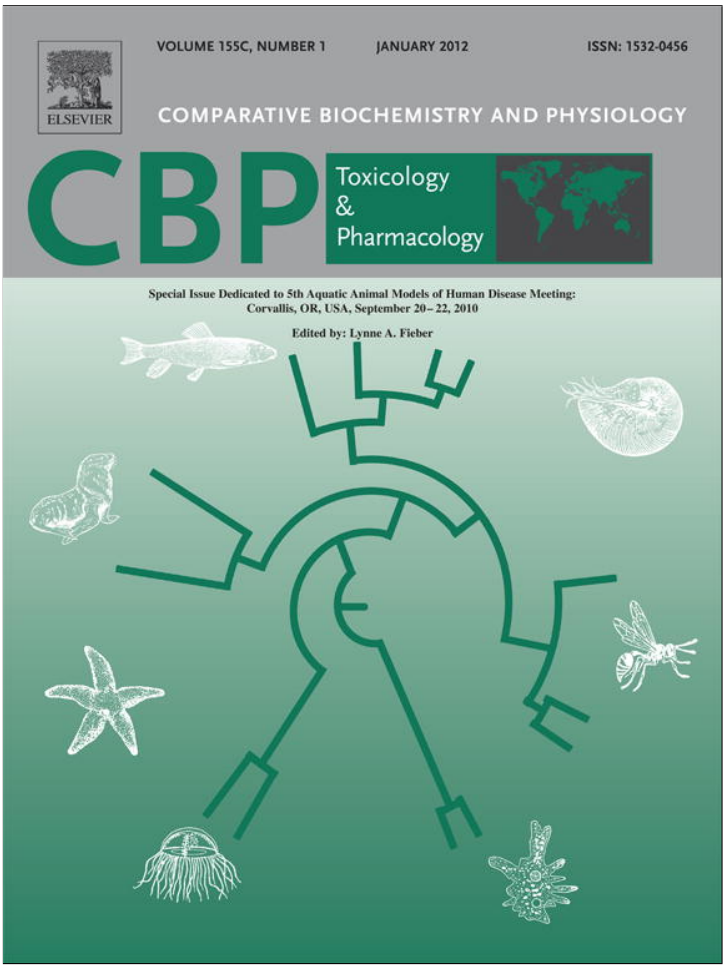

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Behavioral and physiological effects of RDX on adult zebrafish ☆

Larry R. Williams [a,1], Keith Wong [b,1], Adam Stewart [b], Christopher Suciu [b], Siddharth Gaikwad [b], Nadine Wu [b], John DiLeo [b], Leah Grossman [b], Jonathan Cachat [b], Peter Hart [b], Allan V. Kalueff [b,*]

[a] Directorate of Toxicology Health Effects Research Program, US Army Public Health Command, 5158 Blackhawk Rd. Aberdeen Proving Ground, MD 21010-5403, USA
[b] Department of Pharmacology and Neuroscience Program, Zebrafish Neuroscience Research Consortium (ZNRC) and Tulane Neurophenotyping Platform (TNP), Tulane University School of Medicine, New Orleans, LA 70112, USA

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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.

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* Corresponding author at: Department of Pharmacology, Room 3731, Tulane University Medical School, 1430 Tulane Ave., New Orleans, LA 70112, USA. Tel.: +1 504 988 3354.
E-mail address: avkalueff@gmail.com (A.V. Kalueff).

[1] These authors contributed equally to this paper.

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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.

For RT-PCR, 24 zebrafish were exposed to RDX and 24 were exposed to water for 30 min. The brains were dissected, with 4 brains combined per sample. RNA was extracted from all fish brain samples (6 pooled samples per group), and the concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltman, MA). The cDNA was synthesized using random primers and iScript Select cDNA Synthesis Kit (Bio Rad, Hercules, CA, USA). For qRT-PCR, cDNA was amplified with *c-fos* forward primer: 5′-TGCAGCA-CGGCTTCACCGAG and reverse primer: 5′-CGGGCATGAAGAGATCG-CCGT. Amplified products were also compared against a reference gene, *elongation factor 1 alpha*, with forward primer: 5′-CTGGAGGC-CAGCTCAAACAT and reverse primer: 5′-ATCAAGAAGAGTAGTACCGC-TAGCATTAC (Tang et al., 2007).

2.3. Statistical analysis

All data was expressed as mean ± S.E.M., and analyzed using the unpaired Wilcoxon-Mann-Whitney U-test. Significance was set at $P<0.05$.

3. Results

Exposure to 0.1 mM RDX significantly increased locomotion (transitions to top and time in top) and decreased the latency to enter the top in the novel tank test (Fig. 1A). In the OFT, RDX-treated fish displayed marked increases in mean velocity and a trend to reduced freezing duration (Fig. 1B). In the light–dark box, fish treated with 0.1 mM RDX showed no overt behavioral effects (Fig. 1C).

Following exposure to 1 mM RDX, the fish demonstrated reduced novel tank exploratory activity (transitions to top and time in top) as well as increased freezing and robust seizure-like responses (Fig. 2A). Since seizure behaviors were the main focus of this experiment, behavioral analyses for this dose included seizure-related endpoints, and did not include behavioral testing in other paradigms. Computer-aided analysis of traces revealed significantly decreased distance traveled and a trend for reduction in average velocity (Fig. 2B). Manually recorded seizure-like behavior in RDX-exposed fish included spasms, hyperactive bouts, and corkscrew swimming (Figs. 2D and 3). These responses strikingly differed from control fish, but resembled behavioral profiles observed in zebrafish exposed to other known epileptogenic compounds, such as picrotoxin and pentylenetetrazole (Berghmans et al., 2007). Overall, 47 ± 13% high dose (1 mM) RDX-exposed fish displayed bursts of hyperactivity vs. 0 ± 0% control ($P<0.05$), 87 ± 9% spasms vs. 40 ± 13% controls ($P<0.05$), and 40 ± 14% corkscrew swimming vs. 0 ± 0% control ($P<0.05$-0.1, trend). The average cumulative seizure score was high in the RDX-exposed cohort (1.73 ± 0.25) and negligible in control fish (0.4 ± 0.14, $P<0.0005$). Whole-body cortisol was also significantly increased in fish treated with high pro-convulsant 1 mM RDX (Fig. 2C), but not in cohort treated with low "behavioral" doses of 0.1 mM (data not shown). Finally, a significant elevation (3.2 ± 0.6-fold, $P<0.05$) in brain *c-fos* expression was also detected in fish exposed to 1 mM RDX, but not 0.1 mM (data not shown), compared to controls.

4. Discussion

The increasing concerns regarding RDX adverse health effects include its pro-convulsant (Davies et al., 2007; Harrell-Bruder and Hutchins, 1995; Hett and Fichtner, 2002; Kasuske et al., 2009; Kucukardali et al., 2003) and euphoria-inducing action in humans (Stone et al., 1969; Von Oettingen et al., 1949). Rodent models used to examine RDX behavioral effects similarly reveal hyperactivity and convulsions, as well as increased aggression ((ATSDR), 1995). To better understand behavioral profiles of RDX, new models for screening its neuroactive properties are needed. Because of its high degree of homology with rodents and humans, zebrafish represent an effective model for neurobehavioral research with promising translational possibilities (Panula et al., 2010; Panula et al., 2006). For example, neural pathways and mechanism of RDX action, which cannot be studied in humans, can be examined in animals, allowing the

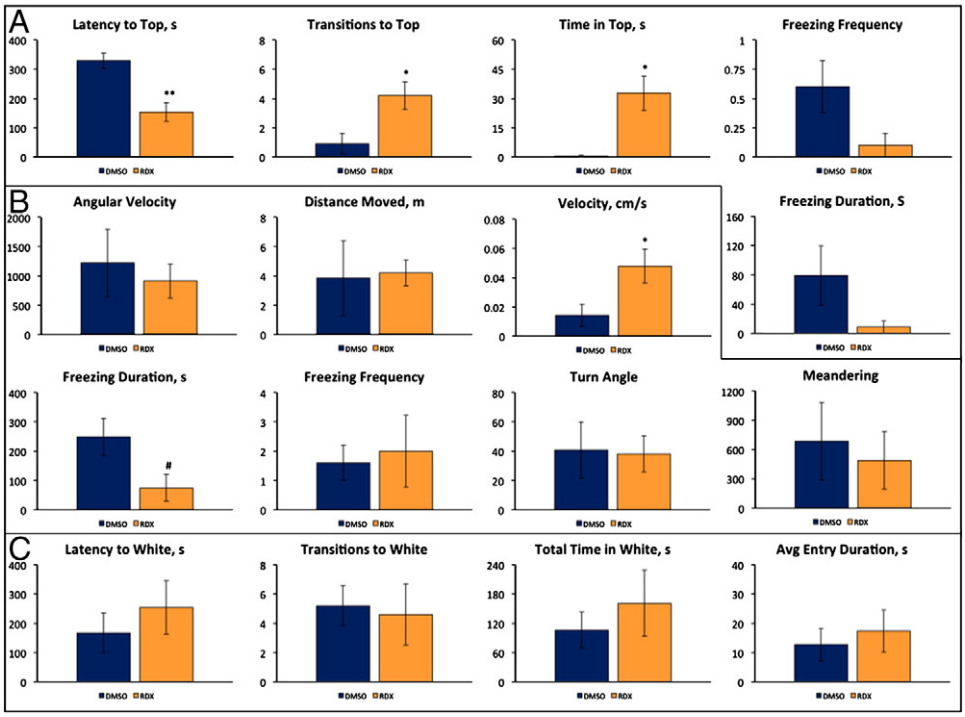

Fig. 1. Behavioral responses in zebrafish following 20 min acute exposure to 0.1 mM RDX. A) Novel tank diving test; B) Open-field test analyzed by Noldus Ethovision; C) Light/dark box. *$P<0.05$, **$P<0.005$, #$P = 0.05$–0.1 (trend), U-test.

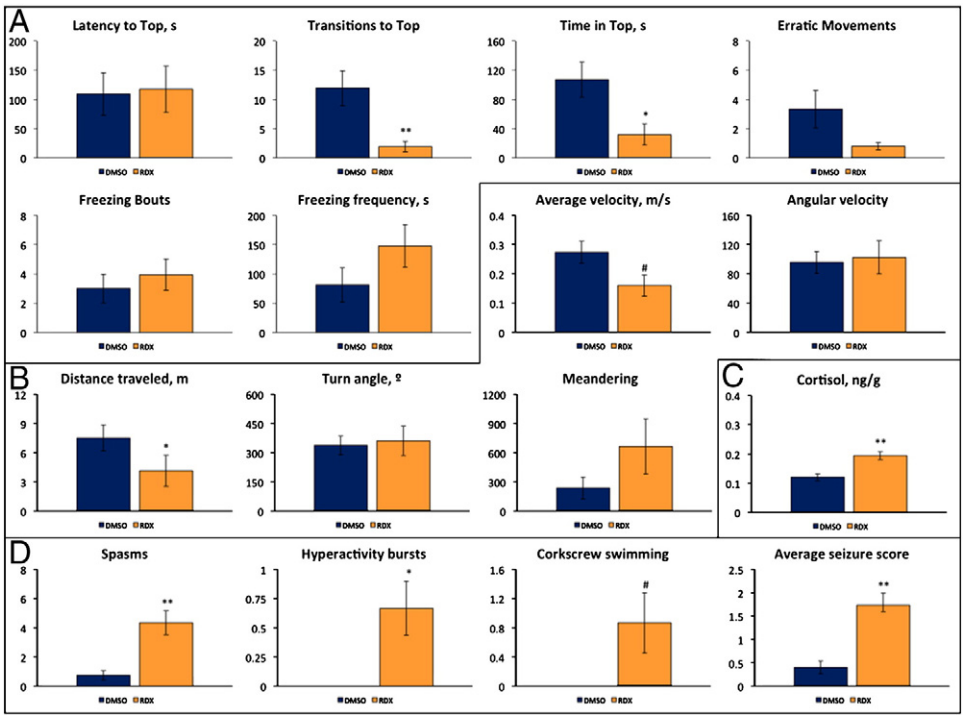

Fig. 2. Seizure behavior in the novel tank diving test following 20 min exposure to 1 mM RDX. A) Manually observed locomotor scores; B) Locomotor scores analysis by Ethovision; C) Cortisol elevations following behavioral testing; D) Seizure-like behavior by exposed zebrafish. *P<0.05, **P<0.005, #P = 0.05–0.1 (trend), U-test.

researchers to expand the species range of validated animal models. Moreover, fish may have alternate neural mechanisms that may complement rodent models and allow for a better understanding of RDX pharmacology. Finally, aquatic species are excellent models for RDX environmental toxicology research, as water contamination with explosives and their transformation products has been reported in munitions production, testing, firing and disposal sites (Lotufo et al., 2010; Mukhi and Patino, 2008).

Our results (Figs. 1–3) show that adult zebrafish can indeed serve as useful screens for behavioral effects of RDX. Specifically, the low dose (0.1 mM) of RDX-evoked hyperactivity and decreased freezing behavior. When exposed to a higher 1 mM dose of RDX, zebrafish displayed reduced exploration (e.g., decreased distance traveled and increased meandering) and increased freezing. Given the convulsant activity observed with this dose, this may parallel the confusion and heightened endogenous stress similar to the post-ictal period seen in seizure patients. Similar to RDX-evoked seizure activity described in clinical and animal literature, the zebrafish demonstrated significant amounts of spasms, hyperactive bursts, and tonus-like corkscrew swimming at 1 mM RDX. In addition, significant increase in cortisol levels were found in high dose RDX-treated zebrafish, in line with previous clinical data on elevated cortisol in epileptic patients (Tunca et al., 2000).

Taken together, our data indicates that RDX exposure may evoke different effects, activating behavior in a low dose, and evoking robust seizure-like activity at a high dose. In previous studies, RDX's metabolites (such as MNX) have been found in adult zebrafish (Mukhi and Patino, 2008) potentially affecting the brainstem and cerebellum (Smith et al., 2007) and causing hyperarousal and seizures (Goddard et al., 1969). In addition, our findings reconfirm previous findings in other species of behavioral activation with low doses of RDX exposure (Stone et al., 1969; Von Oettingen et al., 1949). Interestingly, RDX is not the only pro-convulsant substance that demonstrates behavioral activation in animal models. For example, low doses of benzylpenicillin and PTZ, both known to evoke seizures in humans and animals (Carmody and Brennan, 2010; Prigol et al., 2009), have been shown to produce paradoxical activation of exploratory locomotion in rodents, most likely due to mild non-anxiogenic arousal action of these compounds (Kaluev et al., 1995; Rodgers et al., 1995). Since PTZ and RDX share a similar affinity for the convulsant site on the $GABA_A$ receptor (Coulter et al., 1990; Kalueff, 2007), it is possible that similar pro-arousal psychostimulant-like action was involved in the effect of 0.1 mM RDX reported here. The elevation in brain *c-fos* gene expression following RDX-evoked seizures, observed in the present study, was also consistent with the general excitatory nature of observed seizure-like phenotype. Since *c-*

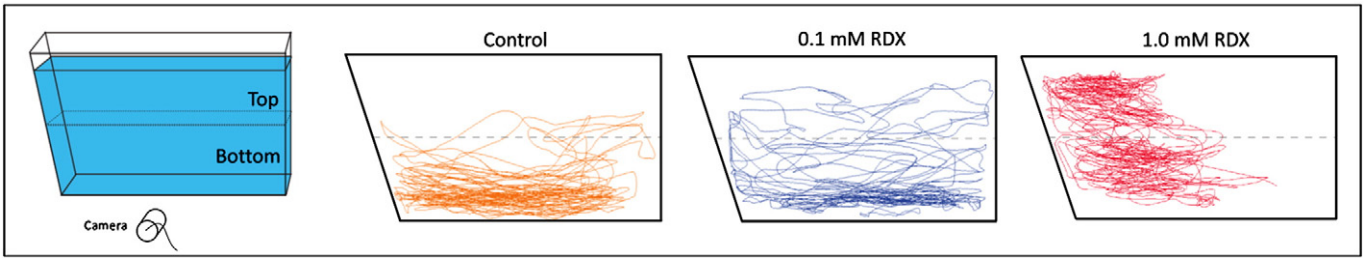

Fig. 3. Representative traces generated by Noldus EthoVision XT7 software in the novel tank diving test following a 30-min exposure to 0.1 or 1 mM RDX.

fos has recently been validated as a potential biomarker of seizures in zebrafish (Baraban et al., 2005), this study applied *c-fos* analysis to high (convulsant) doses of RDX, and performed a whole-brain analysis of its expression. Future studies may explore the ability of the RDX doses to modulate brain *c-fos* expression, as well as focus on region-specific analyses of this and other related marker genes.

5. Conclusions

Although our study demonstrates the biphasic nature of RDX action, more analysis is needed to further develop zebrafish as a model for high-throughput toxicological and neurobehavioral screening. Compared to larval zebrafish (the current established model of zebrafish toxicology), adult zebrafish represent a complementary method of analytical neuropharmacology with the advantage of more robust behavioral and physiological endpoints (Cachat et al., 2010b; Cachat et al., 2011). Our study of the effects of RDX further reinforces the adult zebrafish as a promising aquatic model of various brain disorders with robust behavioral phenotypes.

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