Science of the Total Environment 672 (2019) 121–128

Contents lists available at ScienceDirect

Science of the Total Environment

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

Deciphering the mode of action of pollutants impairing the fish larvae escape response with the vibrational startle response assay

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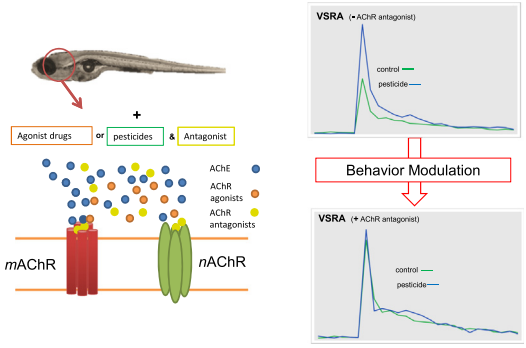
HIGHLIGHTS

- Mechanistic mode of action of neuroactive chemicals was investigated.
- The Vibrational Startle Response Assay was applied using zebrafish larvae.
- Two neurotoxic pesticides, chlorpyrifos-oxon and imidacloprid, were studied.
- Chlorpyrifos-oxon's toxicity was mediated by the activation of both *m* and *n*AChRs.
- Effect of imidacloprid was not mediated by a cholinergic pathway.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 January 2019
Received in revised form 26 March 2019
Accepted 30 March 2019
Available online 01 April 2019

Editor: Henner Hollert

Keywords:
Zebrafish
Pesticide
Startle and habituation
Cholinergic modulation

ABSTRACT

The escape response evoked by vibrational stimuli and its habituation, essential behaviors for fish larvae survival, can be altered by neurotoxic environmental pollutants commonly found in our aquatic ecosystems.
In this study we have analyzed the suitability of the Vibrational Startle Response Assay (VSRA) to obtain mechanistic information about the mode of action (MoA) of the chemicals impairing the escape response and its habituation. As a proof of concept, the pathophysiological mechanisms behind the action of two common neurotoxic pesticides, chlorpyrifos-oxon (CPO) and imidacloprid, over their effects on arousal and habituation of the escape response were studied by using pharmacological antagonists of the nicotinic and muscarinic acetylcholine receptors, mecamylamine (MCA) and scopolamine, respectively. Furthermore, potential changes in the neurotransmitter profile were analyzed. Results revealed that whereas the effect of CPO on arousal was mainly mediated by the activation of nAChRs, its effect on habituation was mainly mediated by mAChRs. On the other hand, imidacloprid only affected larvae arousal which was found to be mediated by a cholinergic independent mechanism. No association between behavioral effects on arousal or habituation in affected larvae was found with their corresponding neurotransmitter profile. These results confirm the suitability of VSRA to provide mechanistic information about the potential MoA of neuroactive compounds.

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https://doi.org/10.1016/j.scitotenv.2019.03.469
0048-9697/

1. Introduction

Predation is one of the main sources of mortality of either marine or freshwater fish at all life stages, but particularly for fish larvae (Fuiman and Magurran, 1994; Houde and Hoyt, 1987). Escape response is a behavioral reflex promoting larval survival to predator's strikes (Anderson et al., 2019; McHenry et al., 2009; Rearick et al., 2018; Zhou and Weis, 1998). However, unnecessary escape responses have a high energetic cost and increase the risk of predation, so habituation of this response to irrelevant stimuli results is also essential for survival (Batabyal et al., 2017; Fields and Yen, 1997; Killen and Brown, 2006).

It has been estimated that up to 30% of all commercially used chemicals (~30,000 chemicals) may have neurotoxic potential (Legradi et al., 2018). Neuroactive chemicals, including neurotoxic pesticides, pharmaceuticals and illicit drugs, are the largest group of micropollutants present in European rivers, where nearly 30% of all detected chemicals were linked to neurotoxicity (Busch et al., 2016). Furthermore it has been shown that fish larvae behavior, including startle response and its habituation may be disrupted by environmental chemicals (Best et al., 2008; Faria et al., 2019; Kinch et al., 2015; Sandahl et al., 2005; Thompson et al., 2017).

Recently we developed the Vibrational Startle Response Assay (VSRA), an automated *in vivo* assay for identifying chemicals impairing the escape response and its habituation in zebrafish larvae (Faria et al., 2019). The assay is based on measuring the distance moved by the larva during the startle responses evoked by repetitive vibrational stimuli. The magnitude of the response to the first vibrational stimulus allows evaluating the level of arousal or alertness of the larvae, whereas the decrease in the motor response resulting from repeated exposure to the same vibrational stimuli provides information on the habituation process of this response in the larvae. By using VSRA the effect of different concentrations of chlorpyrifos-oxon (CPO) and imidacloprid, two neurotoxic pesticides targeting the cholinergic system, on the escape response was characterized.

As a follow-up of our previous work, in this study we intend to test the VSRA's suitability for obtaining mechanistic information about the MoA of the chemicals impairing the escape response of zebrafish larvae. As a proof of principle, the potential involvement of nicotinic and muscarinic acetylcholine receptors (n- and mAChRs) in the impairment of the escape response of CPO and imidacloprid were analyzed. A first set of experiments combining pharmacological modulators of specific mammalian n- and mAChRs (agonists and antagonists) was performed to test the suitability of detecting behavioral modulation with the VSRA. The second set of experiments, aimed to address the potential rescue of the behavioral phenotype induced by each pesticide, was evaluated by co-exposure with mecamylamine (MCA) and scopolamine, pharmacological antagonists of n- and mAChRs, respectively. Finally, potential changes in the neurotransmitter profile associated with the observed neurobehavioral features were examined. The results of this study support the suitability of VSRA not only for screening environmental pollutants impairing arousal and/or habituation, but also to decipher the potential mechanisms of action behind these effects.

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 $CaSO_4$. $2H_2O$] 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 in-tank group breeding with a 5:3, female:male ratio per tank. Breeding tanks are homemade and include a solid external tank and an internal plastic net. Embryos deposited in the bottom of the tank were collected using a 3 mL plastic Pasteur pipette and maintained at a 1 individual/mL density 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 following compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA): nicotine (CAS 54-11-5); pilocarpine (CAS 54-71-7); scopolamine (CAS 55-16-3); and imidacloprid (CAS 138261-41-3). Mecamylamine hydrochloride (MCA; CAS 826-39-1) was purchased from Tocris Bioscience (Minneapolis, MN, USA) and CPO (CAS 5598-15-2) was purchased from Chem Service (West Chester, PA, USA). Whereas stock solutions for nicotine, pilocarpine, imidacloprid, CPO and MCA were prepared in dimethylsulfoxide (DMSO) and diluted to the test concentrations in fish water on the day of the experiment, experimental solutions of scopolamine were directly prepared in fish water. Since some chemicals like scopolamine exhibit very low permeability in zebrafish larvae, 1% DMSO was used to ensure the absorption of the chemicals during the exposure period. Controls were also exposed for 24 h to fish water with 1% DMSO.

The following concentrations of each compound were used, according to those determined in Faria et al. (2019), where only the highest concentrations which did not induce toxicity, evaluated as either death, gross morphology and/or swimming impairment or clear decrease in the escape response evoked by the tapping on the plate, in 8 dpf zebrafish larvae after 24 h of exposure: 25 μM nicotine; 80 μM pilocarpine; 20 μM MCA; 25 μM scopolamine; 5, 25 and 50 nM CPO and 25 and 50 μM imidacloprid.

Before performing the co-exposure experiments with the pesticides, a series of experiments were conducted combining pharmacological agonists and antagonists of AChRs. Thus, in order to test the suitability of this assay to decipher the pharmacological MoA of the tested agonists, 7 days post-fertilization (dpf) zebrafish larvae were treated for 24 h with the following combinations of the selected compounds: (1) nicotine + MCA; and (2) pilocarpine + scopolamine. VSRA was performed immediately after the exposure period.

Then, co-exposure experiments were conducted with the two selected pesticides and the pharmacological antagonists used above. The selected combinations were: (1) CPO + MCA; (2) CPO + scopolamine; (3) imidacloprid + MCA; and (4) imidacloprid + scopolamine.

All the exposures were performed at 28.5 °C (POL-EKO APARATURA Climatic chamber KK350, Poland) with 12 L:12D photoperiod in 48 well plates with 1 larva per well and 1 mL of medium. After 24 h exposure, larvae were directly tested in the VSRA without further manipulation. At least two independent experiments were performed where groups of 48 larvae underwent behavioral testing.

Data of single exposures of the above mentioned chemicals, already reported in the previous work were used to compare with co-exposure results.

2.3. Vibrational startle response assay (VSRA)

Vibrational startle response assay was performed as described in Faria et al. (2019). Videotracking and the escape response were analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands). Trials were performed at 28 °C with near infrared light. Tapping stimulus was selected at the highest intensity (intensity level: 8) and 50 sequences of the vibrational stimuli were delivered at an interstimulus interval (ISI) rate of 1 s. Before delivering the first stimulus, larvae were left in the observation chamber for 15 min to acclimate. Videos were recorded at 30 frames per second and the VSR was

analyzed for each individual larva by measuring the distance moved (cm) over the 1 s period after stimulus.

2.4. Extraction and analysis of neurotransmitters by LC-MS/MS

Pools of 5 zebrafish larvae were used for the extraction and analysis of neurotransmitters following the protocol described by Gómez-Canela et al. (2018). In brief, pools of larvae were spiked with 500 ng of Internal Standard Mixture (ISM) (see supplementary material Section 1.1.1), followed by the addition of 500 μL of MeOH:H_2O (90:10). Three stainless steel beads (3 mm diameter) were placed in each sample and homogenized using a bead mill homogenizer (TissueLyser LT, Qiagen) at 50 oscillations per min during 90 s. Samples were then centrifuged during 20 min at 13,000 rpm at 4 °C. The supernatant was filtered using 0.20 μM PTFE filters (DISMIC -13 JP, Advantec®) and kept in amber chromatographic vials at −80 °C before LC-HRMS analysis. Fifteen neurotransmitters were measured: Acetylcholine (Ach); Serotonin; 5-Hydroxyindoleacetic acid (5-HIAA); 5-Hydroxy-L-tryptophan (5-HTP); Tryptophan (Tryp); Dopamine; L-Phenylalanine; L-Tyrosine; 3,4-Dihydroxyphenylalanine (L-DOPA); 3-Methoxytyramine (3-MT); Epinephrine; Norepinephrine (NE); L-Glutamic acid (Glutamate); γ-Amminobutyric acid (GABA) and Glycine, using liquid chromatography connected to a triple quadrupole detector (Xevo TQD, Waters, USA) (LC-MS/MS). Target compounds were separated using a Synergi Polar-RP 80 Å column (250 mm × 4.6 mm ID, particle size 4 μM, Phenomenex, Torrance, USA). Additional details can be found in Supplementary Methods Section 1.

2.5. Data analysis

Data were analyzed with IBM SPSS 19.0 (Statistical Package 2010, Chicago, IL). Data are presented as the mean ± SEM of all subjects from 2 independent experiments, unless stated otherwise. In this study the original data representation has been modified in order to improve the quality of the information provided on the habituation process. Therefore, behavioral responses are represented as: "*Arousal*": measured as the total distance moved (cm) in response to the first stimulus and as "*Habituation*": represented as: 1) plots of distance moved relative to the response to the first stimulus (S1); and 2) area under the curve (AUC) of plots of distance moved relative to the response to the first stimulus.

One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison tests were used to compare the level differences of each response among experimental groups. Effects over the variance in behavioral responses of the two factors: contaminant concentrations and the presence of AChR antagonist as well as their interaction were analyzed by two-way ANOVA. The statistical test used for each set of results can be found in the text or in the figure caption. Behavioral responses of controls from each experiment and the previous work (Faria et al., 2019) were not found statistically different ($P > 0.05$, Tukey's multiple comparison tests, data not shown).

Levels of neurochemicals in pools were normalized per larva and represented as pg/larvae (Supplementary Material Section 2). Data are presented as the mean ± SEM of 2 independent experiments. One-way ANOVA followed by Dunnett's multiple comparison tests were used to compare the differences in the level neurochemical of each treatment with regard to the control. Significance was set at $P < 0.05$. Control responses in plots and bars graphs represent the mean of all controls.

3. Results

3.1. Pharmacological AChR modulators

In order to test the suitability of using pharmacological modulators of mammalian n- and mAChRs in zebrafish, a set of experiments were initially performed by combining prototypic agonists and antagonists. Thus, nicotine and MCA were selected as agonist and antagonist of the nicotinic AChRs (nAChRs) respectively, whereas pilocarpine and scopolamine were used as agonist and antagonist of the muscarinic AChRs (mAChRs) respectively.

One-way Analysis of Variance indicated significant effects of both groups of pharmacological modulators of the cholinergic system on the larvae escape response, although the effects were stronger for nAChRs modulators. Whereas nAChRs modulators altered both the arousal and the habituation, only the arousal was affected by the mAChR (Table S4, Supplementary Material). While single exposure to nicotine and MCA had no effects on arousal, the co-exposure of these two nAChR modulators resulted in a mild but significant decrease in arousal (Fig. 1A). Pilocarpine significantly increased arousal through the activation of mAChR, as indicated by the full recovery observed during the co-exposure with scopolamine (Fig. 1B). Nicotine significantly delayed habituation, represented by the significant increase of AUC values, and this effect was mediated through the activation of nAChRs, as indicated by the recovery observed during the co-exposure with the specific antagonist MCA (Fig. 1C). However, muscarinic modulators showed no effect over the escape response habituation (Fig. 1D).

3.2. Chlorpyriphos-oxon

Arousal and habituation of the escape response were dramatically affected by CPO. Despite this, cholinergic antagonists were able to significantly recover most of the affected groups. Two-way ANOVA of arousal from single and combined exposure of different CPO concentrations with the selected n- and mAChR antagonists revealed a significant interaction between CPO concentration and cholinergic antagonists (MCA or scopolamine) (Table 1). On the other hand, no interaction was observed for habituation (Table 1). Independent observation of the factors indicates that CPO concentration affected both arousal and habituation, and that the presence of both cholinergic antagonists was significant for arousal, while for habituation only scopolamine displayed important changes over larvae behavior (Table 1).

CPO increased arousal in a concentration-dependent manner (Fig. 2A, C and E). Whereas 5 nM CPO had no effect on arousal, 25 and 50 nM increased the magnitude of the first startle at 36 and 180%, respectively. Co-exposure of 25 nM CPO with MCA and scopolamine were able to rescue the effect on arousal. Despite this, scopolamine only induced a partial rescue of the effect of 25 nM and even less of 50 nM CPO, while MCA was able to induce the full rescue of both concentrations.

Effect of CPO on habituation was significantly delayed in larvae exposed to 5 and 25 nM CPO (Fig. 2B and D) and not to 50 nM CPO (Fig. 2F). Similar to arousal, the effect of CPO on habituation seems to involve the activation of both n- and mAChRs, since behavior recovery could be observed when co-exposing with both antagonists (Fig. 2B and D). However, scopolamine was more efficient than MCA, fully recovering the normal habituation profile of larvae at 5 and 25 nM CPO (Fig. 2D). Curiously, although habituation was unaffected by single exposure to 50 nM CPO, it was significantly increased by co-exposure with scopolamine.

3.3. Imidacloprid

The effects on arousal and habituation of the escape response of zebrafish larvae exposed to 25 and 50 μM imidacloprid are depicted in Fig. 3 and Table 1. Both concentrations of imidacloprid induced a significant decrease in arousal, but no effects on habituation were found. In fact, the distance moved by the treated larvae in response to the first tap was 24–28% lower than that of the control larvae (Fig. 3A and B). Subsequently, the potential involvement of nicotinic and/or muscarinic pathways in the observed effect were tested by co-exposure with MCA and scopolamine. None of the antagonists were able to rescue the imidacloprid effect (Fig. 3 and Table 1), which was confirmed by two-way ANOVA analysis (Table 1) that showed no significant interaction

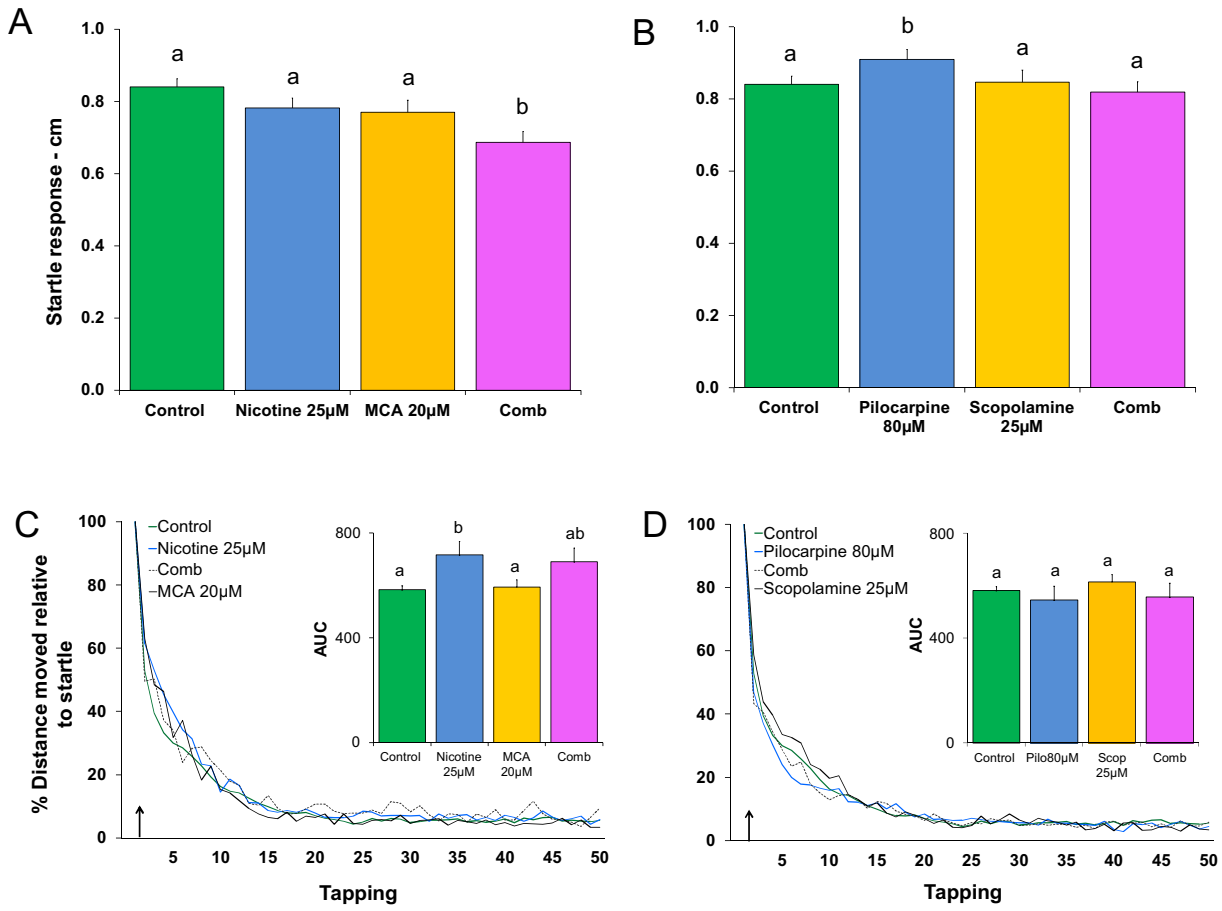

Fig. 1. Zebrafish escape responses and their habituation following pharmacological modulation of nicotinic and muscarinic AChRs. *Upper panel*: **A–B**: Distance moved (cm) during the startle response following delivery of the 1st stimulus in larvae from different experimental groups (mean ± SEM). *Lower panel*: **C–D:** Habituation of zebrafish larvae from different experimental groups, measured as the decrease in the distance moved (percentage) across 50 tapping stimuli delivered at an interstimulus interval of 1 s, relative to the response to the first stimulus delivered (set to 100%). Black arrow indicates the time of delivery of the first stimulus. AUCs (mean ± SEM) of each treatment are shown as an inset to each plot. **A–C**: control (n = 225)/ 25 μM nicotine (n = 89)/ 20 μM MCA (n = 87)/ 25 μM nicotine +20 μM MCA (Comb) (n = 83); **B–D:** control (n = 225)/ 80 μM pilocarpine (n = 83)/ 25 μM scopolamine (n = 91)/ 80 μM pilocarpine +25 μM scopolamine (Comb) (n = 91). Different letters indicate groups that are statistically similar ($p > 0.05$) according to Tukey's multiple-comparison test.

between antagonists and imidacloprid for the arousal ($p = 0.008$). Interestingly, 25 μM imidacloprid co-exposed with MCA did seem to have an impact on habituation (Fig. 3C), despite this, no significant interaction was observed in the ANOVA analysis (Table 1).

3.4. Neurotransmitter profile

It has been demonstrated that different neurotransmitters, including dopamine, serotonin, acetylcholine or glycine, can modulate the vibrational startle response in vertebrates, including fish. In the present study we intended to assess if the observed neurobehavioral changes induced by the exposure to CPO and imidacloprid were linked to changes in the neurotransmitter profile induced by n- or mAChR agonists. Thus, levels of neurotransmitters, as well as their precursors and degradation products, from different neurotransmitter systems potentially involved in the modulation of the escape response have been determined in pools of larvae control and treated with nicotine, pilocarpine, CPO and imidacloprid. Results of the analyses (Supplementary Table S3)

Table 1
Two way ANOVA assessing effects of contaminant concentrations, *n* or *m*AChR antagonist, and the interaction of both, over the variance of zebrafish larvae behavioral responses by. $P < 0.05$.

	Concentration (Con)			Antagonist (Ant)			Con × Ant (interaction)		
	df	F	P	df	F	P	df	F	P
Startle response									
CPO + MCA	3326	44.530	0.000	1326	76.088	0.000	2326	34.641	0.000
CPO + scopolamine	3328	68.573	0.000	1328	21.314	0.000	2328	10.286	0.000
Imidacloprid + MCA	2234	24.429	0.000	1234	7.170	0.008	1234	0.012	0.913
Imidacloprid + scopolamine	2231	27.521	0.000	1231	3.703	0.056	1231	0.415	0.520
AUC									
CPO + MCA	3299	5.869	0.001	1299	1.925	0.166	2299	2.266	0.106
CPO + scopolamine	3306	11.280	0.000	1306	17.882	0.000	2306	0.414	0.662
Imidacloprid + MCA	2229	0.289	0.749	1229	6.702	0.010	1299	1.896	0.170
Imidacloprid + scopolamine	2225	0.112	0.894	1225	1.544	0.215	1225	1.145	0.286

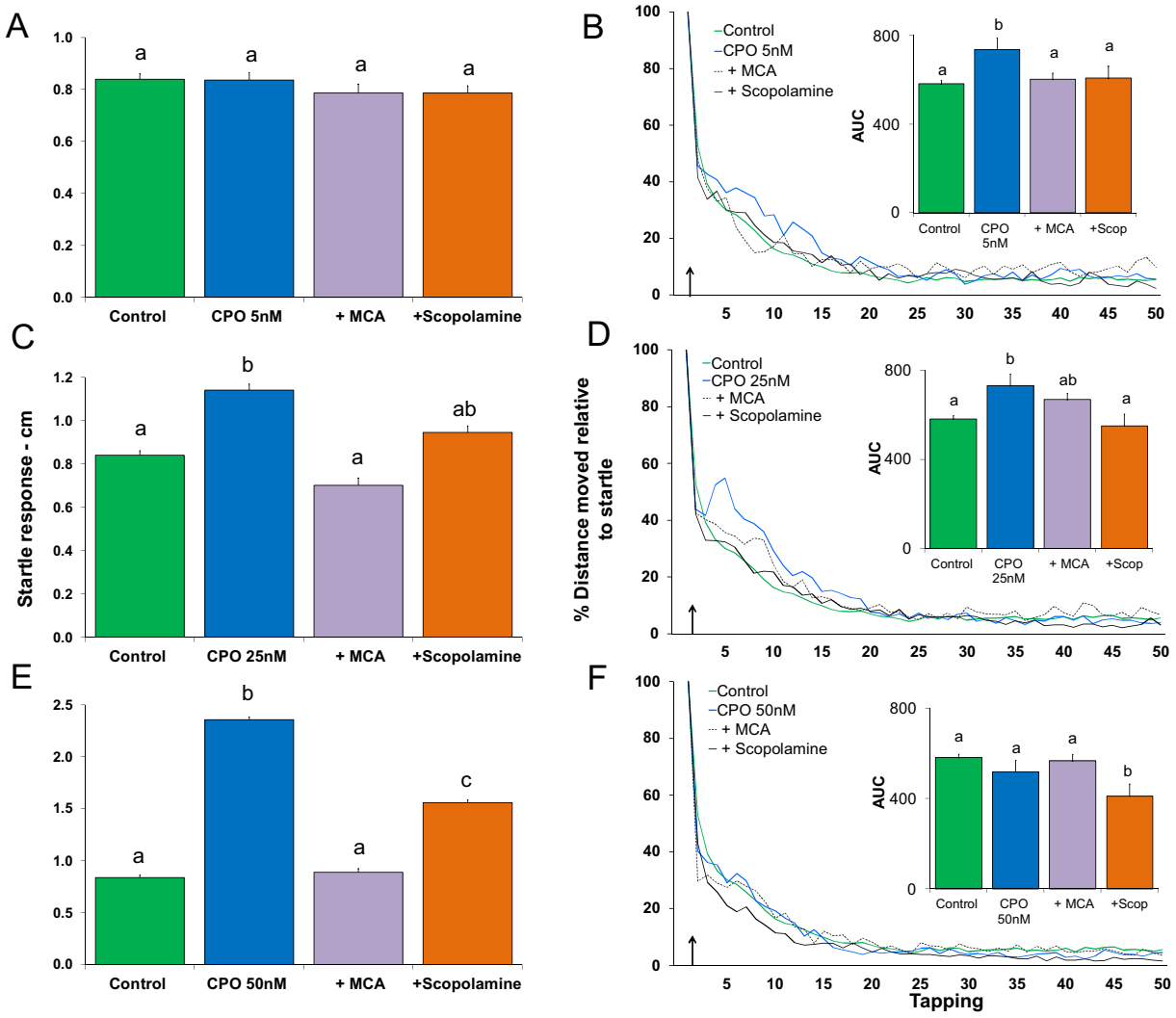

Fig. 2. Effects of different concentrations of CPO over zebrafish larvae startle response and its habitation. *Left panel* **(A, C, E)**: Distance moved (cm) during the startle response following delivery of the 1st stimulus in larvae from different experimental groups (mean ± SEM). *Right panel* (**B, D, F**): Black arrow indicates the time of delivery of the first stimulus. AUCs (mean ± SEM) of each treatment are shown as an inset to each plot. **A–B**: Control (n = 225)/ 5 nM CPO (n = 85)/ 5 nM CPO + 20 μM MCA (+MCA) (n = 90)/ and 5 nM CPO + 25 μM scopolamine (+Scop) (n = 90); **C–D**: Control (n =225)/ 25 nM CPO (n = 79)/ 25 nM CPO + 20 μM MCA (+MCA) (n = 85)/ 25 nM CPO + 25 μM scopolamine (+Scopolamine) (n = 87); **E–F**: Control (n = 225)/ 50 nM CPO (n = 72)/ 50 nM CPO + 20 μM MCA (n = 87)/ 50 nM CPO + scopolamine (+Scop) (n = 83). Different letters represented in each bar indicate groups that are statistically similar ($p > 0.05$) according to Tukey's multiple-comparison test.

show very limited changes in the neurotransmitter profile after the different treatments. Whereas no changes were found in the levels of norepinephrine, serotonin, glycine, GABA and acetylcholine after any treatment, levels of dopamine slightly increased ($p = 0.048$) after treatment with pilocarpine. Levels of glutamate, however, significantly decreased after the treatment with the highest CPO concentration, both imidacloprid concentrations, and pilocarpine. The heat map of hierarchical cluster analysis, in which the magnitude of the first startle (S1) and habituation (AUC) have been also included, showed the absence of a specific profile of neurotransmitters in larvae with similar behavioral effects on arousal or habituation (Fig. 4). However, as neurochemical analyses have been performed on pools of whole larvae, results should be taken with caution, as many of the analyzed chemicals are expressed also in non-neural tissue.

4. Discussion

The aim of the present study was to assess if VSRA is a suitable assay for obtaining mechanistic information about the potential MoA of neuroactive compounds when the assay is performed after a pharmacological approach. Initially, pharmacological agonists and antagonists of n- and mAChRs have been used to assess whether the effects on arousal and/or habituation induced by the agonists were reverted by the corresponding antagonists. We have shown that the decrease in habituation found in larvae treated with nicotine can be only partially rescued by co-exposure with the nAChR antagonist MCA (Fig. 1C). One hypothesis that might explain this occurrence could be due to the differences in absorption, distribution, metabolism and excretion (ADME) of these two chemicals, with MCA requiring a longer time to reach the target tissues. In fact, it has been reported that pre-exposure to MCA prior to co-exposure is more effective in rescuing the behavioral phenotype than direct co-exposure (Levin et al., 2006). Another hypothesis could be the lack of affinity of MCA to bind to nAChRs activated by nicotine. For example, Papke et al., found in zebrafish, that nicotine had high efficacy but low potency for α7 receptors while MCA presented low potency for blocking α7 receptors (Papke et al., 2012). On the other hand, the significant increase in arousal found in larvae exposed to the muscarinic agonist pilocarpine was fully rescued by co-exposure with the muscarinic antagonist scopolamine (Fig. 1B). Indeed, Eddins et al., reported pilocarpine to significantly increase zebrafish larvae startle response (Eddins et al., 2010). Furthermore, modulation of zebrafish behavior by scopolamine has been reported in various studies (Bortolotto et al., 2015;

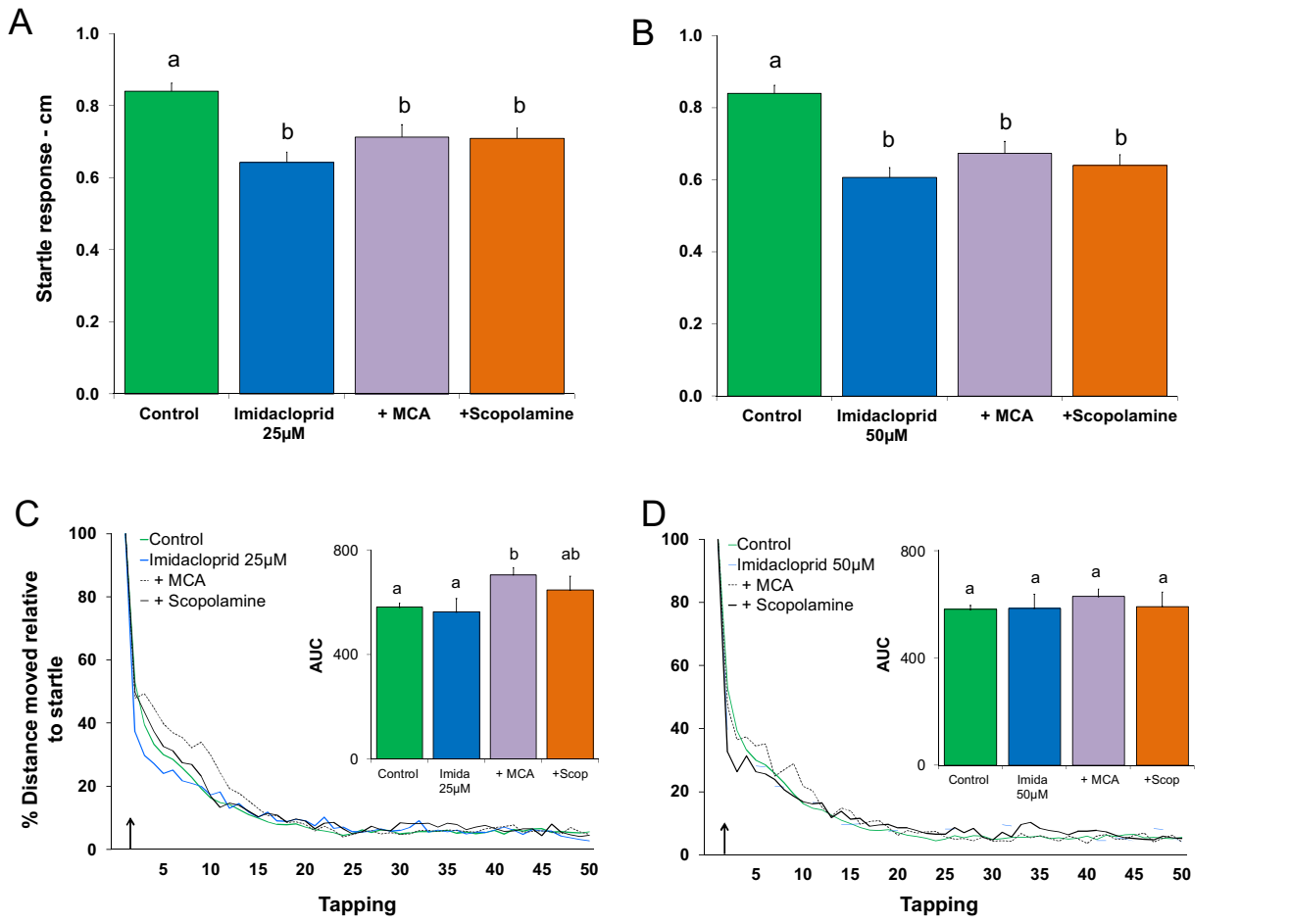

Fig. 3. Effects of two concentrations of imidacloprid over zebrafish larvae startle response and its habitation. *Upper panel*: **A–B**: Distance moved (cm) during the startle response following delivery of the 1st stimulus in larvae from different experimental groups (mean ± SEM). *Lower panel*: **C–D:** Black arrow indicates the time of delivery of the first stimulus. AUCs (mean ± SEM) of each treatment are shown as an inset to each plot. **A–C**: Control (n = 225)/ 25 μM imidacloprid (n = 89)/ 25 μM imidacloprid +20 μM MCA (n = 89)/ 25 μM imidacloprid +25 μM scopolamine (n = 86); **B–D**: Control (n = 225)/ 50 μM imidacloprid (n = 83)/ 50 μM imidacloprid +20 μM MCA (n = 89)/ 50 μM imidacloprid +25 μM scopolamine (n = 88). Different letters represented in each bar indicate groups that are statistically similar ($p > 0.05$) according to Tukey's multiple-comparison test.

Hamilton et al., 2017; Kim et al., 2010), *e.g.*, zebrafish has been proposed as model species to study anxiolytic effects of scopolamine (Hamilton et al., 2017). Thus, our results confirmed that the VSRA is also a suitable assay to test if the behavioral effects induced by cholinergic compounds on arousal and/or habituation are mediated by the specific activation of n- or mAChRs.

CPO, the active metabolite of chlorpyrifos, irreversibly inhibits AChE, resulting in an increase in the levels of acetylcholine in the synaptic clefts and the concomitant overstimulation of AChRs (Faria et al., 2015). The effects of exposure to the same three CPO concentrations used in the present study on arousal and habituation have been recently reported (Faria et al., 2019). In this previous study we found an increase in arousal only for the two higher CPO concentrations. However, a concentration-dependent decrease in habituation, measured by using the AUC values, was reported for all three concentrations. Although the use of AUC values has been proposed as a measure of habituation (Best et al., 2008; Faria et al., 2019), a detailed analysis of the time-course of the responses to the vibrational stimuli clearly shows that for those chemicals inducing a strong modulation of arousal, as 50 nM CPO, the changes found in the AUC values are biased by the first response and do not represent a real effect on the habituation process. To fix this problem we have modified the data treatment, setting to 100% the distance moved after the first stimulus in each treatment group and calculating the responses to the subsequent stimuli relative to the first one. With this adjustment, data of all treatments were normalized, allowing a more refined comparison of the rate of movement decrease to repeated stimuli exposure across all treatments (for further details see Supplementary Results and Discussion, Section 2.2). By using this approach, we have demonstrated here that habituation was equally affected by the two lowest CPO concentrations and unaffected by the highest one (Fig. 2B, D and F). This observed U pattern of response, where effects can be seen at lower doses rather than higher, is similar to that reported in zebrafish and mammalian species exposed to nicotine (Levin and Chen, 2004; Levin and Simon, 1998; Schreiber et al., 2002). Furthermore, we found that the effects of CPO over larvae habituation was mediated through both AChRs, however the outcome by muscarinic modulation by scopolamine was more efficient. On the other hand, similar to habituation, the effect of CPO on the arousal also seemed to involve the activation of both AChRs, as the co-exposure with MCA and scopolamine were able to rescue the effect. However, scopolamine only induced a partial rescue over arousal increase which diminished at the highest concentration of CPO, while MCA was able to induce the full rescue at all affected concentrations. Therefore, the obtained results suggest that nAChRs may play an essential role in the observed effect of CPO on arousal, while mAChRs could be more relevant for the effect on habituation. Consistently, Best et al. (2008) reported that the increase in arousal and decrease in habituation found in zebrafish larvae exposed to donepezil, a reversible AChE inhibitor used in human medicine, was mediated by nAChRs. However, in contrast to our results on CPO, these authors found that MCA, but not atropine, was able to fully rescue the effects of donezepil. The fact that scopolamine is more potent than atropine on CNS (Renner et al.,

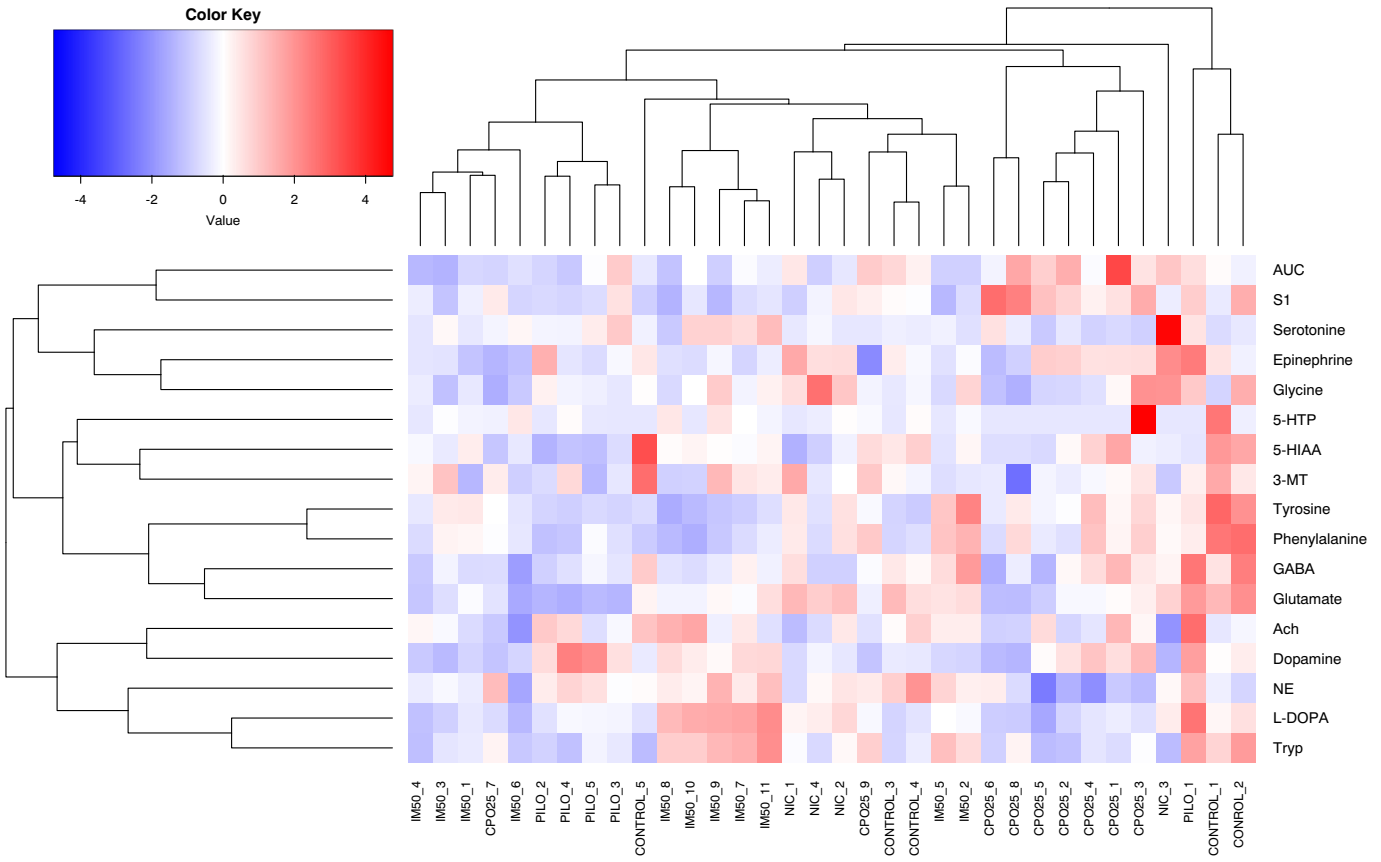

Fig. 4. Hierarchical clustering of behavioral responses and neurotransmitter concentrations (right of the clustering) in zebrafish larvae after the different treatments (bottom of the clustering). Neurotransmitter responses included: The color scale ranges from blue (low concentration) to red (high concentration, normalized values). Dendrograms corresponding to the hierachical clustering of both samples and compounds are shown on the top and on the left of the heatmaps, respectively. Clustering was performed using the *gplots* package in R. Abbreviations list: AUC – area under the curve; S1 – first startle; IM – imidacloprid; NIC – nicotine; PILO – pilocarpine. Neurotransmitter extended name and respective abbreviation can be found in Section 2.4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2005) could explain, at least partially, the observed differences of these drugs on the effect of AChE inhibitors.

Imidacloprid, the most widely used neonicotinoid insecticide in the world, binds and activates nAChRs, exhibiting a much greater affinity for insect than for vertebrate receptors (Tomizawa and Casida, 2005). However, different reports have shown the toxicity of this insecticide towards non-target species including vertebrates, such as birds and rats (Abu Zeid et al., 2019; Kapoor et al., 2014). In the present study, both n- and mAChRs failed in rescuing the imidacloprid effect on arousal (Fig. 3), suggesting the possibility of a non-cholinergic mechanism behind this effect. Interestingly, it has been recently demonstrated that imidacloprid is also a GABA receptor antagonist in insect nervous system (Déglise et al., 2002; Taylor-Wells et al., 2015).

Mauthner cells (M cells) play a central role in the vibrational escape response and its habituation in fish (Burgess and Granato, 2007). The chemical map of the dendrites of these cells has been characterized and includes AMPA, NMDA, GABA, glycine, dopamine and serotonin receptors (Korn and Faber, 2005). Thus, the effects of cholinergic compounds on the escape response and its habituation could involve changes in the neurotransmitter profile of the larvae. However, our results show that changes in arousal and/or habituation after exposure to cholinergic agonists CPO and imidacloprid are not related with changes in the neurotransmitter profile of the whole larvae. By analyzing the neurotransmitter profile in the whole larvae we are integrating potential effects on the central and peripheral nervous system, but at the same time we undergo a loss of sensitivity to small changes in specific areas of the CNS. However, our results may reflect the complexity of the mechanisms involved in the modulatory action of these chemicals.

In summary, the results presented in this manuscript demonstrate that the recently developed VSRA can be a useful tool for obtaining mechanistic information about the potential MoA of neuroactive compounds when the assay is performed after a pharmacological approach.

Author contributions

M.F and E.P performed all the exposure experiments; M.F and J.B performed the behavioral analyses; C.G.C, X.R.G and M.F preformed neurotransmitter analyses; B·P, M.F and D.R. were involved in the interpretation of the data. M.F and D.R. were involved in the conception, design and writing of the manuscript.

Competing financial interests

The authors declare no competing financial interest.

Acknowledgments

This work was supported by the Spanish Government (CTM2017-83242-R) and by the NATO SfP project MD.SFPP 984777. M.F acknowledges financial support from the Beatriu de Pinós programme (grant N°: 2016 BP 00233) provided by the Secretariat of Universities and Research department of the Ministry for Business and Knowledge, Catalonia Government. J.B acknowledges financial support from Bouygues Fundation's bursary, in the frame of the Erasmus Traineeship (FTOULOUS03).

Appendix A. Supplementary Material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.03.469.

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