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Effects of diphenylhydantoin on locomotion and thigmotaxis of larval zebrafish

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ABSTRACT

Antiepileptic effects of diphenylhydantoin (DPH) have been documented in animal studies and clinical research, while little is known about the effects of the drug on basic behaviors and anxiety-related behaviors. In order to understand neuroactivities of DPH deeply and administrate DPH in clinic rationally, it is necessary to study neurobehavioral effects of the drug. In the present study, the effects of DPH on the locomotor activity and thigmotaxis of zebrafish larvae at 5 days post fertilization (dpf) were explored under different illumination conditions. The influence of DPH on zebrafish larval responses to visual stimuli (sudden illumination transition from light to dark) was also investigated. Under light or dark condition, exposure to high concentrations of DPH resulted in decreased locomotor activity and thigmotaxis, whereas DPH treatment at low doses enhanced the locomotor activity. Additionally, sudden illumination transition induced robust increase in the locomotor activity and this phenomenon was not modified by DPH treatment. Our results suggest that DPH has potential stimulatory and inhibitory effects on the locomotor activity and possesses anxiolytic properties. In addition, responses of 5-dpf zebrafish larvae to visual stimuli were not modified by DPH treatment.

1. Introduction

Zebrafish (*Danio rerio*), as an emerging non-mammalian vertebrate model organism, is ideally suited for large-scale analyses of drug-induced behaviors. Hundreds of fertilized eggs can be readily harvested daily and can be immediately and directly treated with external compounds during different developmental stages and their small size allows for easier handling and husbandry (Colwill and Creton, 2011; Richendrfer et al., 2012). Zebrafish develops rapidly into free-swimming larvae. A fertilized egg develops into a larva with eyes, a beating heart, and tail movements after 24 h (Kimmel et al., 1995). By 4–5-dpf, zebrafish larvae demonstrate a broad range of behaviors, such as hunting, avoidance, startle response, scototaxis and thigmotaxis (Fetcho and Liu, 1998; Colwill and Creton, 2011; Schnorr et al., 2012). Thus, many behavioral tests can be carried out in larval zebrafish as early as the first week post fertilization.

Thigmotaxis, as an index of anxiety, is the unconditioned preference of an animal for the boundaries of an environment. This behavior is evolutionarily conserved and performed by a variety of species, including humans (Kallai et al., 2005; Kallai et al., 2007), rodents (Treit and Fundytus, 1988; Prut and Belzung, 2003; Sousa et al., 2006; Belzung and Philippot, 2007) and fish (Peitsaro et al., 2003; Lopez-Patino et al., 2008; Sharma et al., 2009; Champagne et al., 2010; Colwill and Creton, 2011). Zebrafish larvae display clear thigmotaxis behavior as well. Many factors are able to influence the thigmotaxis of zebrafish larvae, such as feeding and neuroactive drugs. The fed 7-dpf larvae displayed a decreased thigmotaxis, compared with the unfed 7-dpf larvae (Clift et al., 2014). The thigmotaxis of 5-dpf larvae was significantly attenuated by the anxiolytic agent diazepam and significantly enhanced by the anxiogenic drug caffeine (Schnorr et al., 2012). In addition, Zebrafish larvae at 5-dpf exhibited reduced thigmotaxis when treated with yohimbine at 25 mg/L, while higher concentrations of yohimbine had no effect on the thigmotaxis in 5-dpf larvae (Li et al., 2014).

DPH, a sodium channel blocker, is one of the most popular antiepileptic drugs (Munro et al., 2007). It is supposed to act by modification of glutamatergic transmission (Bremner et al., 2005). Although DPH has good pharmacological efficacy, its clinical use is often limited because of side effects such as teratogenicity and neurotoxicity. Teratogenicity effects of DPH have been documented in studies with rodents and humans (Cohn et al., 1978; Yang et al., 1978; Kim et al., 2012), while more attention should be paid on functional deficiencies study, such as neurobehavioral function, as behavioral analysis is perhaps the best way to investigate the function of the brain (Gerlai, 2003; Tierney, 2011). The neuroactivities of DPH have been studied in rodents. Pregnant Sprague–Dawley (SD) rats orally administered DPH (200 mg/kg) on days 7–18 of gestation showed increased early locomotor activity in offspring (Minck et al., 1991). The intraperitoneal injection of DPH

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at 5–10 mg/kg blocked the increased open-field locomotion that was induced by methylphenidate (Tonelli et al., 2013). The daily pretreatment of the DPH (120 mg/kg, i.p.) abolished the locomotor hyperactivity in the open-field test in rats of seizures induced by repeated electroconvulsive shock (ECS) (Hidaka et al., 2008). Adult SD rats that were exposed to therapeutic levels of DPH throughout prenatal development and the postnatal pre-weaning period showed increased rates of acquisition and performance in both the appetitive and avoidance learning paradigms and a substantial impairment in avoidance learning following the transfer from appetitive to aversive conditioning (Mowery et al., 2008). Male Wistar rats that were exposed to DPH (75 mg/kg, i.p.) for 21 days displayed significant deficits in learning/memory as indicated by a significant increase in the retention transfer latency in an elevated plus maze test and a significant decrease in the retention latency in the passive avoidance paradigm (Reeta et al., 2009).

In addition to the work in rodents, there are relatively few reports on the locomotion effects of DPH in zebrafish and most were relative to seizure. The seizure-related behavioral alterations in adult zebrafish that were induced by pentylenetetrazole (PTZ) treatment were suppressed by pretreatment with DPH at 450 μM for 1 h (Siebel et al., 2013). The seizure-like swimming pattern in zebrafish larvae that was induced by 2 h exposure to ginkgotoxin was significantly reversed by simultaneously adding 1 mM of DPH (Lee et al., 2012).

AEDs have been successfully used in the treatment of mood disturbances including anxiety disorders, such as tiagabine (Baetz and Bowen, 1998), gabapentin (Pollack et al., 1998) and pregabalin (Stahl, 2003). DPH treatment (30 mg/kg, i.p.) for 15 days significantly improved the depressive like behavior along with its anticonvulsant effect in Male Swiss Albino mice that were successfully kindled by pentylenetetrazole (Choudhary et al., 2013). In male ddY mice at 7–8 weeks of age, DPH treatment (10 mg/kg, i.p.) enhanced effects of (+)-SKF-10,047 and dextromethorphan on the stress response, while the effects could be blocked by the dopamine D1 and D2 receptor antagonists (Kamei et al., 1996). DPH has been demonstrated to exert prophylactic effect in bipolar disorder patients, one of the most common mood disorders (Mishory et al., 2003).

It has been implied from the experimental literature that DPH exposure could influence behaviors, such as learning- and seizure-related behaviors, while little is known about its effects on basic behaviors and anxiety-related behaviors. Study of neurobehavioral effects of the drug is necessary for better understanding of neuroactivities of DPH and beneficial for the rational administration of DPH in clinic. In this current study, we explored the effects of DPH on the locomotor activity and thigmotaxis of zebrafish larvae at 5-dpf under light or dark conditions, because behaviors of zebrafish can be influenced by environmental factors (Irons et al., 2010; de Esch et al., 2012). The influence of DPH on zebrafish larval responses to visual stimuli was also investigated. It was hypothesized that DPH could modify the locomotor activity and anxiety-related behavior in zebrafish larvae, which would be concentration-dependent.

2. Materials and methods

2.1. Animals and housing

AB wild type zebrafish was maintained at 28.5 °C and kept on a 14:10-hour light:dark cycle (lights on at 08:00 AM). The eggs were obtained by random mating between sexually mature individuals and were raised in groups of 50 in an incubator at 28.5 °C. The eggs and larvae were kept under the same lighting schedule as that of adult zebrafish. Larvae were allowed to develop under this condition until behavioral testing at 5-dpf and the larvae that were used in the experiments were not fed. All of the animal experimental procedures complied with local and international regulations. All of the protocols were approved by the institutional animal care committee, Children's Hospital of Fudan University.

2.2. Drugs

5, 5-Diphenylhydantoin sodium salt (D4505-25G, Sigma-Aldrich) was dissolved in a 30 mM stock solution with sterilized water and stored at −80 °C. DPH working solution was freshly diluted from stock solution to appropriate concentrations with zebrafish system water before the experiments.

2.3. Drug treatment and behavior tests

Behavior tests were carried out with the zebrafish larvae at 5-dpf in 24-well plates. All the experiments were performed 2 h after the beginning of the light cycle and 2 h before the beginning of the dark cycle. The experiments were arranged in a way that all concentration groups were equally presented in each 24-well plate to avoid any inter-treatment variations due to differences in experiment timing during the day.

The zebrafish larvae were carefully transferred to a 24-well plate with one single larva in each well. Excessive fluid was removed, and 500 μL of fresh system water was loaded into each well immediately. Subsequently, 500 μL of DPH working solution was quickly added into the wells; therefore, each well contained 1 mL liquid. The final tested DPH concentrations were 0 (control), 1, 4, 20, 100 and 500 μM. The plate was then placed into Zebrabox (ViewPoint Life Sciences) equipped with a recorder to record the video of zebrafish larvae activities. The current protocol and experimental procedure are shown in Fig. 1A.

2.3.1. Locomotor activity

The quantification of zebrafish larvae locomotor activity was achieved using the tracking mode of Zebralab software with recorded videos. The videos of zebrafish larvae were taken at the rate of 25 fps, and were pooled into 1 min time bins. Only the total distance traveled was obtained for analysis. The distance moved by the larvae in the whole well was acquired for the analysis of general locomotor activity.

2.3.2. Thigmotaxis

A round-shaped center arena that occupied half of the area of a single well was defined in each well (Fig. 1B). Thigmotaxis was presented as the percentage (%) of the total distance moved (TDM) in the outer zone of the test apparatus as previously described by Schnorr et al. (2012). The percentage of TDM in the outer zone was obtained by multiplying this ratio by a factor of 100 as depicted in the formula below. This calculation was performed to correct for individual differences in the locomotor activity as recommended by Bouwknecht and Paylor (2008).

$$\text{Thigmotiaxis}\,(\%\text{TDM in outer zone}) = \left[\frac{\text{TDM outer}}{\text{TDM outer} + \text{inner}}\right] \times 100$$

2.4. Data presentation and statistics analysis

Data are presented as the mean ± SEM. Statistical analyses and graphs were performed using GraphPad Prism software (version 5.0).

One-way ANOVA followed by Dunnett's multiple comparison post hoc tests was performed to compare the DPH-treated groups with the controls to assess the effects of DPH on the locomotor activity and thigmotaxis, and a probability level of 5% was used as the minimal criterion of significance.

Student's t-tests (two-tailed) were performed to analyze the behavioral changes in response to light/dark challenges within each concentration group (light vs. dark). The minimal criterion of significance was set at 5%.

Correlation analysis was performed between the locomotor activity and thigmotaxis in the same DPH-treated groups and corresponding control groups.