, 25, 50 μ M); FG-7142 (1, 10 μ M); *S*-MEPH (10, 100, 300 μ M); bupropion (10, 100, 300 μ M); fluoxetine (0.5, 1, 10 μ M); or ethanol (0.001, 0.01, 1%)]. To confirm effects using a natural stimulus, an additional experiment testing the effect of predator odor (*i.e.*, frog scent) on light/dark response was conducted using concentrations of 0.001 and 0.01%.

2.3. Data analysis

For each concentration of specific test compound, time spent in the ambient light was normalized to its respective water control and expressed graphically as percentage of water control (S.E.M.). Using this approach, a matching water control group was included for each substance tested. The absolute time spent in the light (s) during the 10-min (600 s)

observation interval was determined for each condition (water or substance) and then converted to percentage of time spent in the light using the following formula: (absolute time spent in light[s]/600 s) $\times$ 100. Data were analyzed by one-way ANOVA. In cases of a significant main effect, differences between treatment groups and water control were determined by a Dunnett's post-hoc analysis. Statistical significance was set at $p < 0.05$.

3. Results

Experiments conducted in spring water at 4 different times of day (12am, 6am, 12pm and 6pm) revealed the following (n=12 planarians per time point) (time of day, percentage of time spent in light compartment $\pm$ SEM): (6am, 34.29 ± 5.17); (12pm, 37.10 ± 7.91); (6 pm, 45.58 ± 5.18); and (12am, 60.75 ± 4.95). One-way ANOVA identified a significant main effect [$F(3, 44) = 4.023$, $p < 0.05$], and post-hoc analysis indicated a significant difference in time spent in the light at 6am and 12am.

Fig. 1 presents effects on light/dark inclination (expressed as percentage of respective water control) for different classes of compounds. For the 6 respective water control groups (*i.e.*, an individual water control group was included for each test compound), the percentage of time spent in the light was not significantly different (one-way ANOVA; [$F(5, 76) = 1.815$, $p > 0.05$]) (data not shown). Panel A shows effects of a benzodiazepine agonist (clorazepate) and benzodiazepine inverse agonist (FG-7142). For clorazepate, one-way ANOVA indicated a main effect [$F(3, 44) = 3.877$, $p < 0.05$]. Planarians treated with 10 μ M clorazepate spent more time in the light than water-exposed controls ($p < 0.01$). For the benzodiazepine inverse agonist FG-7142, one-way ANOVA indicated a main effect [$F(2, 33) = 12.48$, $p < 0.0001$]. Time spent in the light was enhanced in planarians treated with 1 or 10 μ M FG-7142 relative to planarians treated with water ($p < 0.001$). Panel B shows effects of two synthetic cathinone compounds (*S*-MEPH and bupropion). For *S*-MEPH, one-way ANOVA indicated a main effect [$F(3, 30) = 8.640$, $p < 0.0001$]. Planarians treated with 300 μ M *S*-MEPH spent more time in the light than water-exposed controls ($p < 0.001$). For bupropion, a significant main effect was not detected with a one-way ANOVA [$F(3, 74) = 1.463$, $p > 0.05$].

Effects of fluoxetine (SSRI) and ethanol are shown in Panels C and D, respectively. One-way ANOVA indicated a main effect for fluoxetine [$F(3, 44) = 4.732$, $p < 0.01$] (C). Treatment of planarians with 1 μ M fluoxetine increased time spent in the light relative to water-exposed planarians ($p < 0.01$). For ethanol (D), one-way ANOVA indicated a main effect [$F(3, 33) = 4.656$, $p < 0.01$]. Compared to water-treated controls, planarians treated with 1 % ethanol spent greater time in the light ($p < 0.01$).

For frog scent experiments (0, 0.01, 0.001%) there was a significant main effect [$F(2, 29) = 4.822$, $p < 0.05$] (data not shown). Post-hoc analysis revealed that planarians exposed to 0.01% frog scent spent a greater amount of time in the dark compared to water-treated controls ($p < 0.01$).

4. Discussion

The simplicity of the planarian brain (Pagan et al., 2014), combined with planarians expressing mammalian-like neurotransmitter systems and displaying quantifiable behaviors in response to stimuli, offers an invertebrate model for studying stimulus/response events and developing predictive assays to screen small quantities of essentially any compound for potential therapeutic activity. The goals here were to determine if behavioral responses to anxiolytic or anxiogenic stimuli can be reliably quantified using a planarian light/dark test (PLDT). We found that the time planarians spend in a light compartment increases during exposure to drugs that reduce anxiety in humans (*e.g.* clorazepate, fluoxetine, and ethanol) and decreases during exposure to an agent that produces anxiety in mammals (*e.g.* FG-7142). Previous findings indicate that drugs of abuse, when administered in conditioning or withdrawal paradigms, alter negative phototaxis in planarians. Negative phototaxis is reduced (*i.e.*, planarians spend more time in the light compartment) following conditioning sessions in which an addictive substance is paired with the light compartment (Hutchinson et al., 2015; Tallarida et al., 2014). However, following withdrawal from cocaine or ethanol exposure, planarians display enhanced negative phototaxis (*i.e.*, spend less time in the light compartment) (Nayak et al., 2016).

The aforementioned studies did not investigate unconditioned anxiety-like responses in planarian, especially in the context of how acute exposure to known anxiolytic and anxiogenic compounds might affect light/dark responses in planarians. Here, using the PLDT test, we show that clorazepate, a benzodiazepine receptor agonist approved to treat anxiety disorders, increased the percentage of time that planarians spent in the light compartment at doses below those that reduce motility, suggesting that clorazepate efficacy in the PLDT test was not due to generalized behavioral depression. Clorazepate and related benzodiazepine agonists are not only efficacious against anxiety disorders in humans but also show broad-range efficacy in most preclinical models including the EPM and LBD tests (Ennaceur, 2013). In contrast to clorazepate, the β -carboline compound FG-7142, an inverse benzodiazepine receptor agonist, produced the opposite phototactic effect, decreasing time spent in the light compartment compared. FG-7142 is frequently used to induce an anxiogenic state in mammalian models of anxiety, including the EPM, LBD, and self-injury (Venault and Chapouthier, 2007). One limitation of our study is an inability to specifically identify receptor sites of action as the planarian genome remains poorly elucidated. It should be noted that planarians do express and utilize the neurotransmitter GABA, as well as elements of the GABA system such as glutamic acid decarboxylase (GAD), the enzyme that converts glutamate into GABA (Nishimura et al., 2008).

Two synthetic cathinones – bupropion and *S*-MEPH (*S* enantiomer of MEPH) were also tested in the PLDT test, with *S*-MEPH displaying efficacy. Mephedrone (4-methylmethcathinone, MEPH) is among the class of synthetic cathinones (*i.e.*, beta-ketone amphetamine) designated as controlled substances (Schedule I) but originally sold legally under labels such as “plant food”, “bath salts”, and “research chemicals”. Notably, MEPH has a chemical composition that includes R and S enantiomers—constituent compounds in racemic mixture much like other controlled substances including methamphetamine (METH), cocaine and MDMA. The stereochemistry of enantiomers allows us to separate

addictive and therapeutic effects of a drug, as each enantiomer can produce disparate CNS effects. Gregg et al. (2015) showed that both enantiomers of MEPH display similar potency as substrates at DAT, but *S*-MEPH is about 50-fold more potent than *R*-MEPH in promoting 5-HT release by its substrate action at SERT. Dose-response experiments using intracranial self-stimulation (ICSS) to compare rewarding effects in rats demonstrated that *R*-MEPH produces greater maximal facilitation of ICSS than equivalent doses of *S*-MEPH (Gregg et al., 2015). In rat CPP studies, *R*-MEPH produces place preference whereas equivalent doses of *S*-MEPH do not have rewarding effects (Gregg et al., 2015). Importantly, *S*-MEPH may have some therapeutic potential, as it displays anxiolytic and antidepressant effects in cocaine-withdrawn rats (Philogene-Khalid et al., 2017). In the present experiments, *S*-MEPH, during acute exposure in otherwise naïve planarians, increased the percentage of time spent in the light compartment in the PLDT test, indicating a reduction in negative phototaxis and defensive responding. Previous work has shown that *S*-MEPH, when tested over concentrations similar to the concentration that produced efficacy here in the PLDT test, does not produce environmental place conditioning (i.e., CPP) or robust stereotypical responses in planarians (Vouga et al., 2015). Taken together, these data suggest that *S*-MEPH efficacy in the PLDT test was due more to its anxiolytic properties than rewarding efficacy or extraneous behavioral effects. It will be important in future studies to assess the efficacy of *S*-MEPH on unconditioned anxiety-like responses in rats.

Since *S*-MEPH is structurally similar to bupropion, with both compounds sharing the cathinone structural signature, it was somewhat surprising that bupropion was ineffective in the PLDT test. The differences in efficacy could be due to underlying differences in mechanism between *S*-MEPH and bupropion. While *S*-MEPH is a substrate-type releaser at monoamine transporters with strong 5-HT- and NE-releasing effects (Gregg et al., 2015), bupropion is a monoamine transporter blocker that preferentially inhibits DA and NE uptake with minor effects on 5-HT. The stronger 5-HT releasing effect of *S*-MEPH relative to bupropion may have contributed to its greater efficacy in the PLDT test. It should be noted that bupropion efficacy in mammalian models of anxiety are inconsistent and dependent on factors such as dose of the drug and age of the animals (Carrasco et al., 2013).

Fluoxetine, the prototypical SSRI, and ethanol, the world's most commonly abused drug, also increased percentage of time spent in the light compartment. Considering the inconsistent and contradictory results obtained with SSRIs in standard mammalian models of anxiety (EPM, LDB) (Ennaceur, 2013), the marked efficacy of fluoxetine in the planarian test is especially interesting and suggests the PLDT test could be a useful complement to established mammalian models such as the EPM and LDB tests, which are often more predictive for GABA-based compounds (Ennaceur, 2013). The underlying mechanism of fluoxetine in planarians is unclear, though elements of a robust 5-HT system, including 5-HT neurons and receptors (5-HT1A), have been identified (Saitoh et al., 1996).

In summary, we show that defensive responding by planarians is altered by anxiolytic or anxiogenic drugs and identify the PLDT as an invertebrate assay for studying and teaching anxiety-like responses in a living organism. Our evidence suggests the model is predictive, as a benzodiazepine agonist and antagonist produced directionally opposite effects on negative phototaxis, and sensitive, as different classes of drugs (benzodiazepines,

cathinones, SSRIs, and ethanol) were efficacious. In addition to investigating predator odor and a benzodiazepine antagonist, other anxiogenic stimuli, including alterations in home-water pH, unconditioned acute responses to light shock, and more intense light exposure, will be evaluated in future studies to further validate the planarian assay. While defensive responding in planarians was indeed altered by anxiolytic and anxiogenic substances, one apparent liability of the invertebrate assay, akin to rodent assays, is an inability to capture innate motivated behaviors that contribute to the cognitive and emotional features of anxiety.

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Highlights

- We propose planarians to study defensive responding and anxiety-like effects.
- The invertebrate model is designated as the PLDT (planarian light/dark test).
- Clorazepate, fluoxetine, ethanol and a synthetic cathinone decreased defensive responding.
- An inverse benzodiazepine agonist and predator odor increased defensive responding.
- PLDT may be as a predictive, cost-effective screen to study anxiety-like effects.

Fig. 1. Effects of benzodiazepines (A), cathinones (B), SSRI (C) and ethanol (D) on time spent in the light compartment

Planarians were pretreated with a specific drug for 30 min and then placed into a Petri dish (split evenly into light and dark compartments) containing that same drug for 10 min during which time spent in the light environment was determined. A matching water control was run for each drug. Percentage of time spent in the light for each respective water control was (Drug, % time in light): (Clorazepate, 37%); (FG-1742, 45%); (Fluoxetine, 26%); (*S*-mephedrone, 41%); (Bupropion, 27%); and (Ethanol, 38%). Data are expressed as percentage of water control time spent in the light (+S.E.M.). ** $P < 0.01$ or *** $P < 0.001$ compared with respective water control.