Comparative Biochemistry and Physiology, Part C 155 (2012) 33–38

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Behavioral and physiological effects of RDX on adult zebrafish ☆

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ARTICLE INFO

Article history:
Received 8 December 2010
Received in revised form 25 February 2011
Accepted 26 February 2011
Available online 5 March 2011

Keywords:
Seizures
Epilepsy
Pro-convulsant action
Behavioral activation
Cortisol
Zebrafish

ABSTRACT

1,3,5-Trinitroperhydro-1,3,5-triazine (RDX) is a nitroamine explosive, with common toxic effects including seizures. Here, we explore the behavioral effects of acute RDX exposure in adult zebrafish *Danio rerio*, a rapidly developing model in neuroscience and neurotoxicology research. Overall, a 30-min exposure to RDX low dose of 0.1 mM evoked behavioral activation in zebrafish, while a higher dose of 1 mM markedly reduced exploration, increased freezing and evoked seizure-like responses (*i.e.*, bouts of hyperactivity, spasms, and corkscrew swimming). Likewise, whole-body cortisol levels were also significantly elevated in fish exposed to 1 mM (but not 0.1 mM) RDX. In line with clinical and animal data, our study demonstrates the dose-dependent behavioral activation and pro-convulsant effects of RDX in zebrafish-based models.

1. Introduction

1,3,5-Trinitroperhydro-1,3,5-triazine (RDX, Royal Demolition Explosive) is a high-energy cyclic trinitramine compound and a common ingredient in military and industrial explosives including C4. Over-exposure to RDX is a known cause of dizziness, confusion and seizures (Goldberg et al., 1992; Kasuske et al., 2009; Kucukardali et al., 2003; Woody et al., 1986). RDX-induced seizures are observed clinically after inadvertent or deliberate exposure (Davies et al., 2007; Harrell-Bruder and Hutchins, 1995; Hett and Fichtner, 2002; Kasuske et al., 2009; Kucukardali et al., 2003; Stone et al., 1969), and have been characterized experimentally in animals (Burdette et al., 1988; Meyer et al., 2005; Schneider et al., 1978; Smith et al., 2007).

Recently, Williams et al. (2011) found that RDX has a significant affinity for the convulsant site on the gamma amino butyric acid ($GABA_A$) receptor. RDX readily crosses the blood brain barrier, alters the expression of multiple brain genes, and evokes pronounced seizure-like responses in a wide range of species, from lizards to non-human primates (Bannon et al., 2009; Burdette et al., 1988; Davies et al., 2007; Gust et al., 2009; Gust et al., 2011; Kasuske et al., 2009; Kucukardali et al., 2003; Martin and Hart, 1974; Quinn et al., 2009; Zhang and Pan, 2009).

Zebrafish (*Danio rerio*) are becoming increasingly popular in biomedical research, as they share substantial genetic and physiological homology with humans, rodents and other vertebrate species (Brittijn et al., 2009; Egan et al., 2009). Recently, epilepsy-like behavior has been reported in *larval* zebrafish (Baraban et al., 2007; Baraban et al., 2005; Berghmans et al., 2007; Winter et al., 2008), which are emerging as high-throughput screens for various drugs (Berghmans et al., 2007; Goldsmith, 2004; Langheinrich, 2003). Several limitations of larval models, however, include underdeveloped neural and endocrine systems, small body size and primitive locomotor responses (Ingham, 2009; Kari et al., 2007; Penberthy et al., 2002; Stewart et al., 2010a). Furthermore, larval and adult zebrafish differ in their locomotory patterns, swimming biomechanics and muscular physiology (Muller and van Leeuwen, 2004), all of which may affect their seizure phenotypes. Adult zebrafish have recently been introduced as a model of epilepsy, sensitive to various GABA-ergic convulsants, including pentylelenetetrazole, picrotoxin (Wong et al., 2010) and benzylpenicillin (own unpublished data). Adult zebrafish have also been validated as a useful model to study the effects of various compounds on fish behavior and anxiety (Cachat et al., 2010a; Egan et al., 2009; Levin et al., 2007).

In the present study, we exposed adult zebrafish to various systemic doses of RDX to assess the behavioral and physiological responses evoked by this compound, and support the notion that zebrafish may be useful in modeling epilepsy (Baraban et al., 2007;

Abbreviations: RDX, 1,3,5-trinitroperhydro-1,3,5-triazine; GABA, Gamma-amino butyric acid; DMSO, Dimethyl sulfoxide; OFT, Open-field Test; LDT, Light–dark Test.

☆ This paper is based on a presentation given at the *5th Aquatic Annual Models of Human Disease* conference: hosted by Oregon State University and Texas State University-San Marcos, and convened at Corvallis, OR, USA September 20–22, 2010.

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doi:10.1016/j.cbpc.2011.02.010

Wong et al., 2010). Paralleling results of clinical and rodent studies, our data also implicates zebrafish as an emerging experimental model to investigate the behavioral and physiological effects of RDX.

2. Methods and materials

2.1. Animals and housing

A total of 86 adult (5–7 month-old; ~50:50 male:female ratio) wild type *short-fin* zebrafish (*D. rerio*) were used in this study. The animals were obtained from a local commercial distributor (50 Fathoms, Metairie, LA, USA) and acclimated for at least 20 days to the animal facility. The fish were housed in groups of approximately 30 fish per 40-L tank, filled with filtered facility water maintained at approximately 25 °C and pH of 7.0–8.0. Illumination was provided by ceiling-mounted fluorescent light tubes on a 12:12-h cycle (on: 6:00 h, off: 18:00 h), according to the zebrafish standard of care (Westerfield, 2007). All animals used in this study were experimentally naïve and fed Tetramin Tropical Flakes (Tetra USA, VA) twice daily.

2.2. Experimental manipulations

The animals (n = 12–15 in each group) were individually exposed to 0.1 mM (22 mg/L) and 1 mM (222 mg/L) RDX for 30 min in a 0.5-L plastic beaker. RDX (>99.5% purity) was obtained from the Department of the Navy (Naval Ordnance and Security Activity, Indian Head, MD, USA; (Williams et al., 2011)). RDX doses were dissolved with a 100 mM DMSO (>99.7% purity, Sigma-Aldrich, St. Louis, MO) solution prepared as a safe, concentrated stock solution. Due to the limited solubility of RDX in water, DMSO was used to dilute this compound, and this mix was further diluted in facility water immediately prior to immersing zebrafish in the exposure tanks. Doses and pre-treatment time were selected based on our own pilot studies using a wide spectrum of RDX concentrations (0.01–1 mM; data not shown) as well as pro-convulsant doses of RDX used to model epilepsy in rodents (Smith et al., 2007). In behavioral experiments, control fish tanks were exposed to equivalent volumes of facility water (water control) or DMSO-treated facility water (DMSO control). Since the DMSO control did not evoke seizures (own systematic observations), DMSO control group was not used for testing epilepsy-like behavior for the high dose 1 mM of RDX. Behavioral testing was performed using a standard observation tank, representing a 1.5-L trapezoidal tank (15 height × 7 width × 28 top × 23 cm bottom length; Aquatic Habitats, Apopka, FL, USA) maximally filled with aquarium water. The observation tanks rested on a level, stable surface and were divided into two equal horizontal portions, pre-marked by a line on the exterior (Cachat et al., 2010b; Egan et al., 2009).

Behavioral testing took place between 11:00 and 16:00 h, to ensure consistency and minimize circadian variation in behavioral and endocrine responses. Following pre-treatment, the animals were transferred to the observation tank and recorded for 6 min by two trained observers (inter-rater reliability >0.85, determined using Spearman correlation). The manually recorded endpoints, traditionally used to describe behavioral zebrafish activity in novel tanks (Levin et al., 2007), included time spent (s) in the upper half/top of the tank, number of transitions to the top, number of erratic movements, and frequency and duration (s) of freezing bouts. Erratic movements were defined as sharp changes in direction and/or velocity, representing rapid darting behaviors. Freezing was defined as a total absence of movement, except for the gills and eyes, for>2 s. A significant decrease in exploration (increased latency to reach the top, fewer entries to the top, longer freezing) or elevated erratic movements represent behavioral profiles indicative of high stress and anxiety (Barcellos et al., 2007; Levin et al., 2007). In addition to manually scoring fish behavior, we also performed automated registration of behavior. Trials were recorded to a computer using a USB webcam (2.0-Megapixel, Gigaware, UK) for the 6-min observation period, and subsequently analyzed using EthoVision XT7 (Noldus IT, Wageningen, Netherlands), as described elsewhere (Cachat et al., 2010c). 'Top' and 'bottom' zones were established and event rules set to precisely and consistently register behavioral profiles. Additional endpoints, such as distance traveled (m), velocity (m/s), meandering and turn angle (°) were also analyzed for in this study. In addition to traditional locomotory endpoints, the frequency of the following seizure-related endpoints (Wong et al., 2010) was recorded by the observers: bursts of hyperactivity, spasms, and corkscrew swimming. Hyperactivity, in contrast to erratic movements, was defined as prolonged (>3 s) periods of sharp changes in direction and/or velocity. Sudden overt twitches or small jerks of the body (that may or may not result in propulsion) were recorded as spasms. Corkscrew swimming was defined as swimming in a helical path, and circular swimming episodes were recorded as rapid bouts of swimming in a circle (Wong et al., 2010). In addition, we utilized the open-field test (OFT; a white plastic box 14 height × 29 - width × 37 cm length) and light–dark box test (LD, 16 height × 24 - width × 52 cm length) to more fully characterize the behavioral effects of a non-convulsant dose (0.1 mM) of RDX. OFT data was analyzed by computer to calculate the distance traveled (m), meandering (°/m), velocity (m/s), turn angle (°) and freezing behavior (see (Cachat et al., 2010b) for details). Light–dark box data was manually analyzed to examine the latency and transitions to the light half, and time spent there. Zebrafish LD was a rectangular tank, modified from the mouse light/dark box, and maximally filled with aquarium water (Stewart et al., 2010b). The box rested on a level, stable surface and was divided into two equal vertical portions, demarcated by black and white coloration. The following endpoints were recorded for a 6 min latency to enter the white half, time spent in the white half, and the number of transitions (entries) to the white half. A significant decrease in exploration (longer latency to enter and fewer entries to the white half as well as longer freezing) was indicative of higher anxiety in this test (Egan et al., 2009).

Seizure endpoints for the experimental and control groups were further evaluated using two additional scoring systems (Wong et al., 2010), recorded by two trained observers blinded to the treatments. First, the fish were assigned a score of 0 or 1 for each seizure-like phenotype (hyperactivity, spasm, and corkscrew swimming) based on whether the particular behavior was exhibited during the 6-min observation period. The percentage of fish demonstrating the respective seizure-like phenotype was then calculated. For the second system, cumulative seizure scores on a scale of 0 to 3 were obtained for each fish (as the sum of seizure scores obtained using a 0-or-1 system described above) for individual types of seizure-like behavior, in order to assess the spectrum of different seizure-like phenotypes displayed by each individual animal. The average cumulative seizure scores were calculated for each experimental cohort and compared with their respective controls, providing a quantitative analysis of seizure severity similar to the Racine scale widely utilized in experimental murine models of epilepsy (Racine, 1972, 1975), where greater values result in greater severity.

In addition to manual observation, video-tracking tools (EthoVision XT7) were used to analyze zebrafish activity. Zebrafish swimming behavior was recorded with a webcam connected to a computer (side-view), and analyzed for total distance travelled (m), average meandering, velocity (m/s) and turn angle (°). In addition, traces were generated for each fish, to visualize the patterns of their locomotion in the observation tank (Wong et al., 2010).

Immediately after testing, the animals were euthanized using 500 mg/L Tricaine (Sigma-Aldrich). The cortisol analysis was as described previously (Cachat et al., 2010a; Egan et al., 2009) using human salivary cortisol ELISA kit (Salimetrics LLC, State College, PA). Whole-body cortisol levels were calculated based on the absorbencies of standardized concentrations, and presented as relative concentrations per gram of body mass for each fish (Egan et al., 2009).

RT-PCR was performed against *c-fos* mRNA, to assess the expression of this early proto-oncogene, serving as a marker of neuronal activation.