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

Melissa Faria[1], Eva Prats[2], Karen Adriana Novoa-Luna[3], Juliette Bedrossiantz[1,4], Cristian Gómez-Canela[1,5], Leobardo Manuel Gómez-Oliván[3], Demetrio Raldúa[1].

[1]IDAEA-CSIC, Jordi Girona 18, 08034, Barcelona, Spain;

[2]CID-CSIC, Jordi Girona 18, 08034, Barcelona, Spain;

[3]Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan s/n. Col. Residencial Colón, 50120 Toluca, Estado de México, Mexico

[4]University of Toulouse III, Route de Narbonne, 31330 Toulouse, France

[5]Department of Analytical Chemistry and Organic Chemistry, Campus Sescelades, Faculty of Chemistry, Universitat Rovira i Virgili, Marcel·lí Domingo s/n, Tarragona, Spain

*Correspondence author e-mail address: mdfqam@cid.csic.es

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.

Keywords

Zebrafish; behavior; escape response; habituation; neurotransmitters.

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 $CaSO_4$. $2H_2O$] 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 hours (from 7-8 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 28.5ºC 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 hours 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.

2.3. Vibrational startle response assay (VSRA)

The vibrational startle response assay (VSRA) was based in the automatized delivery of the vibrational stimuli using the DanioVision Tapping Device DVTD-0010, installed in a DanioVision Observation Chamber (DVOC-0040). Videotracking and the escape response were analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands) (Fig. S1 – Supplementary material). A DanioVision Temperature Control Unit (DVTCU-0011) guaranteed that all trials were performed at 28ºC. All, tapping stimulus was selected at the highest intensity (intensity level: 8), and then, sequences of

the vibrational stimuli were delivered during fixed time periods referred to as interstimulus interval (ISI). Trails were conducted in 48 well plates, with one 8 dpf zebrafish larvae in each well containing 1 mL of exposure medium. Before delivering the first stimulus, larvae were left in the DVOC for 30 min to acclimate. Videos were recorded at 30 frames per second and the VSR was analysed for each individual larva by measuring the distance moved (cm) over the 1s period after stimulus.

2.4. Assessment of habituation criteria

In order to determine if a sequence of vibrational stimulus is able to induce a true habituation, different criteria were evaluated. Briefly, 8 dpf zebrafish larvae were placed individually in 48 well plates containing 1mL of fish water medium. For induction of habituation, tapping stimulus was delivered at 1s, 5s or 20s ISI at sessions that lasted up to 100 taps. Subsequently, the recovery of tapping startle was elicited by submitting 48 larvae to two series of tapping sessions (1s ISI) separated by 15 min. Finally, to confirm that the VSR attenuation was indeed habituation and not the result of sensory adaptation or motor fatigue, the effect of a cross-modal stimulation was tested. Thus, larvae were subjected to a 5s ISI regime and a 2s white light pulse, at a white light intensity of 200 lux (5% intensity level in DanioVision settings) was delivered after the 80^{th} tapping, recording larvae movement for the remaining 20 tapings. To confirm dishabituation, larvae were subjected to a 5s ISI regime and a 2s white light pulse was introduced after the 80^{th} tapping (400s), recording larvae movement for the remaining 100s (time interval equivalent to 81-100^{th} tapping) with no further vibrational or visual stimuli.

196

197 ### 2.5. Concordance analysis

198 For the concordance analysis, 7 dpf larvae were exposed for 24 hours to a
199 battery of pharmacologically active compounds added to fish water medium
200 after which were then placed in the DVOC system for tracking and analysis.
201 Startle responses were evoked in 8 dpf control (DMSO) and treated larvae
202 (n=96-240 larvae per condition from two independent experiments) by
203 producing a series of vibrating stimulus at 1s ISI, for a total of 50 stimuli,
204 synchronized by EthoVision software.

205 ### 2.6. Data analysis

206 Data were analyzed with IBM SPSS 19.0 (Statistical Package 2010, Chicago,
207 IL), using Student's t-test or one-way ANOVA followed by Tukey's multiple
208 comparison test. The statistical test used for each set of results can be found in
209 the text or in the figure caption. For further clarification, Student's t-test analysis
210 comparing mean distance moved of treated with that of the paired control
211 larvae, was used for each point of plots of larvae movement during stimuli
212 delivery trials. Data are presented as the mean ± SEM of 2 independent
213 experiments, unless otherwise stated. Significance was set at $P < 0.05$. The
214 area under the curve (AUC) was calculated using Y=0. To simplify data
215 representation, responses (distance moved) from the first 20 stimuli are
216 represented in graphs plotting the VSR of each neurotransmitter modulator,
217 while the results representing the AUC were calculated for all 50 stimulus.

218

3. Results and discussion

3.1. Escape response and habituation can be determined in the VSR assay

The VSRA uses zebrafish larvae instead of embryos. Although zebrafish is used at unlicensed stages for developmental neurotoxicity studies, we decided to select 7-8 dpf larvae for the development of the assay because, while they are still suitable for high-throughput screening of chemical libraries, the potential confounding factor of neurodevelopmental processes is strongly reduced at this developmental stage. In fact, by 7-8 dpf neuronal proliferation is limited to only a few particular regions, with most regions of the brain comprised of post-mitotic neurons with well-elaborated neuronal arbors (Fero et al., 2011). Moreover, complex behaviors such as responses to visual and acoustic/vibrational stimuli are only apparent in larvae (Fleming, 2007).

Response decrements resulting from repeated stimulation can be produced not only by habituation, but also by other mechanisms including receptor adaptation and effector fatigue (Thompson, 2009). Thus, any method proposed for habituation analysis needs to meet a number of hallmark criteria (Best et al., 2008; Thompson and Spencer, 1966) including: (1) the more rapid the frequency of stimulation the more rapid and/or more pronounced is habituation; (2) habituated responses exhibit spontaneous recovery and (3) instant recovery from habituation by delivery of a second stimulus, also called dishabituation. To test the first criteria of habituation, VSR was analyzed in 8 dpf zebrafish larvae

using different ISIs (1s, 5s and 20s), for a total of 50 tapping stimulus (Fig. 1A). In all groups, the presentation of the first tapping stimulus dramatically evoked escape response (see Video 1 – supplementary material), determined here as the distance moved during 1 s after stimulus (Student's t-test: $P<0.001$). Although the distance moved during the first startle response was similar across the different ISIs, ranging around 0.7 cm (one-way ANOVA: $F_{(2,128)}=1.443$; $P>0.05$), AUC increased significantly with the interval of the stimulus (one-way ANOVA: $F_{(2,149)=}129.742$; $P<0.001$). Thus, AUCs reached for 1s, 5s and 20s ISIs were 6.16 ± 0.86, 15.42 ± 1.50 and 20.25 ± 1.76, respectively. At the shortest interval of stimuli used (ISI: 1s) larvae responses decreased to baseline levels (pre-trial phase) by the 14th tap. For the 5s ISI regime, larvae responses reached a steady state by the 8th tap, although this new established baseline was 4 fold higher than the 1s ISI routine. On the other hand, during the 20s ISI regime, larvae movement following the 1st tap remained relatively high and varied between 0.5 and 0.22 cm, throughout the rest of the trial. For testing the second criteria for habituation, spontaneous recovery, larvae were tested with two series of 20 tapping stimulus delivered at 1s ISI rate separated by a period of 15 min (Fig. 1B). Whereas an iterative reduction in startle response was found across the first series of tapping stimulus, reaching the steady state by the 19th tap ($P=0.237$), the startle response was fully recovered in the second series of tapping stimulation. The increase in the larvae movement after the first tap was similar in both series (0.640 ± 0.036 cm vs 0.570 ± 0.031 cm for the first and second series of tapping stimulation, respectively), and the magnitude of the movement across both series was within the same range (Student's t-test: $P>0.05$). Finally, in order to assure that the iterative decrease

in the response observed in VSRA corresponds to true habituation and not to fatigue nor sensory adaptation (Thompson and Spencer, 1966), dishabituation of the larvae was analyzed. For inducing dishabituation, a different type of stimulus, a 2s white-light pulse, was used. A series of 100 tapping stimulus delivered at 5s ISI was used to cause habituation. Four trial setup combinations (Fig. 1C-F) of tapping and white-light pulse were used to establish dishabituation of habituated larvae. As previously demonstrated for 5s ISI routine, larvae movement increased significantly in response to the first tap and then, an iterative reduction in the responses was observed until the 8-9th tap, when motor activity reached a steady state (Fig. 1C). However, when a 2s white-light pulse was introduced after the 80th tap, the 81st tap evoked an escape response with a similar magnitude to that of the first tap ($P>0.05$). Interestingly, escape response remained elevated for the rest of the trial following the startle restart (Fig. 1D). On the other hand, the white-light pulse by itself did not evoke an escape response. In fact, when no more tapping stimuli were delivered after the 2s light pulse, larvae movement significantly decreased ($P<0.001$) at the time corresponding to the 81st tapping stimulus and then, locomotor activity increased gradually, returning to pre-light pulse levels at the time corresponding to the 90th tapping stimulus (Fig. 1F). The results presented above demonstrate that the proposed assay not only allows quantifying the distance moved for each larva during the VSR, but also meets the criteria proposed for habituation analysis.

3.2. Concordance analysis of VSR assay

An important criterion for the validation of VSRA is that whereas the assay is performed on zebrafish larvae, results should be predictive of the responses in other vertebrate species, including other fish species and mammals. In order to assess the VSRA predictivity, the concordance of the results obtained with this assay with preexisting data in fish and mammals has been analyzed. For this study of concordance analysis, a group of neuroactive compounds modulating three different neurotransmitter systems have been selected (Best et al., 2008; Roberts et al., 2013; Roberts et al., 2011; Wolman et al., 2011).

3.2.1. Cholinergic system

The cholinergic system plays a pivotal role in learning and memory (Robinson et al., 2011), with cholinergic agonists improving memory (Mattson, 2004). In the present study, the effects of 25 µM nicotine, 20 µM MLA, 80 µM pilocarpine, 25 µM scopolamine and 10 µM donepezil on the distance moved during the escape response and the habituation to VSR have been analyzed using the VSRA (Fig. 2). Nicotine and pilocarpine, agonists of nicotinic and muscarinic acetylcholine receptors (AChR), respectively, significantly reduced habituation of VSR, as indicated by the significant increase in the AUC values found in treated larvae respect to the control (Fig. 2A,B). A detailed analysis of the responses across the assay showed that the nicotine effect was restricted to the responses elicited by the 2^{nd} to 6^{th} tapping stimuli ($P<0.05$; Fig. 3A). Pilocarpine increased significantly the magnitude of the first startle response ($P=0.005$; Fig. 2B) as well as the responses elicited from the 15^{th} tapping stimulus onwards. In contrast to the effect of cholinergic agonists, no clear effects of MLA and scopolamine, antagonists of nicotinic and muscarinic AChRs, respectively, on

315 the VSR magnitude and habituation were found (Fig. 2C,D), as indicated by the
316 similar values of the AUC between treated larvae respect to the control (Fig.
317 2C,D; P>0.05). Finally, the effect of donepezil, an acetylcholinesterase (AChE)
318 inhibitor used as cognitive enhancer in Alzheimer's disease patients, was
319 consistent with its AChRs agonist role (Fig. 2E,E). On one hand, donepezil
320 induced a significant increase in the magnitude of the first startle response
321 (P<0.001). Moreover, donepezil significantly reduced habituation to VSR, as
322 indicated by the significant increase observed in the AUC values (Fig. 2E) as
323 well as in the higher response found after all the vibrational stimuli delivered in
324 the assay (Fig. 2E).

325 These results with the modulators of the cholinergic system obtained by using
326 the developed VSR assay are consistent with those reported in the literature.
327 Thus, a significant increase of magnitude of the startle response evoked by
328 acoustic/vibrational stimulus has been reported in rodents and zebrafish
329 exposed to nicotine and pilocarpine (Acri et al., 1991; Acri et al., 1994; Eddins
330 et al., 2010; Kumari et al., 2001; Schreiber et al., 2002). Moreover, the effect of
331 10 µM donepezil on the startle response evoked by acoustic stimulation on 7
332 dpf zebrafish larvae has been recently characterized (Best et al., 2008).
333 Consistent with the results obtained in this study using vibrational stimuli,
334 donepezil enhanced acoustic startle response and significantly reduced
335 habituation. In the same study, authors did not find any effect on habituation
336 using MLA and atropine (muscarinic AChR antagonist), a result also consistent
337 with that obtained using the VSRA. Finally, scopolamine failed to induce any
338 significant effect on the VSR magnitude and habituation in adult zebrafish

(Levin and Cerutti, 2009), a result also consistent with the results presented in this study using VSRA.

3.2.2. Serotonergic system

Serotonin (5-HT) is a monoamine neurotransmitter involved in the control of mood, cognition and memory. Serotonergic neurons are known to modulate responses of the organism to environmental stimulation (Conner et al., 1970; Pittman and Lott, 2014; Quednow et al., 2004). To determine the effect of well-known modulators of the serotonergic system on the VSR magnitude and habituation, larvae were incubated for 24h with 2.5 mM PCPA (tryptophan-5-hydroxylase inhibitor), 0.5 µM fluoxetine (selective serotonin reuptake inhibitor), 5 µM deprenyl (monoamine oxidase inhibitor) and 2.5 µM buspirone (5-HT1A receptor agonist; Fig. 3). Changes in the escape response of the larvae were evaluated during 50 consecutive tapping stimulus delivered at 1s ISI rate. Decreasing serotonin synthesis with PCPA significantly reduced habituation of VSR, as indicated for the significant increase in the AUC values found in the treated larvae respect to the control ($P<0.001$; Fig. 3A). Interestingly, the modulatory effect of PCPA on habituation was restricted to the responses elicited between the 4^{nd} and 14^{th} tapping stimulus ($P<0.001$; Fig. 3A). The effect of PCPA in the VSRA is consistent with the reduced habituation of ASR found in rats exposed to PCPA (Carlton and Advokat, 1973; Conner et al., 1970; File, 1977). In contrast to the effects of the tryptophan hydroxylase inhibitor PCPA, increasing of serotonin levels at the serotonergic synapses with fluoxetine and deprenyl significantly increased habituation to VSR, specially reducing the

escape responses to the first 8-10 tapping stimulus (Fig. 3B,C). This increase in habituation found after treatment with fluoxetine and deprenyl was confirmed by the significant decrease found in the AUCs when compared to control ($P<0.001$; Fig.s 3B,C). Interestingly, only fluoxetine was able to reduce VSR magnitude after the first tapping stimulus ($P<0.001$). Results of fluoxetine and deprenyl in the VSRA are also consistent with the reported role of serotonin promoting habituation of the startle response by enhancing glycinergic inhibition of the Mauthner cells(Fero et al., 2011; Mintz et al., 1989). No clear effects were found in the VSR magnitude or habituation with the 5-HT1A receptor agonist buspirone. This absence of modulatory effect of buspirone might be related to the fact that 5-HT5 and 5HT6, but not 5-HT1, are expressed in Mauthner cells (Whitaker et al., 2011).

3.2.3. Glutamatergic system

Glutamatergic system also plays an important role in learning and memory in vertebrates. When larvae were incubated for 24 hours with 40 µM memantine, an NMDA receptor antagonist, and recorded during 50 consecutive tapping stimulus delivered at 1s ISI rate, a significant reduction in the habituation of VSR was found ($P<0.05$), as indicated by a significant increase of the AUC of treated larvae ($P<0.001$; Fig. 4). Additionally, response to the first tap in memantine-treated larvae was higher than in controls, and the response remained higher during the following 3 stimulus (Fig. 4). Consistent with the results obtained with VSRA in this study, previous reports on the effect of the NMDA receptor antagonists on the ASR in mice and zebrafish showed also

388 reduced habituation compared to controls (Best et al., 2008; Klamer et al.,
389 2004). Three different NMDA receptor antagonists also reduced short-term
390 habituation in response to vibrational stimuli in zebrafish larvae (Wolman et al.,
391 2011). Moreover, the higher magnitude of the escape response after the first
392 tap found with the VSRA is also consistent with the reported effects on the ASR
393 in larval zebrafish exposed to 30 µM memantine (Best et al., 2008).

394 ### 3.2.4. Environmentally relevant compounds

395 The last part of this work was to assess the suitability of VSRA for dealing with
396 environmental relevant compounds. Imidacloprid and chlorpyrifos-oxon (CPO),
397 two neurotoxic pesticides disrupting normal cholinergic signaling, were selected
398 as a proof of concept. Imidacloprid belongs to the group of nicotine-related
399 insecticides referred to as neonicotinoids, which act as agonists of the insect
400 postsynaptic nicotinic acetylcholine receptors (nAChRs) (Matsuda et al., 2001)
401 resulting in the impairment of normal nerve function. CPO, the active metabolite
402 of chlorpyrifos, is a prototypic organophosphate (OP) with a toxic underlying
403 mechanism that involves the irreversible inhibition of acetylcholinesterase
404 (AChE) and overstimulation of both AChRs (Faria et al., 2015). To test the
405 suitability of VSRA to assess quantitative concentration-response relationship,
406 zebrafish larvae were exposed to three concentrations of each pesticide for 24
407 hours. We found that both contaminants disrupted normal VSR magnitude and
408 habituation in a dose dependent matter (Fig.s 5 and 6). Likewise, 25 and 50 µM
409 of imidacloprid decreased the VSR magnitude and increased habituation (Fig.
410 5B-D) to similar levels ($P<0.05$), and no effect was observed at 5 µM ($P=0.174$;
411 Fig. 5A,D). Despite this, analysis of AUC reported significant effects across

concentrations (one-way ANOVA: $F_{(3,186)}$=14.280; P<0.001, (Fig. 5D), which indicates that concentration of imidacloprid had a significant effect over VSR response. Interestingly, these results are not within the same line as that observed for nicotine, another nAChR agonist. Although the effects of imidacloprid on habituation have been well-established in honeybee (Decourtye et al., 2004; Goñalons and Farina, 2018), the effects of this compound in fish have not been well characterized.

In the only available report addressing the effect of imidacloprid on startle response and habituation in fish, an increase in the VSR magnitude and a decrease in habituation was found in adolescent zebrafish exposed to 45-60 µM imidacloprid during the first 5 days of development (Crosby et al., 2015). The differences found between that study and our results using VSRA could be related with the different experimental design used, including different exposure time, developmental stage at the time of exposure and time between the end of exposure to the behavioral testing.

Chlorpyrifos-oxon evoked a dramatic dose dependent impairment of the VSR in larvae after 24h of exposure (one-way ANOVA: $F_{(3,182)}$=35.163, P<0.001). VSR magnitude and habituation were respectively higher and lower than control (Fig. 6A-D). After the first tap, VSR magnitude in larvae treated with 25 and 50 nM CPO were already 1.4- and 3-fold higher than the corresponding controls, respectively, and remained significantly elevated throughout most of the first 20 stimuli. On the other hand, 5 nM CPO seemed to have a delayed effect over VSR magnitude, presenting significant differences from the 6-14th tapping stimuli. Eventually, habituation of the VSR was reached for all treatments before

436 the 20th tapping stimulus. AUC values were already notably significant at 5 nM
437 (P=0.014) (Fig. 6D). Impairment of the startle response by chlorpyrifos has
438 been reported in different fish species and rodents (Carlson et al., 1998; Eddins
439 et al., 2010; Levin et al., 2002; Sledge et al., 2011). Additionally, these
440 responses, though much more potent, resemble those reported for the two
441 AChR agonists and for the cholinergic drug tested in this study, which
442 emphasizes the robustness of the proposed method and reinforces this
443 approach as a promising behavioral monitoring tool for screening and
444 differentiating chemicals present in the environment.

445 Zebrafish larvae are ideally suited for large-scale analysis of vertebrate
446 behavior, including learning and memory (Colwill Ruth and Creton, 2011; Levin
447 and Chen, 2004). For medium- to high-throughput analyses of behavior, it is
448 crucial to use robust assays that can be automated. Indeed, easy to use
449 automated systems, designed as a plug and play system, including both
450 software and equipment, have been recently developed and are currently
451 commercially available for imaging and analyzing zebrafish larvae behavior in
452 multi-well plates. However, to our knowledge, implicit learning evaluation
453 methods have not yet been validated for any commercially available platform. In
454 this study we have demonstrated that an automated system using vibrational
455 stimulation was able to fulfill a number of criteria that have been established to
456 determine true habituation (Brown, 1998; Thompson, 2009; Thompson and
457 Spencer, 1966). In this study, zebrafish larvae demonstrated the basic
458 response of habituation, an iterative reduction of the distance moved in
459 response to repetitive vibrational stimuli, fulfilling the main criteria proposed for
460 habituation. Aiming to analyze the predictivity of the results of this assay

performed with zebrafish larvae to other vertebrate species inhabiting aquatic ecosystems, a group of modulators of the cholinergic, serotonergic and glutamatergic systems was tested, and the results were in agreement with the data available in the bibliography for fish and rodents. This concordance of the results in the developed assay with others developed in the same and different vertebrate species demonstrate the results from this assay can be easily extrapolated to other aquatic vertebrates. The results regarding CPO and imidacloprid also demonstrated that the developed assay is suitable for the analysis of the concentration-response relationship of environmental pollutants that impair the escape response elicited by vibrational stimuli. This result is highly relevant, since the concentration-response relationships obtained in this assay might provide an estimation of the potency (EC50 or EC10) of the disrupting effect of each tested pollutant on the predator avoidance behavior. Moreover, the combination of concentration-response data from VSRA with data about lethality should allow to determine the associated hazard (LC50/EC50) to any tested compound (Thienpont et al., 2011). Additional efforts should be done to assess the suitability of this new assay to determine the potency and hazard of environmental pollutants.

4. Conclusion

We have developed a new simple and automated assay in zebrafish larvae, for *in vivo* medium to high-throughput assessment of the escape response and its habituation. We were able to demonstrate the assay's predictive and feasible qualities for the potential development of a systematic approach able to screen chemical compounds present in the

environment with specific effects over fish escape behavior, as well as, establish fish species more sensitive or resistant to such chemicals.

5. Acknowledgements

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 Nº: 2016 BP 00233) provided by the Secretariat of Universities and Research department of the Ministry for Business and Knowledge, Catalonia Government. K.A.N.L was supported by the grant 291212 from the Mixed Fund programme for mobility (CONACYT-2017). J.B acknowledges financial support from Bouygues Fundation's bursary, in the frame of the Erasmus Traineeship (FTOULOUS03).

6. References

Acri JB, Grunberg NE, Morse DE. Effects of nicotine on the acoustic startle reflex amplitude in rats. Psychopharmacology 1991; 104: 244-248.

Acri JB, Morse DE, Jon Popke E, Grunberg NE. Nicotine increases sensory gating measured as inhibition of the acoustic startle reflex in rats. Psychopharmacology 1994; 114: 369-374.

Babin PJ, Goizet C, Raldua D. Zebrafish models of human motor neuron diseases: advantages and limitations. Progress in neurobiology 2014; 118: 36-58.

511 Batabyal A, Balakrishna S, Thaker M. A multivariate approach to understanding
512 shifts in escape strategies of urban lizards. Behavioral Ecology
513 and Sociobiology 2017; 71: 83.
514 Best JD, Berghmans S, Hunt JJ, Clarke SC, Fleming A, Goldsmith P, et al. Non-
515 associative learning in larval zebrafish.
516 Neuropsychopharmacology 2008; 33: 1206.
517 Brown GD. Nonassociative learning processes affecting swimming probability in
518 the seaslug Tritonia diomedea: habituation, sensitization and
519 inhibition. Behavioural brain research 1998; 95: 151-165.
520 Carlson RW, Bradbury SP, Drummond RA, Hammermeister DE. Neurological
521 effects on startle response and escape from predation by medaka
522 exposed to organic chemicals. Aquatic Toxicology 1998; 43: 51-
523 68.
524 Carlton PL, Advokat C. Attenuated habituation due to parachlorophenylalanine.
525 Pharmacology Biochemistry and Behavior 1973; 1: 657-663.
526 Colwill Ruth M, Creton R. Imaging escape and avoidance behavior in zebrafish
527 larvae. Reviews in the Neurosciences. 22, 2011, pp. 63.
528 Conner RL, Stolk JM, Barchas JD, Levine S. Parachlorophenylalanine and
529 habituation to repetitive auditory startle stimuli in rats. Physiology
530 & Behavior 1970; 5: 1215-1219.
531 Crosby EB, Bailey JM, Oliveri AN, Levin ED. Neurobehavioral impairments
532 caused by developmental imidacloprid exposure in zebrafish.
533 Neurotoxicology and teratology 2015; 49: 81-90.
534 Decourtye A, Armengaud C, Renou M, Devillers J, Cluzeau S, Gauthier M, et al.
535 Imidacloprid impairs memory and brain metabolism in the

honeybee (Apis mellifera L.). Pesticide biochemistry and physiology 2004; 78: 83-92.

Eddins D, Cerutti D, Williams P, Linney E, Levin ED. Zebrafish provide a sensitive model of persisting neurobehavioral effects of developmental chlorpyrifos exposure: comparison with nicotine and pilocarpine effects and relationship to dopamine deficits. Neurotoxicology and teratology 2010; 32: 99-108.

Faria M, Prats E, Padrós F, Soares AM, Raldúa D. Zebrafish is a predictive model for identifying compounds that protect against brain toxicity in severe acute organophosphorus intoxication. Archives of toxicology 2017; 91: 1891-1901.

Fero K, Yokogawa T, Burgess HA. The behavioral repertoire of larval zebrafish. Zebrafish models in neurobehavioral research. Springer, 2011, pp. 249-291.

Fields DM, Yen J. The escape behavior of marine copepods in response to a quantifiable fluid mechanical disturbance. Journal of Plankton Research 1997; 19: 1289-1304.

File SE. Effects of parachlorophenylalanine and amphetamine on habituation of exploration. Pharmacology Biochemistry and Behavior 1977; 6: 151-156.

Fleming A. Zebrafish as an alternative model organism for disease modelling and drug discovery: implications for the 3Rs. NC3Rs, Iss. 10, National Centre for the Replacement, Refinement and Reduction of Animals in research, 2007.

Gómez-Canela C, Tornero-Cañadas D, Prats E, Piña B, Tauler R, Raldúa D. Comprehensive characterization of neurochemicals in three zebrafish chemical models of human acute organophosphorus poisoning using liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry 2018: 1-14.

Goñalons CM, Farina WM. Impaired associative learning after chronic exposure to pesticides in young adult honey bees. Journal of Experimental Biology 2018; 221: jeb176644.

Houde E, Hoyt R. Fish early life dynamics and recruitment variability. Trans. Am. Fish. Soc 1987.

Kandel ER. Cellular mechanisms of learning and the biological basis of individuality. Principles of neural science 1991; 3: 1009-1031.

Killen SS, Brown JA. Energetic cost of reduced foraging under predation threat in newly hatched ocean pout. Marine Ecology Progress Series 2006; 321: 255-266.

Klamer D, Pålsson E, Revesz A, Engel JA, Svensson L. Habituation of acoustic startle is disrupted by psychotomimetic drugs: differential dependence on dopaminergic and nitric oxide modulatory mechanisms. Psychopharmacology 2004; 176: 440-450.

Kumari V, Soni W, Sharma T. Influence of cigarette smoking on prepulse inhibition of the acoustic startle response in schizophrenia. Human Psychopharmacology: Clinical and Experimental 2001; 16: 321-326.

583 Leussis MP, Bolivar VJ. Habituation in rodents: A review of behavior,
584 neurobiology, and genetics. Neuroscience & Biobehavioral
585 Reviews 2006; 30: 1045-1064.
586 Levin ED, Addy N, Baruah A, Elias A, Christopher NC, Seidler FJ, et al.
587 Prenatal chlorpyrifos exposure in rats causes persistent
588 behavioral alterations. Neurotoxicology and teratology 2002; 24:
589 733-741.
590 Levin ED, Cerutti DT. Behavioral neuroscience of zebrafish. 2009.
591 Levin ED, Chen E. Nicotinic involvement in memory function in zebrafish.
592 Neurotoxicology and Teratology 2004; 26: 731-735.
593 Marsden KC, Granato M. In vivo Ca2+ imaging reveals that decreased dendritic
594 excitability drives startle habituation. Cell reports 2015; 13: 1733-
595 1740.
596 Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB.
597 Neonicotinoids: insecticides acting on insect nicotinic
598 acetylcholine receptors. Trends in pharmacological sciences
599 2001; 22: 573-580.
600 Mattson MP. Pathways towards and away from Alzheimer's disease. Nature
601 2004; 430: 631-639.
602 Mintz I, Gotow T, Triller A, Korn H. Effect of serotonergic afferents on quantal
603 release at central inhibitory synapses. Science 1989; 245: 190-
604 192.
605 Painter MM, Buerkley MA, Julius ML, Vajda AM, Norris DO, Barber LB, et al.
606 Antidepressants at environmentally relevant concentrations affect
607 predator avoidance behavior of larval fathead minnows

608 (Pimephales promelas). Environmental Toxicology and Chemistry
609 2009; 28: 2677-2684.
610 Park C, Clements KN, Issa FA, Ahn S. Effects of Social Experience on the
611 Habituation Rate of Zebrafish Startle Escape Response: Empirical
612 and Computational Analyses. Frontiers in Neural Circuits 2018;
613 12.
614 Pittman JT, Lott CS. Startle response memory and hippocampal changes in
615 adult zebrafish pharmacologically-induced to exhibit
616 anxiety/depression-like behaviors. Physiology & Behavior 2014;
617 123: 174-179.
618 Quednow BB, Kühn K-U, Stelzenmueller R, Hoenig K, Maier W, Wagner M.
619 Effects of serotonergic and noradrenergic antidepressants on
620 auditory startle response in patients with major depression.
621 Psychopharmacology 2004; 175: 399-406.
622 Roberts A, Bill B, Glanzman D. Learning and memory in zebrafish larvae.
623 Frontiers in Neural Circuits 2013; 7.
624 Roberts AC, Pearce KC, Choe RC, Alzagatiti JB, Yeung AK, Bill BR, et al.
625 Long-term habituation of the C-start escape response in zebrafish
626 larvae. Neurobiology of Learning and Memory 2016; 134: 360-
627 368.
628 Roberts AC, Reichl J, Song MY, Dearinger AD, Moridzadeh N, Lu ED, et al.
629 Habituation of the C-start response in larval zebrafish exhibits
630 several distinct phases and sensitivity to NMDA receptor
631 blockade. PLOS one 2011; 6: e29132.

Robinson L, Platt B, Riedel G. Involvement of the cholinergic system in conditioning and perceptual memory. Behavioural Brain Research 2011; 221: 443-465.

Schreiber R, Dalmus M, De Vry J. Effects of α 4/β 2-and α 7-nicotine acetylcholine receptor agonists on prepulse inhibition of the acoustic startle response in rats and mice. Psychopharmacology 2002; 159: 248-257.

Sledge D, Yen J, Morton T, Dishaw L, Petro A, Donerly S, et al. Critical duration of exposure for developmental chlorpyrifos-induced neurobehavioral toxicity. Neurotoxicology and Teratology 2011; 33: 742-751.

Thienpont B, Tingaud-Sequeira A, Prats E, Barata C, Babin PJ, Raldúa D. Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. Environmental science & technology 2011; 45: 7525-7532.

Thompson RF. Habituation: a history. Neurobiology of learning and memory 2009; 92: 127.

Thompson RF, Spencer WA. Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychological review 1966; 73: 16.

Whitaker KW, Neumeister H, Huffman LS, Kidd CE, Preuss T, Hofmann HA. Serotonergic modulation of startle-escape plasticity in an African cichlid fish: a single-cell molecular and physiological analysis of a vital neural circuit. Journal of neurophysiology 2011; 106: 127-137.

Wolman MA, Jain RA, Liss L, Granato M. Chemical modulation of memory formation in larval zebrafish. Proceedings of the National Academy of Sciences 2011; 108: 15468-15473.

Author contributions

M.F and E.P performed all the exposure experiments; M.F, C.G-C, K.A.N-L and J.B performed the behavioural analyses; D.R. was involved in the conception; M.F and D.R were involved in the design and interpretation of the data and the writing of the manuscript with input from L.M.G-O.

Competing financial interests

The authors declare no competing financial interest.

Supporting Information.

List of the neuroactive compounds and environmental contaminants used for this study.

Figure captions

Figure 1. **Vibrational Startle Response Assay (VSRA) meets the main habituation criteria**. Plots of results of mean distance moved ± SE (n=48) against tapping stimulus in zebrafish larvae 8 dpf. Black arrow indicates the beginning of tapping stimuli. (**A**) Results of 50 tapping stimulus delivered at three different interstimulus interval (ISI): 1s (red triangle), 5s (blue squares) and 20s (green circles); (**B**) Larvae show a spontaneous recovery of startle response from a first session of 20 tapping stimulus at 1s ISI (solid black line, black dots) after 15 minute period (straight brackets). The spontaneous recovery is observed when larvae undergo a second session of 20 tapping stimulus of 1s ISI (dashed black line, black dots); (**C-F**) Cross-modal stimulation. Plotted results of mean distance moved ± SE (n=48) against tapping stimulus under a 5s ISI regime with a total of 100 stimuli. Black arrow indicates the beginning of tapping stimulus. White arrow indicates 2s light pulse (5% intensity, according to DanioVision equipment). (**C**) 100 tapping stimulus; (**D**) 100 tapping stimulus with two seconds light pulse interposed between the 80th and 81st tapping; (**E**) no stimuli given for an equivalent time to 80 tapping stimulus (5s ISI, total time of 400 seconds) followed by a two seconds light pulse, followed by the first (corresponding to tapping stimulus 81^{th}) of 19 consecutive tapping stimulus; (**F**) 80 tapping stimulus with followed by two seconds light pulse and no further tapping stimulus.

Figure 2. Effect of cholinergic modulators on the visual startle response and habituation. Plots of average distance moved ± SE against 20 tapping stimulus and corresponding bar graphs of calculated AUC (mean ± SE) of 50

tapping stimuli at 1s ISI. The results presented refer to control larvae (blue squares/bar) and larvae exposed to compounds that modulate the cholinergic system: (A) – 25 µM nicotine (red triangles/bar) (n=89/point); (B) – 80 µM pilocarpine (Pilo; olive green triangles/bar) (n=88/point); (C) – 20 µM methyllcaconitine (MLA; light blue triangles/bar) (n=86/point) (D) – 25 µM scopolamine (Scop; orange triangles/bar) (n=86/point); and (E) 10 µM of donepezil (green triangles/bar) (n=48/point). Each point of control DMSO 0.1 and 1%(*) corresponds to n=328 and n=91 respectively. Black arrow indicates the beginning of tapping stimulus trial.***P<0.001; **P<0.01 and *P<0.05 vs corresponding control value (Student's t-test) of plots and bar graphs.

Figure 3. Effect of serotonergic modulators on the visual startle response and habituation. Plots of average distance moved ± SE against 20 tapping stimulus and corresponding bar graphs of calculated AUC (mean ± SE) of 50 tapping stimuli at 1s ISI. The results presented refer to control larvae (blue squares/bar, n=328) and larvae exposed to compounds that modulate the serotonergic system: (A) 2.5 mM PCPA (dark purple triangles/bar, n=94); (B) 0.5 µM fluoxetine (black triangles/bar, n=93); (C) 5 µM deprenyl (grey triangles/bar, n=94) and (D) 2.5 µM buspirone (green triangles/bar, n=91). Black arrow indicates the beginning of tapping stimuli trial. ***P<0.001; **P<0.01 and *P<0.05 vs corresponding control value (Student t-test) of plots and bar graphs.

Figure 4. Effect of memantine, a NMDA receptor antagonist, on the vibrational startle response and habituation. Plot of average distance moved ± SE against 20 tapping stimulus at 1s ISI for control larvae (blue squares; n=328) and larvae exposed to 40 µM memantine (dark yellow

triangles; n=91/point). Black arrow indicates the beginning of tapping stimulus trial. Inset: AUC (mean ± SE) corresponding to 50 tapping stimulus at 1s ISI of 40µM of memantine and control. ***P<0.001; **P<0.01 and *P<0.05 vs corresponding control value (Student t-test) of the plots and bar graphs.

Figure 5. Effects of imidacloprid on the vibrational startle response and habituation. (**A-C**) Plots of average distance moved ± SE against 20 tapping stimulus at 1s ISI for control larvae (blue squares; n=96) and larvae exposed to increasing concentrations of imidacloprid (circles with different shades of marine blue): 5 µM (**A**; n=47/point), 25 µM (**B**; n=94/point) and 50 µM (**C**; n=89/point). Black arrow indicates the beginning of tapping stimuli trial; (**D**) AUC (mean ± SE) corresponding to 50 tapping stimulus at 1s ISI of control and 5, 25 and 50 µM imidacloprid. The following symbols represent static differences for plots: ***P<0.001; **P<0.01 and *P<0.05 vs corresponding control value, Student's t-test. For the bar graph, different letters indicate significant (P<0.05) differences following one-way ANOVA and Tukey's multiple-comparison test.

Figure 6. Effects of chlorpyrifos-oxon (CPO) on the vibrational startle response and habituation. (**A-C**) Plots of average distance moved ± SE against 20 tapping stimulus at 1s ISI for control larvae (blue squares; n=96) and larvae exposed to increasing concentrations of CPO (triangles with different shades of pink): 5 nM (**A**; n=88/point), 25 nM (**B**; n=86/point) and 50 nM (**C**; n=88/point). Black arrow indicates the beginning of tapping stimulus; (**D**) AUC (mean ± SE) corresponding to 50 tapping stimulus at 1s ISI of control and 5, 25 and 50 nM CPO. The following symbols represent statistic differences for plots: ***P<0.001; **P<0.01 and *P<0.05 vs corresponding control value, Student's t-

test. For the bar graph, different letters indicate significant ($p<0.05$) differences following one-way ANOVA and Tukey's multiple-comparison test.

Figure 1.

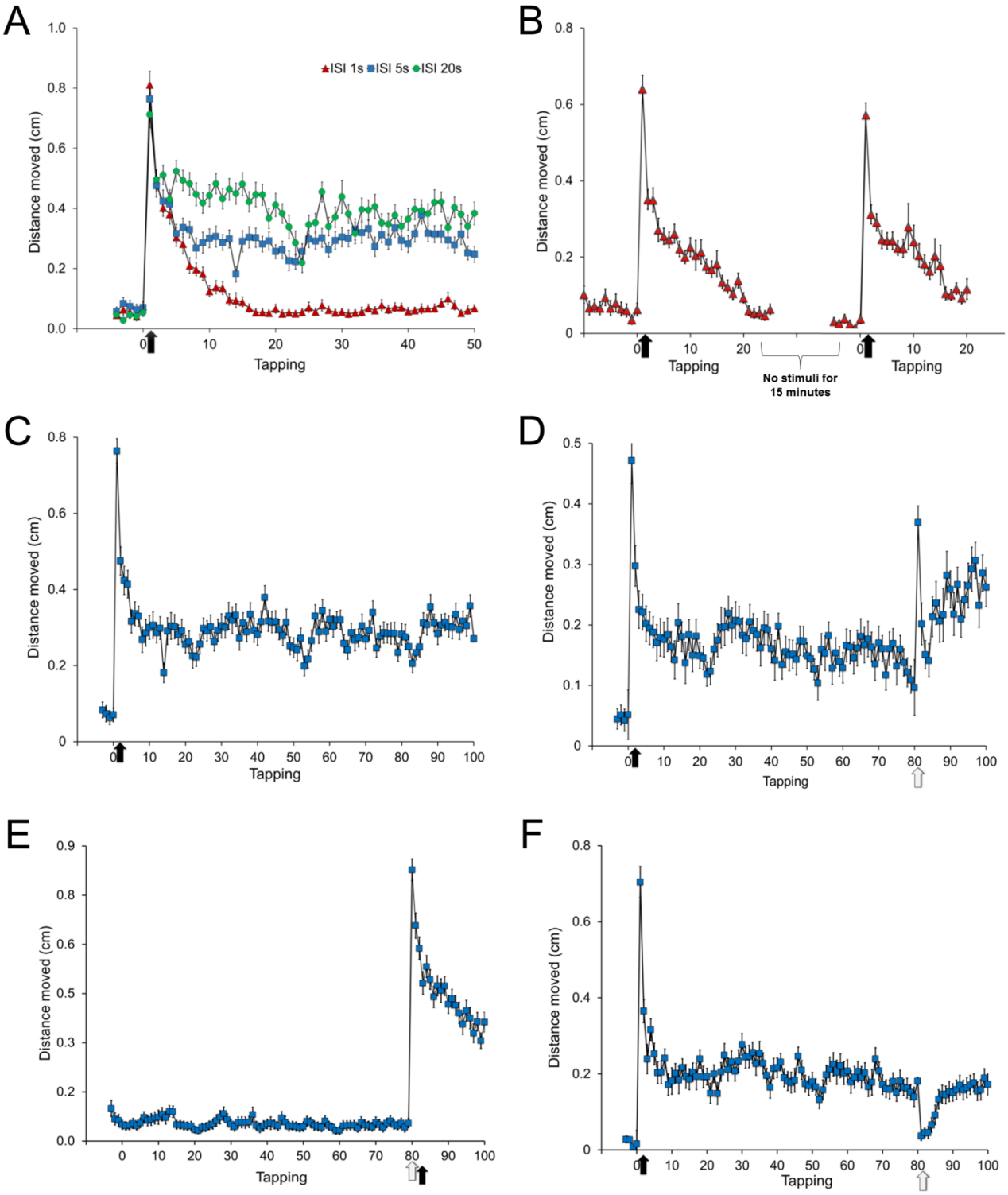

A
ISI 1s ISI 5s ISI 20s
Distance moved (cm)
Tapping
B
Distance moved (cm)
Tapping
No stimuli for
15 minutes
Tapping
C
Distance moved (cm)
Tapping
D
Distance moved (cm)
Tapping
E
Distance moved (cm)
Tapping
F
Distance moved (cm)
Tapping

Figure 2.

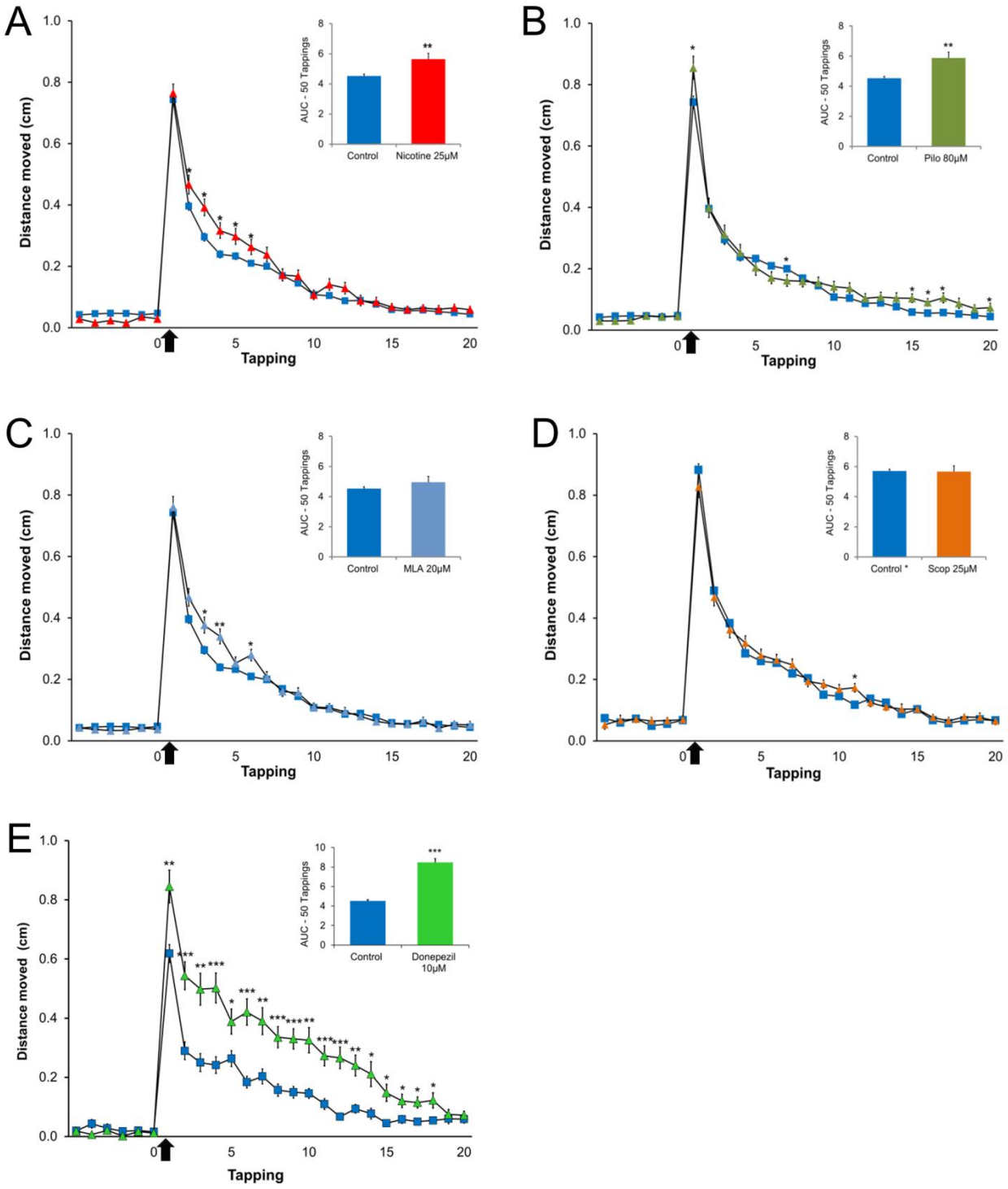

A
Distance moved (cm)
Tapping
AUC - 50 Tappings
Control
Nicotine 25μM
B
Pilo 80μM
C
MLA 20μM
D
Control *
Scop 25μM
E
Donepezil 10μM

Figure 3.

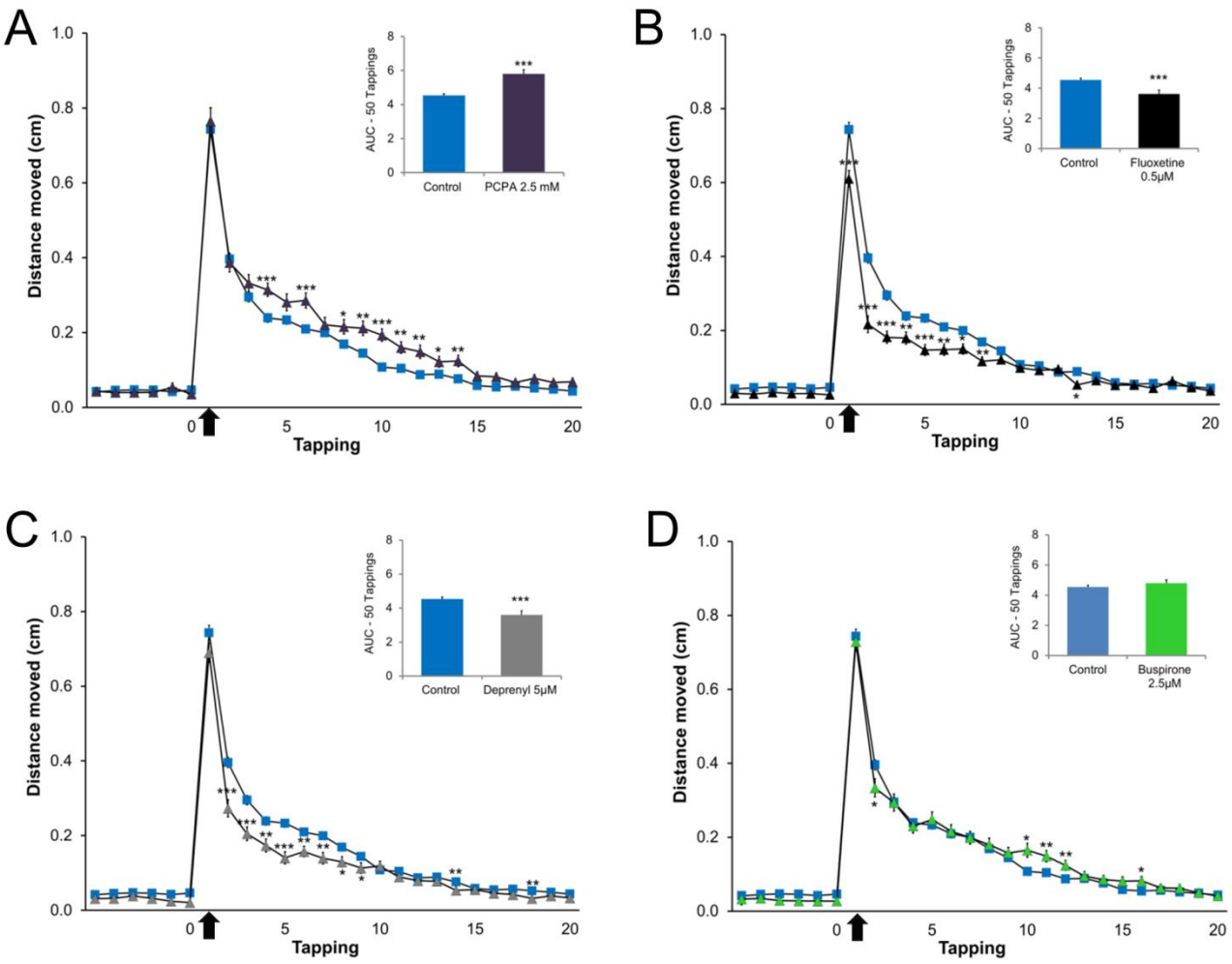

A
B
C
D
Distance moved (cm)
Tapping
AUC - 50 Tappings
Control
PCPA 2.5 mM
Fluoxetine 0.5μM
Deprenyl 5μM
Buspirone 2.5μM

Figure 4.

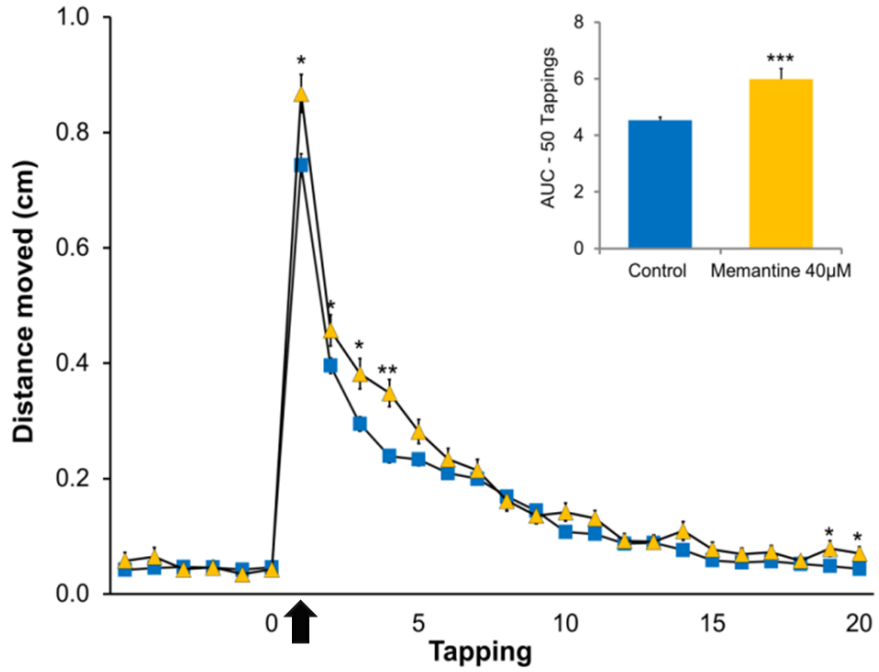

Distance moved (cm)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Tapping
*
*
*
**
*
*
AUC - 50 Tappings
8
6
4
2
0
***
Control
Memantine 40μM

Figure 5.

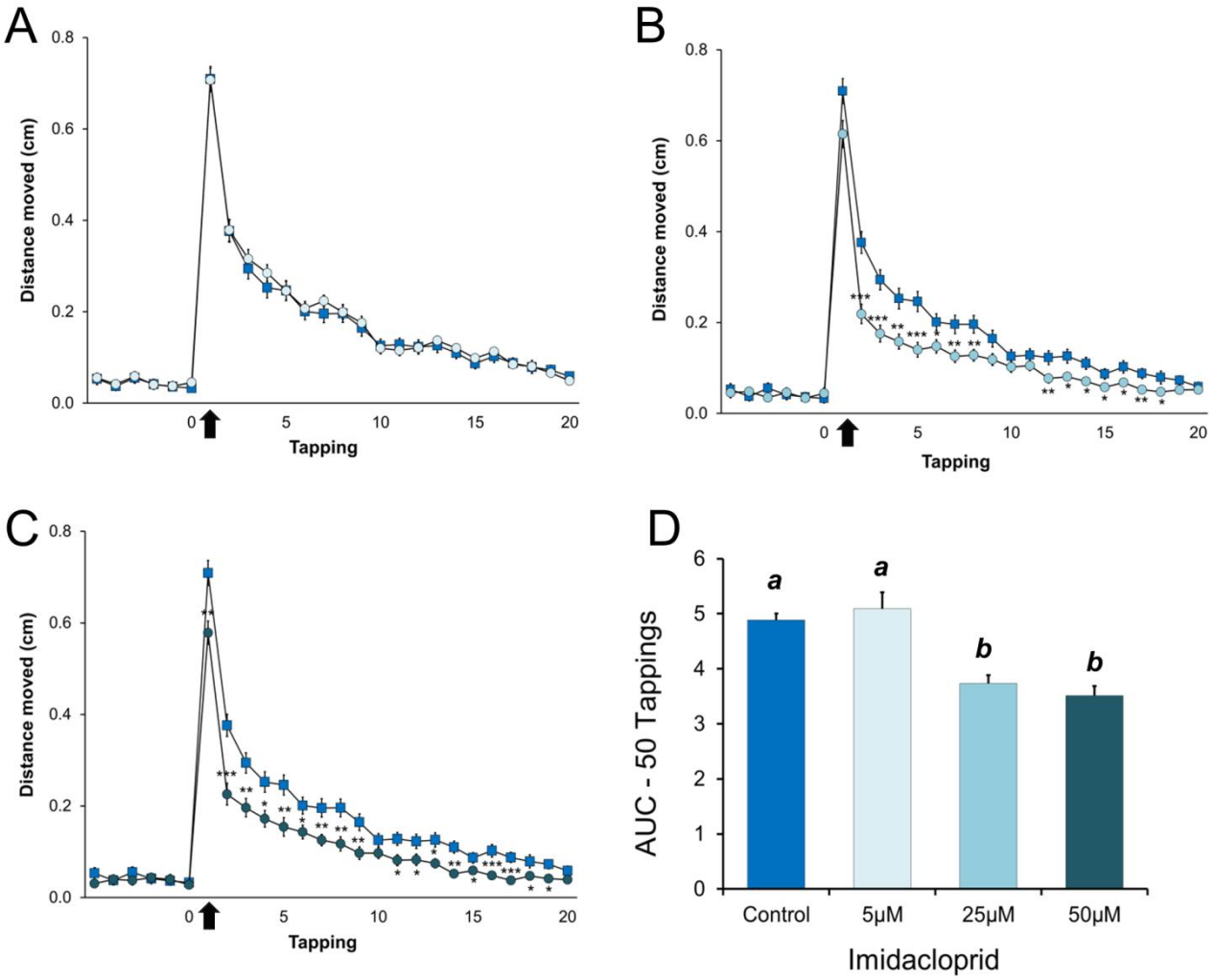

A
B
C
D
Distance moved (cm)
Tapping
AUC - 50 Tappings
Imidacloprid
Control
5μM
25μM
50μM
a
a
b
b

Figure 6.

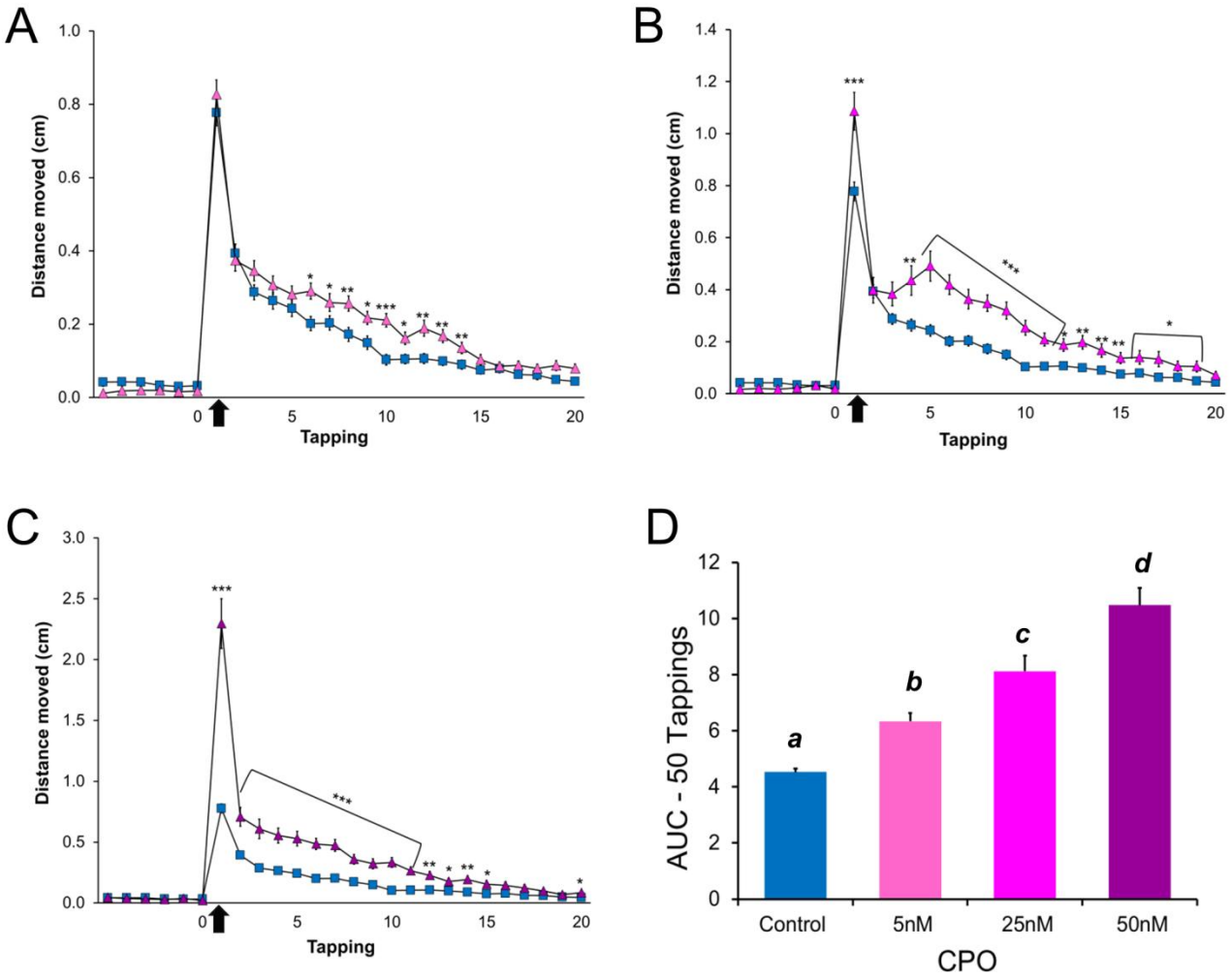

A
Distance moved (cm)
Tapping
B
Distance moved (cm)
Tapping
C
Distance moved (cm)
Tapping
D
AUC - 50 Tappings
a
b
c
d
Control
5nM
25nM
50nM
CPO

Figure 1

Click here to download high resolution image

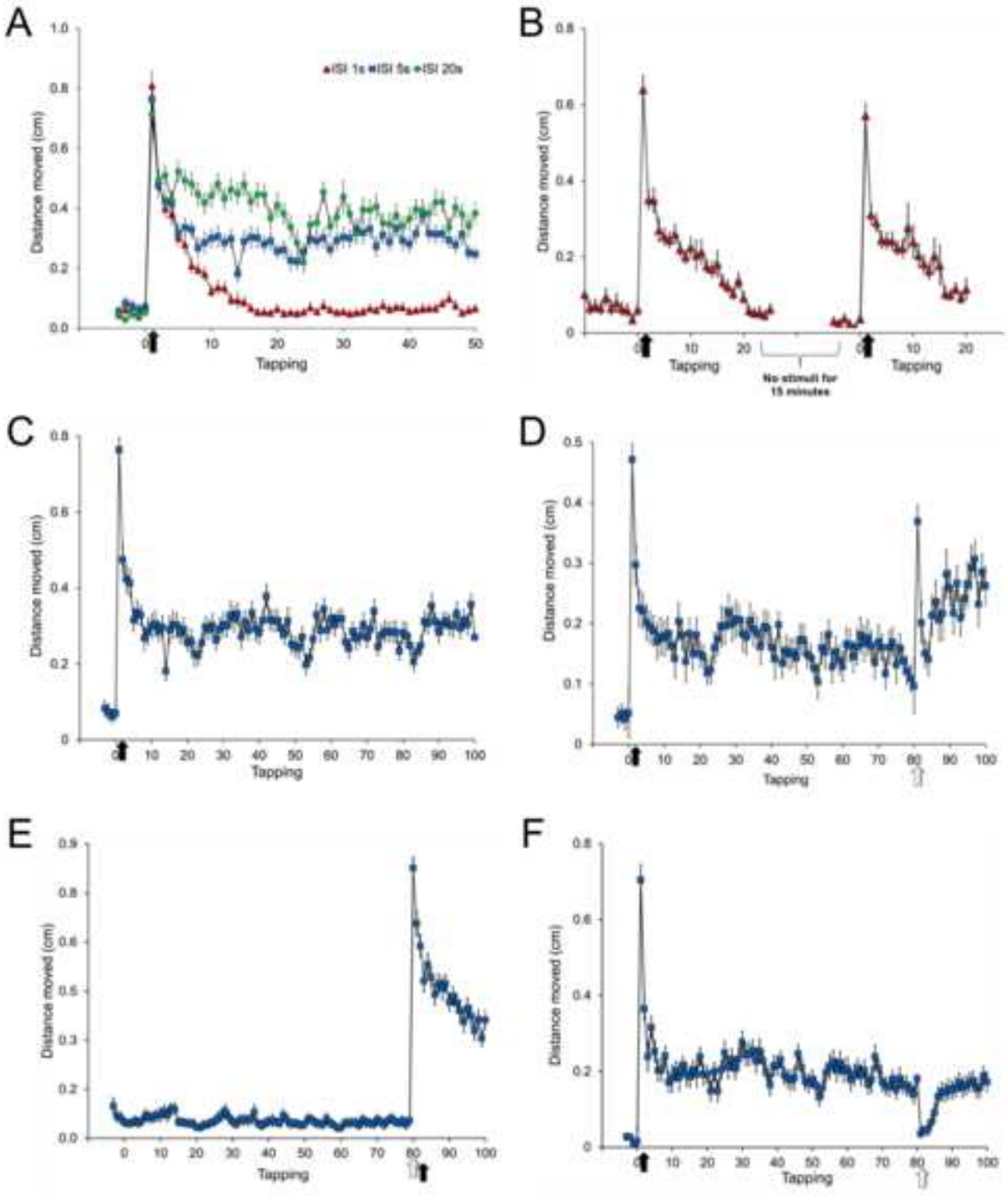


Figure 2

Click here to download high resolution image

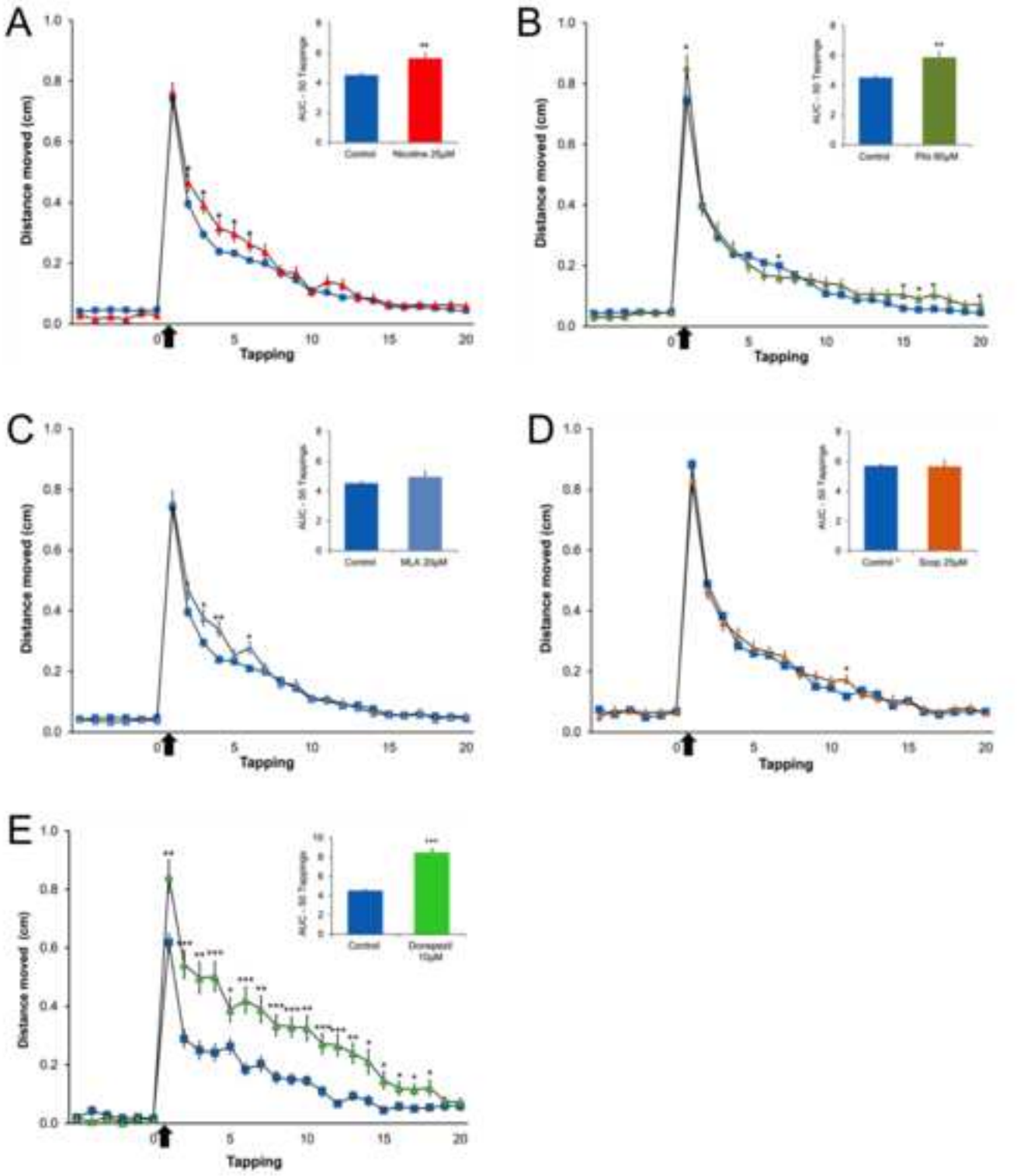


Figure 3

Click here to download high resolution image

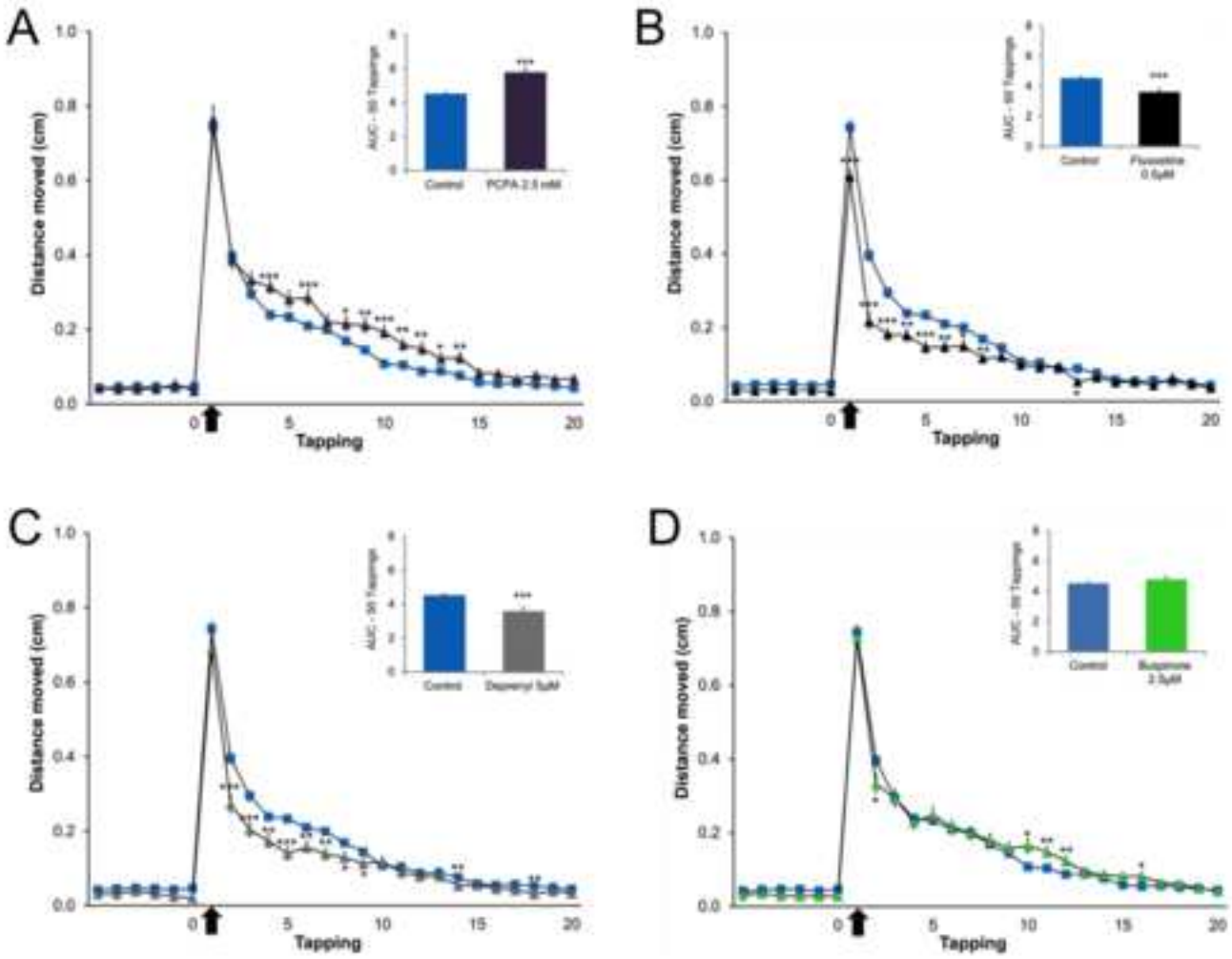


Figure 4

Click here to download high resolution image

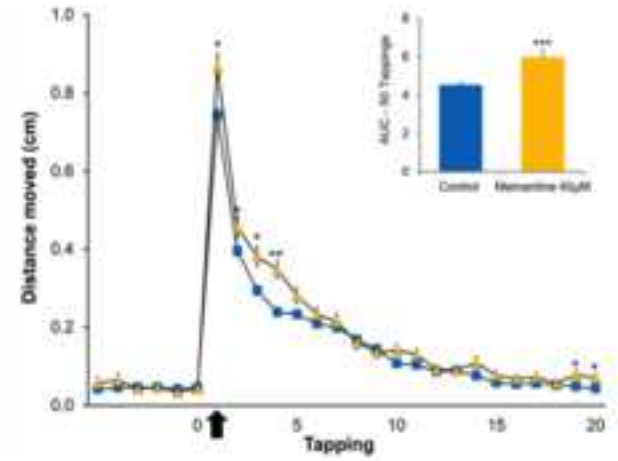


Figure 5

Click here to download high resolution image

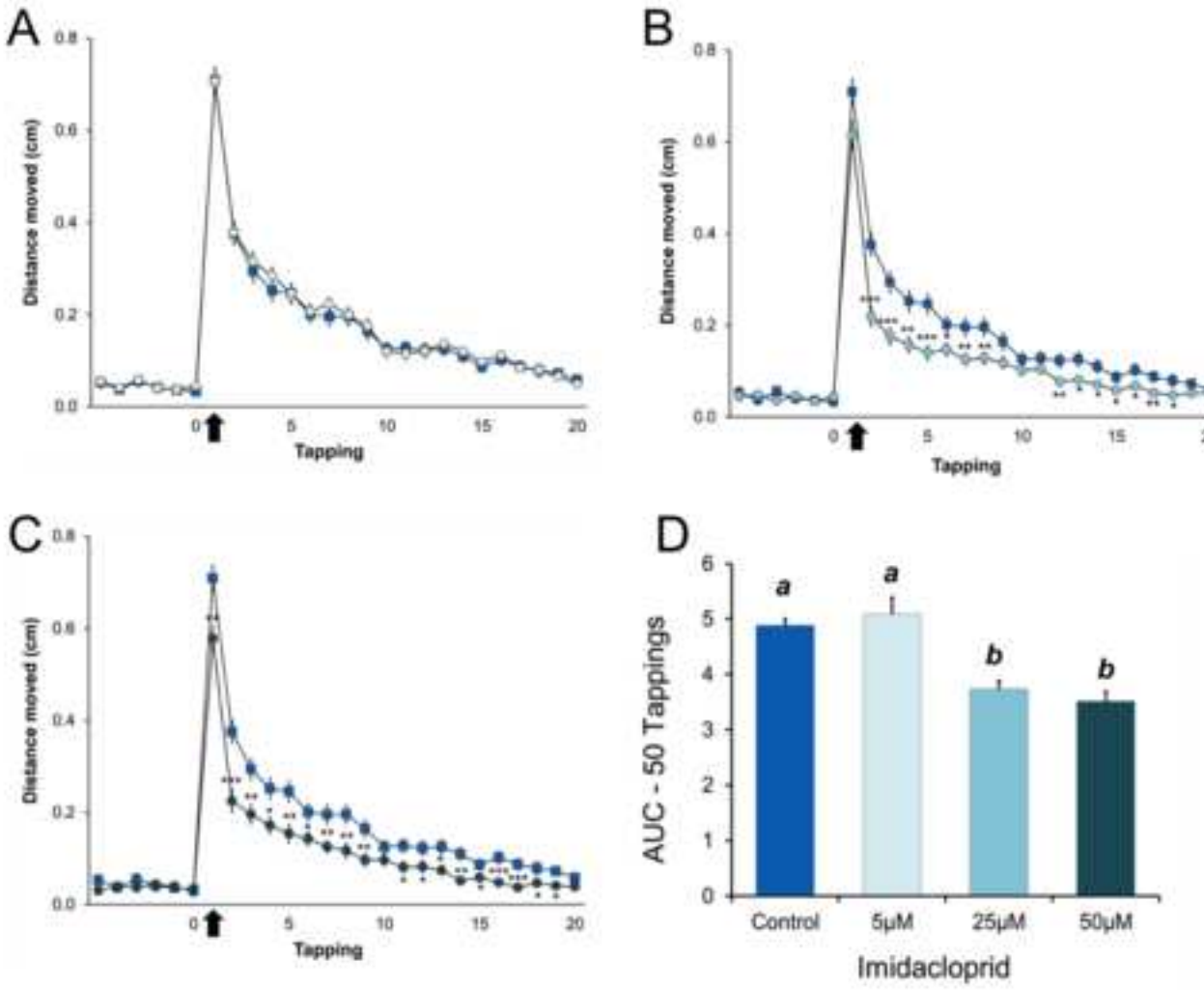
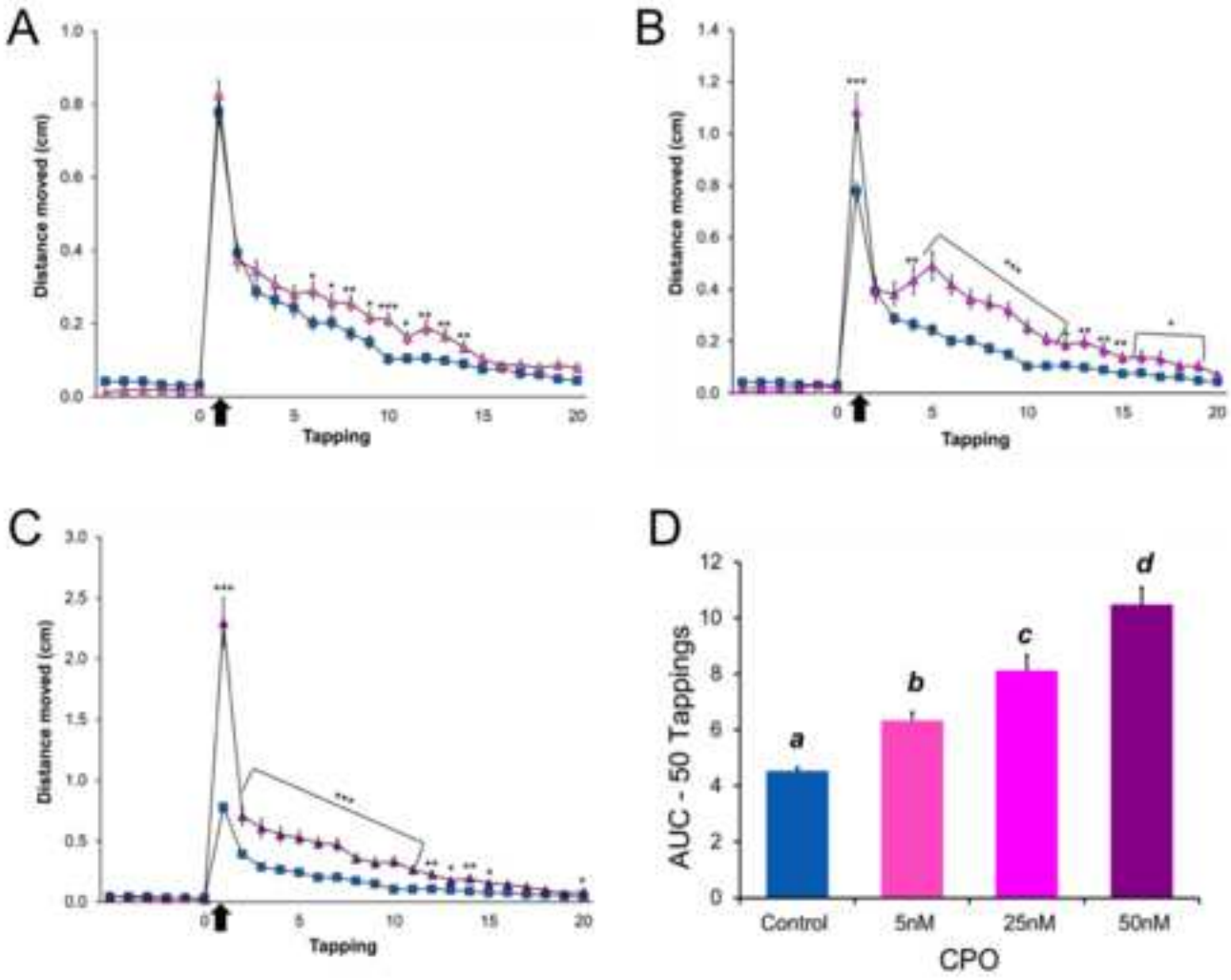


Figure 6

Click here to download high resolution image



Supplementary material for on-line publication only

Click here to download Supplementary material for on-line publication only: Supplementary material_R.docx

Video 1 of supplementary material

Click here to download Video: Video 1.mp4