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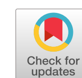
Development of a vibrational startle response assay for screening environmental pollutants and drugs impairing predator avoidance



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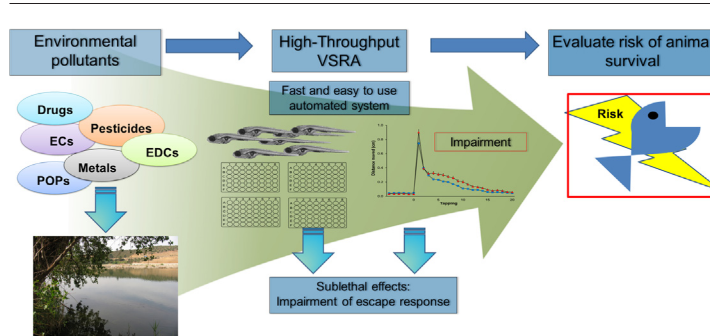
HIGHLIGHTS

- The new method enables in vivo medium- to high-throughput screening of pollutants.
- Hallmark criteria for escape response habituation were met.
- Pharmacological modulation of escape response habituation similar to mammal species
- The new method proves sensitive to different concentrations of environment pollutants.

GRAPHICAL ABSTRACT



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ABSTRACT

The present paper describes the vibrational startle response assay (VSRA), a new robust, simple and automated in vivo medium- to high-throughput procedure for assessment of the escape response and its habituation in zebrafish larvae. Such behaviors enable fish larvae to escape from predator strikes in aquatic ecosystems. The assay is based on measuring the distance moved by each larva during the startle response evoked by repetitive vibrational stimuli. The iterative reduction observed in the response to a series of tapping stimulus in VSRA met the main criteria of habituation. Subsequently, the analysis of concordance using a battery of neuroactive compounds modulating different neurotransmitter systems demonstrated that the results of VSRA are highly predictive of the effects on other vertebrates. Finally, as a proof of concept, VSRA was used to test two relevant environmental pollutants at different concentrations. The results demonstrated that VSRA is suitable for concentration-response analysis of environmental pollutants, opening the possibility to determine the potency and the associated hazard of impaired escape response for the different compounds. Therefore, we suggest that VSRA could be a valuable tool for screening of chemical compounds capable of compromising predator avoidance behavior.

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1. Introduction

In natural conditions predation is one of the main causes of mortality in feral fish, especially during the larval stage (Houde and Hoyt, 1987). As a part of an innate behavioral repertoire enabling larvae to escape from predator strikes, they respond to abrupt acoustic/vibrational stimuli with a fast C-bend followed by a bout of high-amplitude and low frequency fast swimming (Fero et al., 2011). Two modes of C-bend response have been identified according to latency. Whereas short latency C-bend (SLC) occurs within 15 ms of the stimulus, long latency C-bend (LLC) is initiated 20–60 ms after the stimulus (Fero et al., 2011). SLC response is regulated by a sensory motor axis that integrates auditory and vibrational information and transduces these stimuli into musculoskeletal activation via a bilateral pair of giant reticulospinal neurons in the hindbrain, the Mauthner cells (Painter et al., 2009). Because of their short latency and explosive speed of the movement, SLC responses are similar to the startle responses in higher vertebrates (Fero et al., 2011).

Habituation is a primitive form of implicit learning. The animal first responds to a new stimulus and, if the stimulus is neither beneficial nor harmful, animal learns, after repeated exposure, to ignore it (Kandel, 1991). Habituation of the escape response results essential for aquatic organisms, as repeated unnecessary escape responses reduce foraging and result in an increase in the predation risk by at least two different ways (Fields and Yen, 1997). On one hand, escape response supposes a high energetic cost, and repeated escape responses will result in exhausted organisms, making them more susceptible to predation. Moreover, unnecessary escape responses attract the attention of both visual and mechanoreceptive predators (Batabyal et al., 2017; Fields and Yen, 1997; Killen and Brown, 2006). Short-term habituation of C-startle response occurs when larvae is exposed to repeated stimulation at short interstimulus intervals (ISIs), with the corresponding Mauthner cell responding only to the few first stimuli, and failing then to elicit a Mauthner spike (Park et al., 2018). As a result, SLC responsiveness to the acoustic/vibrational stimuli diminishes extremely rapidly during short-term habituation.

Currently, the available information about potential adverse effects of environmental pollutants present in aquatic ecosystems on the C-startle response and habituation in fish larvae is very scarce. To our knowledge, only eight environmental pollutants have been tested to determine the effects on the C-startle in fish, and the results indicated that fish exposed to seven of these chemicals were more susceptible to predation (Carlson et al., 1998). Moreover, although it has been demonstrated that exposure to some drugs alters habituation of C-startle evoked by acoustic stimulus in fish larvae (Best et al., 2008; Marsden and Granato, 2015; Roberts et al., 2016; Wolman et al., 2011), information about the potential effect of environmental pollutants of this form of implicit learning is still missing. Thus, the development of medium- and high-throughput assays suitable for identifying environmental pollutants altering escape response and habituation in fish larvae is urgently needed.

Zebrafish is a cyprinid increasingly used as a vertebrate model for the study of the molecular mechanisms of brain function (Babin et al., 2014; Faria et al., 2017; Gómez-Canela et al., 2018), with the key advantage of being suitable for in vivo high-throughput screening of chemical libraries for pharmacological and/or toxicological effects. An assay to assess short-term habituation in zebrafish larvae, based on determining the motor activity of the larvae after the delivery of repetitive acoustic stimuli, was recently developed (Best et al., 2008). By using this assay, the modulation of the C-startle and habituation by different cognitive enhancers has been demonstrated. However, the fact that the above mentioned assay used a homemade setup for video-recording and the delivery of the acoustic stimuli, makes it difficult to implement in other labs and to compare results among different labs.

In this study, a new high-throughput assay for identifying compounds able to impair the vibrational C-startle response and the short-term habituation has been developed in zebrafish larvae. The vibrational startle response assay (VSRA) is based on measuring the distance moved by each larva in response to repetitive vibrational stimuli generated by a tapping device on a 48-wells microplate. Although VSRA has been developed using a commercial platform for automatizing the stimuli delivery, videotracking and further data analysis, it can be easily adapted to other existing zebrafish platforms. The first step after developing VSRA was to determine if the progressive reduction observed in the motor response after repeated stimulation met the main criteria established for habituation (Best et al., 2008; Brown, 1998; Thompson and Spencer, 1966). Then, VSRA was used to determine startle and habituation in a battery of 10 neuroactive compounds modulating cholinergic, serotonergic and glutamatergic systems, in order to analyze the concordance of VSRA with the existing data in fish and rodents (Table S1) (Leussis and Bolivar, 2006). Finally, the developed assay was used to analyze the effect of chlorpyrifos oxon and imidacloprid, as a proof of concept of the applicability of this assay to test environmental pollutants (Table S1).

2. Methods

2.1. Fish husbandry and larvae production

Adult wild-type zebrafish, purchased from Piscicultura Superior SL, Parets del Vallès, Barcelona, were maintained in fish water [reverse-osmosis purified water containing 90 μg/mL of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4 \cdot 2H_2O$] at 28 ± 1 °C in the Research and Development Centre of the Spanish Research Council (CID-CSIC) facilities under standard conditions. Embryos were obtained by natural mating and maintained in fish water at 28.5 °C on a 12 light:12 dark photoperiod. Larvae were not fed during the experimental period. All procedures were approved by the Institutional Animal Care and Use Committees at the CID-CSIC and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

2.2. Experimental procedure

The chemicals used for this study were of certified laboratory high quality grade and can be found enlisted in the Supplementary material document under Section S1.1 of Supplementary methods. Stock solutions of nicotine, pilocarpine, buspirone, chloro DL phenylalanine (PCPA), deprenyl donezepil, imidacloprid, chlorpyrifos oxon (CPO) and Methyllycaconitine (MLA) were prepared in DMSO on the day of the experiment. Whereas experimental solutions for these compounds were prepared in fish water from the stock solutions, those for memantine, fluoxetine and scopolamine were directly prepared in fish water. The final concentration of DMSO in all the exposure solutions was 0.1%, except for scopolamine. As this compound exhibits very low permeability in zebrafish larvae, DMSO concentration 1% in order to increase the permeability. Solvent controls containing 0.1% or 1% DMSO were used.

Zebrafish larvae were treated with selected compounds for 24 h from 7 to 8 dpf (days post fertilization). Experiments were conducted in 48 well plates with 1 larva per well and 1 mL of medium. Plates were placed in a POL-EKO APARATURA Climatic chamber KK350 (Poland) at 28.5 °C and 12L:12D photoperiod. Larvae were never fed throughout the experimental period.

At least two independent experiments were preformed where groups of 48 larvae underwent behavioral testing. Compounds were initially evaluated for toxicity before habituation testing. Briefly, toxicity was ascertained in 8 dpf zebrafish larvae after 24 h of exposure and was established either by death, gross morphology and/or swimming impairment or clear decrease in the escape response evoked by the tapping on the plate. The highest concentration, which did not induce toxicity, was used in the subsequent VSR assay.

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