Journal of Neuroscience Methods 293 (2018) 284–288

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Short communication

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

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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.

ARTICLE INFO

Article history:
Received 8 July 2017
Received in revised form
19 September 2017
Accepted 13 October 2017
Available online 16 October 2017

Keywords:
Planarian
Anxiety
Invertebrate
Benzodiazepine
Clorazepate
Fluoxetine
Mephedrone
Cathinone
Carboline

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 μM) increased time spent in the light whereas an inverse benzodiazepine agonist (FG-7142; 1, 10 μM) produced the opposite response. Fluoxetine (1 μM) or ethanol (1%), as well as the 'bath salt' cathinone *S*-mephedrone (300 μM), 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.
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.

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

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https://doi.org/10.1016/j.jneumeth.2017.10.010
0165-0270/

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. 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.

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 μM); FG-7142 (1, 10 μM); S-MEPH (10, 100, 300 μM); bupropion (10, 100, 300 μM); fluoxetine (0.5, 1, 10 μM); 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) x 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 ± 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 μM 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