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Evaluation of the effects of acetylcholinesterase inhibitors in the zebrafish touch-evoked response: quantitative vs. qualitative assessment

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Abstract

Background: The difficulty of finding new treatments for neurological diseases with great impact in our society like Alzheimer's disease can be ascribed in part to the complexity of the nervous system and the lack of quick and cost-effective screening tools. Such tools could not only help to identify potential novel treatments, but could also be used to test environmental contaminants for their potential to cause neurotoxicity. It has been estimated that 5–10% of the anthropogenic chemicals are developmental neurotoxic (DNT) and exposure to DNT compounds has been linked to several neurological diseases. Within this study we were testing the applicability of a quick and cost-effective behavioural test using zebrafish embryos: the touch-evoked response assay, in this case, an assay evaluating the swimming response to a tap in the tail. Two acetylcholinesterase (AChE) inhibitors positive controls (paraoxon and huprine Y), as well as 10 huprine-derivative compounds were tested and the results were evaluated using 2 different methods, a quantitative and a qualitative one.

Results: We could show that the methodology presented is able to detect behavioural effects of AChE inhibitors. A good correlation between the results obtained with the quantitative and the qualitative method was obtained ($R^2 = 0.84$).

Conclusions: Our proposed method enables combination of screening for new drugs with toxicity screening in a whole embryo model alternative to animal experimentation, thereby merging 2 drug development steps into one.

Keywords: Behaviour, Neuromotor, Developmental neurotoxicity, Alternative organism, Methodology, Zebrafish, Drug screening

Background

The zebrafish (*Danio rerio*) is a very promising model to study the effects of compounds at a neurological level, either in the neuropharmacological or in the neurotoxicological field. This is because it allows the study of mechanisms of action and the assessment of morphological effects, as well as the evaluation of functional effects like behavioural changes representing the pharmacological effect or the adverse outcome of the test compounds [1–4]. Zebrafish embryos and larvae display several behavioural patterns correlating very well with those of mammals, from a mechanistic point of view [5, 6]. For instance, the different neuron types and neurotransmitters are well conserved between zebrafish and humans [5, 7]. According to the animal welfare law in most regions, zebrafish are considered an alternative organism model

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to animal experimentation up to 120 h post-fertilization (hpf) [8, 9]. For this reason and because of the possibility to obtain a large number of embryos at a low cost and in a short period of time, zebrafish represents a valuable tool for screening purposes. Besides, behavioural changes in zebrafish embryos and larvae are in general easy to detect, therefore, the number of behavioural assays performed in zebrafish up to 120 hpf has been increasing in the last years [5, 10].

Although many different behavioural tests in zebrafish have been proposed, there is still no harmonization on the experimental parameters to perform them or on how to evaluate them, which hinders inter-laboratory comparison of results [5, 11]. One of the proposed tests, which is quite simple but very informative, is the touch-evoked response (TER) assay. The assay evaluates the response to a tactile stimulus, the TER, which is defined as "A large body angular acceleration and displacement in response to a startling stimulus" [12]. To perform this assay, a correct neuromuscular function of the embryos is needed. It has been proved that touch-evoked motility is severely impaired in acetylcholinesterase (AChE) mutant embryos whose AChE activity is completely abolished [13], as well as in embryos exposed to cholinergic blockers like α-bungarotoxin or d-tubocurarine, which suppress neuromuscular transmission in the zebrafish [14], but so far, it has not been used to evaluate the behavioural effects of new synthetic AChE inhibitors with potential pharmacological activity. As AChE is the enzyme responsible for finalizing cholinergic activity in the synaptic cleft, AChE inhibition leads to a synaptic accumulation of the neurotransmitter acetylcholine (ACh) and results in an increased stimulation of cholinergic neurons. This mechanism of action has been related to different pharmacological and toxicological effects: it is a desired effect in new potential treatments for Alzheimer's disease because it is related with memory and learning processes or in new potential treatments for myasthenia gravis because of its functional effects at the neuromuscular junction. Unfortunately, a high percentage of irreversibly inhibited molecules of AChE leads to numerous toxic effects, like those displayed by organophosphates, including among others, seizures and prolonged muscular contractions [15]. Thus, AChE inhibitors can be medical drugs or toxic compounds depending on their potency, their therapeutic index and the dose of exposure.

To evaluate the effects of AChE inhibitors in the TER assay in zebrafish embryos, it is important to perform the test at an adequate time-point. The onset of the touch-response occurs at 21 hpf but at this time it consists of only fast coilings, while at 26 hpf, the response is "a clear forward movement of the embryo by at least one body length" [14]. As mentioned before, there is no consensus on how to perform or how to evaluate this assay and some authors perform it at 48, 72, 96 or 120 hpf [16–18]. Besides, some perform it touching the head and some touching the tail of the embryo, and some evaluate the response by using a pattern of concentric rings under the plate [16], by giving a 1/0 score (response/no response) [18] or by video-taping and measuring the exact swimming distance [19].

In this study, our aim is to propose a specific experimental protocol that allows the observation and quantification of the TER assay after developmental exposure to AChE inhibitors and to compare 2 evaluation methods, a quantitative and a qualitative one (Fig. 1). We expose zebrafish embryos to paraoxon-methyl (paraoxon), a classical irreversible AChE inhibitor reference organophosphate [20], to the highly potent reversible AChE inhibitor huprine Y ((±)-12-amino-3-chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinoline), and to 10 newly synthesized huprine-based compounds with potent AChE inhibitory activity, which are drug candidates for the treatment of Alzheimer's disease (see the general chemical structure and some molecular properties of the tested compounds in Additional file 1: Figure S1 and Table S1). Our analysis shows that the qualitative method is as valid as the quantitative method to detect the effects in the zebrafish TER assay, while having the advantage that it saves evaluation time. Based on our results, we provide recommendations which will help to improve the use of this behavioural assay in zebrafish embryos for compounds with AChE inhibitory activity, and will facilitate future inter-laboratory comparison of results.

Materials and methods

Zebrafish maintenance and egg production

Adult zebrafish (*Danio rerio*) were obtained from a pet distributor (PET strain; BCN Piscicultura Iberica; Terrassa, Spain) were maintained in 2 different tanks with a closed flow-through system in water ISO 7346-3 (ISO, 1996; 2 mM $CaCl_2{\cdot}2H_2O$ [CAS: 10035-04-8; purity ≥ 99%; Fisher Scientific]; 0.5 mM $MgSO_4 \cdot 7H_2O$ [CAS:10034–99-8; purity ≥ 99.5%; Fisher Scientific]; 0.75 mM $NaHCO_3$ [CAS: 144–55-8; purity ≥ 99.7%; Fisher BioReagents]; 0.07 mM KCl [CAS: 7447–40-7; purity ≥ 99%; SigmaUltra]). Fish were kept in a room with controlled temperature (26 ± 1 °C) and photoperiod (14 h light and 10 h dark). Water temperature was maintained between 26.5 and 28 °C and water parameters (temperature, pH, nitrates and nitrites) were controlled twice a week. Water parameters were maintained at: pH = 7.4–8, NO_3^-: < 10 mg/L, NO_2^- and NH_4^+ < 1 µg/L. Fish were fed twice a day, in the morning with brine shrimp and in the afternoon with dry flake food.

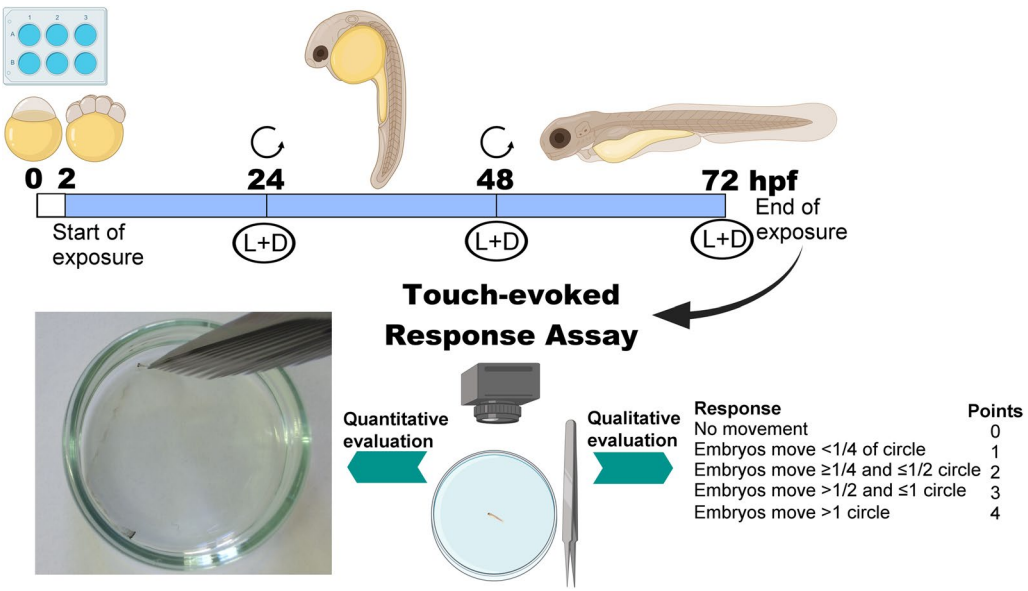

Fig. 1 Graphical summary of the methodological procedure. Fertilized eggs were exposed at 2 hpf and incubated in a 6-well plate. At 24 and 48 hpf the medium was renewed, and at 24, 48 and 72 hpf lethality and dysmorphogenesis (L + D) were evaluated. At 72 hpf the touch-evoked response assay was performed and the results were evaluated either quantitatively or qualitatively. Icons of zebrafish embryos and culture plates/dishes are created with BioRender.com

The day before the test, adult females and males (1:1; approximately 40 fish in total) were transferred into breeding tanks with marbles and artificial plants to induce spawning. Thirty minutes after lighting fertilization took place, and the eggs were collected and cleaned with water ISO 7346-3 diluted 1:5.

Test compounds

Paraoxon-methyl (CAS: 950-35-6) was purchased from Sigma-Aldrich. Huprine Y (CAS: 206259-88-3) and the new huprine derivatives 1–10 were synthesized at the Laboratory of Medicinal Chemistry of the Faculty of Pharmacy and Food Sciences of the University of Barcelona.

Zebrafish embryotoxicity assay

A schematic representation of the assay is depicted in Fig. 1. Test substances were diluted daily in Danieau's solution 0.3 × [21] with DMSO 1% (Sigma-Aldrich; ≥ 99.5%). Additional file 1: Figure S2 provides a comparison of controls with 1% and without DMSO, proving no significant differences in distance swam among the 2 groups ($p = 0.867$), in agreement with previous descriptions [22, 23]. However, with this DMSO percentage, it cannot be excluded that DMSO modifies the permeability of the compounds through the chorion [24]. Fertilized eggs with synchronous cell divisions were selected under a stereomicroscope and randomly distributed into 6-well plates (Thermo Fisher Scientific; 10 embryos/well; layout one compound per plate) with 5 mL of Danieau's 0.3 × with DMSO 1% for the solvent control or 5 mL of the freshly prepared test solution. Plates were incubated at 26 ± 1 °C.

Every 24 h mortality and dysmorphogenesis were recorded and the medium was renewed with 5 mL of freshly prepared solutions (semi-static procedure). A maximum of 10% mortality in the control group was considered as validity criteria for the embryotoxicity test experiments. At the end of the test, the dysmorphogeneses observed were categorized into 2 groups: structural dysmorphogeneses (including those which could interfere with the swimming response, as dysmorphogeneses in the tail, fins, or abnormal flexion of the embryo) and non-structural dysmorphogeneses (including alterations in pigmentation, dysmorphogeneses in the jaw, and oedemas among others) and the mean % of dysmorphogeneses of at least 3 independent experiments was calculated. Two positive control compounds were included in this study: paraoxon, as a classical irreversible AChE inhibitor [20] and the highly potent reversible AChE inhibitor huprine Y due to its structural similarity to the other 10 tested compounds [25, 26].

All huprine-related compounds including huprine Y were tested in the zebrafish embryotoxicity assay at a concentration range starting at 100 μM, and including 4 more concentrations with a dilution factor of 2. Paraoxon

was tested at a single concentration, 10 μM, previously described by our group and in the literature as non-teratogenic [20, 27]. Only for those compounds inducing a mortality percentage equal to 100% in at least 4 out of the 5 tested concentrations, or for those presenting solubility problems, the concentration range was selected with a maximum concentration of 10 μM and dilution factor of 2.

Behavioural evaluation: TER assay

At 72 hpf one embryo was transferred to a glass Petri dish of 2.9 cm of diameter filled with 2 mL of Danieau's solution 0.3 ×. The Petri dish was placed under a video camera and during 1 min the embryo was allowed to acclimatize to the new environment. After this minute, video recording (Casio EX-H30 camera, Japan) was started and at the 10th second the tail of the embryo was touched with the tip of a forceps (FST by Dumont #5, Switzerland). The same procedure was repeated leaving 10 s after each touch, up to a total of 3 touches per embryo. The total length of the video was then 40 s (36 embryos could be recorded in ~1 h). A total of 6 embryos per concentration were evaluated, preferably choosing the ones that already hatched and had no dysmorphogenesis from the 10 embryos/group included in the zebrafish embryotoxicity assay (see previous section). In case not enough embryos hatched, they were dechorionated manually. This test was only performed in concentrations where no precipitation of the compounds occurred during the 72 h assay and the accumulated lethality at 72 hpf was lower than 20%. The evaluation time of 72 hpf was because it allowed longer exposure periods than 48 hpf and to have a broader test range. The whole test was repeated at least 3 times on different days (3 independent experiments); therefore, a minimum of 18 embryos per concentration were tested.

Video analysis

All recorded videos were analysed using two different methods, a quantitative one (results in mm) and a qualitative one (results in points). In order to make a comparison between the 2 methods and to avoid researcher-related biases, the qualitative and quantitative evaluation were always performed by a different researcher.

Quantitative method

Videos were analysed using ImageJ 1.47v (32-bit). Briefly, videos were transformed into stacks. Stacks corresponding to the 10 s after the moment of each touch were projected as a single image (z project function). Then, the experimenter drew a manual line following the track of the zebrafish and measured the length of this line in pixels (see Additional file 1: Table S2 and Figure S3 for a more detailed description). Finally, the value obtained was converted into millimetres. The same procedure was repeated for all touches of the video. A quality criterion was established to consider the experiment as valid: the mean swimming distance of the 6 controls should be at least 20 mm, otherwise, the whole experiment was discarded.

Qualitative method

Videos were visualized using QuickTime Player. Each response to a touch stimulus received a value depending on the estimated swimming distance after the touch. These values were based on the values proposed by [28] and were adapted to: 0 points, the embryo did not move after the stimulus; 1 point, the embryo was swimming less than a quarter of a circle; 2 points, the embryo was swimming between a quarter and half of a circle (both included); 3 points, the embryo was swimming between half and a circle; and 4 points, the embryo was swimming more than one circle (see Table 1). In accordance with the quality criteria established in the quantitative method, the minimum mean score of the 6 controls required to accept an experiment as valid was 0.85.

Statistical analysis

Statistical analysis of the TER assay was performed with GraphPad Prism v.8. Individual (embryo) measurement was the average of 3 responses (distance or qualitative score) evoked by 3 consecutive touching stimuli. The mean of individual measurements for each group of treatment and replication was calculated. Statistical analyses were done comparing the means of 3 independent experiments ($n=3$). As means are normally distributed, parametric tests were applied. Statistical comparisons were made with one-way ANOVA followed by Bonferroni test (applied to analyse the results of huprine Y, and compounds 2, 3, 4, 5 and 9) or 2-tailed *t*-test (applied to analyse the results of paraoxon, and compounds 1, 6, 7, 8, and 10) depending on the number of groups of concentrations (more than two or only two, respectively). A

Table 1 Summary of the qualitative scoring system used in the TER assay

Points	Movement
0	No movement
1	< 1/4 circle
2	≥ 1/4 to ≤ 1/2 circle
3	> 1/2 to ≤ 1 circle
4	> 1 circle

p value $p \leq 0.05$ was established as threshold for statistical significance.

Human AChE inhibition assay

The inhibitory activity of the selected compounds against human recombinant AChE (Sigma, Milan, Italy) was evaluated spectrophotometrically by the method of Ellman et al. [29]. The stock solutions of the enzyme were prepared by dissolution of the enzyme lyophilized powder in 0.1% Triton X-100/0.1 M potassium phosphate, pH 8.0, and the stock solutions of the compounds (1 mM) were prepared by dissolution in MeOH. The assay solution contained 340 µM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.02 unit/mL hAChE, and 550 µM acetylthiocholine iodide as the substrate, in 0.1 M potassium phosphate, pH 8.0. Before the addition of the substrate, the assay solutions with and without the compounds were preincubated at 37 °C for 20 min. Blank solutions containing all components but the enzyme were prepared in parallel to correct for non-enzymatic hydrolysis of the substrates. Initial rate assays were performed at 37 °C with a Jasco V-530 double beam spectrophotometer. At least five increasing concentrations of the compounds, which led to 20–80% enzyme inhibition, were tested. IC_{50} values were calculated using Microcal Origin 3.5 software (Microcal Software, Inc.).

Results

Quantitative analysis of the TER assay at 72 hpf allows the detection of specific behavioural effects of AChE inhibitors

Both positive controls induced a significant decrease in the mean swimming distance ($p_{Paraoxon} = 0.0193$; $p_{HuprineY} < 0.0001$; Fig. 2; Quantitative column of graphs). Huprine Y also induced structural dysmorphogenesis at one of the tested concentrations (6.25 µM) but only in 28% of the embryos, while the mean swimming distance of this group showed a significant reduction of 89% from the control ($p < 0.0001$), indicating that the behavioural effect is much larger than the dysmorphogenic one. Besides, the zebrafish embryotoxicity test was performed with 10 embryos per group, while the TER assay was always performed with 6 out of these 10 embryos per group, selecting the ones without dysmorphogenesis when possible (see Materials and methods section for more details). Moreover, in the other tested concentration of huprine Y (12.5 µM) the percentage of dysmorphogenesis was 0 and the swimming distance was also significantly reduced ($p < 0.0001$). In this case, a significant reduction of 94% from the control value was observed.

All tested compounds structurally related to huprine Y also induced a significant decrease in swimming distance ($p_1 = 0.0019$; $p_2 < 0.0001$; $p_3 < 0.0001$; $p_4 = 0.0161$; $p_5 < 0.0001$; $p_6 = 0.0084$; $p_7 = 0.0048$; $p_8 = 0.0044$; $p_9 = 0.0012$; $p_{10} = 0.0163$), six of them at concentrations producing less than 10% of structural dysmorphogenesis: compounds 2, 3, 5, 6, 7, 9 (Figs. 3 and 4; Quantitative column of graphs and Additional file 1: Table S3) and in all cases at concentrations lower than the concentration inducing 20% of mortality (Additional file 1: Table S4), showing that this endpoint (touch-evoked swimming distance) is much more sensitive than the detection of morphological or lethal effects. Besides, differences in potency were detected among the 10 test compounds, as four of them induced a significant decrease in swimming distance at concentrations ten times lower than the others: compounds 1, 2, 9, and 10. However, this difference in potency did not correlate with their relative human AChE inhibitory activities (Table 2). Nevertheless, a concentration–effect relationship could be detected in five of the test compounds: compounds 2, 3, 4, 5 and 9, and in the cases of 5 and 9, in concentration groups with less than 10% dysmorphogenesis. These results indicate that the assay is useful to detect behavioural effects related with AChE inhibitory activity at lower concentrations than morphological alterations occur, and therefore that the effects observed are not unspecific.

Quantitative and qualitative analysis of the TER assay provide comparable results for all tested compounds

Regarding the methodology used to assess the swimming distance, both analyses, the quantitative in mm and the qualitative using the 4-point scoring system (Table 1), provided similar results for all compounds with only one slight difference for compound 4: the swimming distance in the 12.5 µM concentration was significantly decreased in the quantitative but not in the qualitative analysis ($p_{Quantitative} = 0.0326$; $p_{Qualitative} = 0.0606$). In all other cases, including the positive controls, significant concentrations matched precisely between both analyses, although it is true that the reductions in the mean values of treated groups were in general slightly smaller in the qualitative analysis. For example, following the qualitative analysis, huprine Y 6.25 µM produced a significant reduction in swimming distance of 78% from the control, instead of the 89% given in the quantitative analysis. This indicates that only in those cases where the difference between treated and control embryos is very close to the significance threshold, the effect of the compound could be underestimated.

Besides comparing the quality of the results obtained, it is important to mention that the qualitative analysis is approximately 10 times faster than the quantitative one (see Materials and methods section for more details). Thus, for further experiments, the analysis strategy

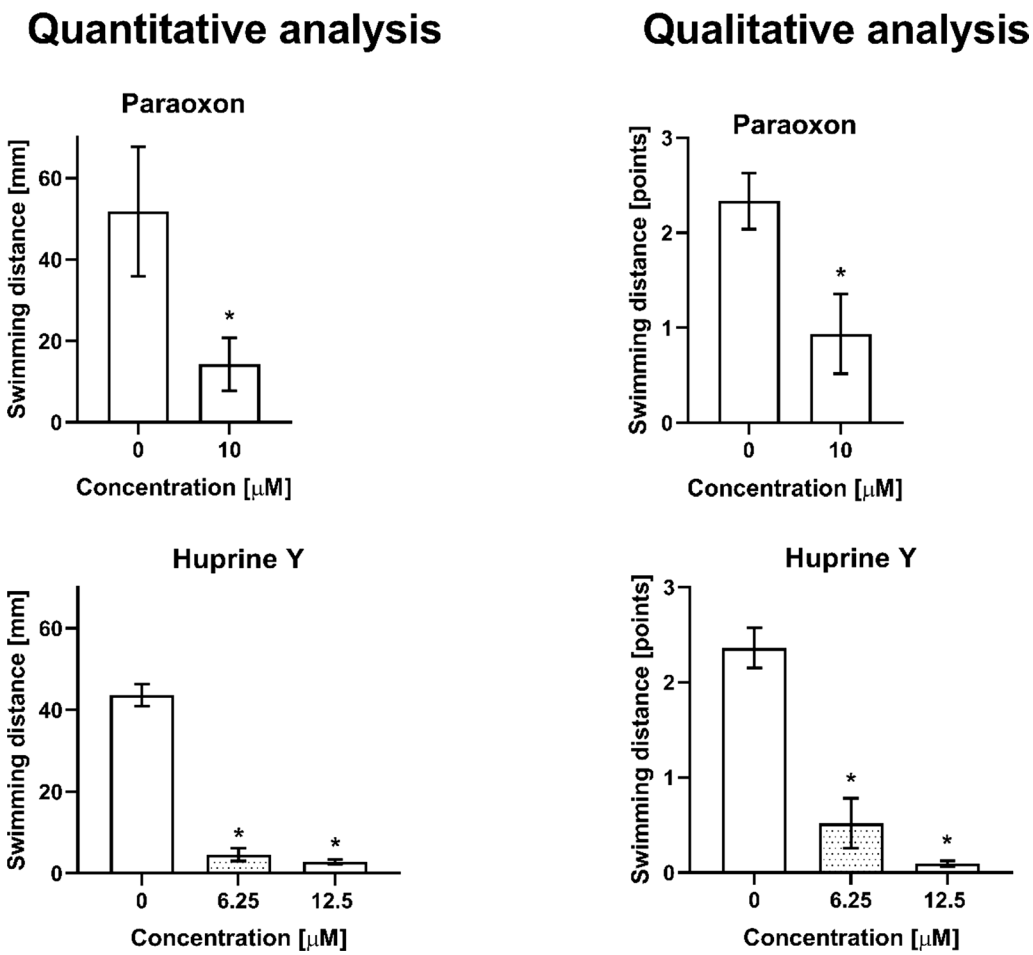

Fig. 2 Evaluation of the TER after exposure to paraoxon and huprine Y. Results are expressed as mean ± standard deviation of swimming distance in mm or points for quantitative or qualitative evaluation, respectively. Each experiment was repeated at least 3 times with 6 embryos/concentration (minimum 18 embryos in 3 independent experiments) and performing 3 tactile stimuli to each embryo. Structural dysmorphogeneses recorded during the experiments are represented as follows: white bars ≤ 10% of embryos with dysmorphogeneses, dotted bars ≥ 11% & ≤ 50% and striped bars > 50%. See Additional file 1: Table S3 for more details. T-test (paraoxon, compounds 1, 6, 7, 8, and 10) or one-way ANOVA followed by Bonferroni test (huprine Y, compounds 2, 3, 4, 5, and 9) were used to statistically compare groups with the control. *Indicates significant differences with the control ($p < 0.05$)

should be chosen depending on the throughput and the precision needed.

Global comparison of the quantitative and qualitative analysis

To determine if the results obtained with both evaluation methods were comparable, not only when looking at the mean of 3 experiments, but also at the level of each individual embryo, the distribution of control values of all experiments was compared ($n = 214$) and a correlation analysis comparing the individual results obtained with both methods in all embryos of this study (controls and treated; total $n = 599$) was performed (Fig. 5).

The distribution of control values was very similar with both methods. With the quantitative method a mean swimming distance of 39 mm ± 8 (SD) was obtained, while with the qualitative method the mean ± SD was 2 ± 0.3. However, with the qualitative method a higher percentage of embryos was accumulated in one score group, as 41% of controls were classified in the 2 to < 2.5 group. The correlation analysis showed a very good correlation between both methods ($R^2 = 0.81$; Fig. 5c). However, as swimming distances above 100 mm received always 4 points (> 1 circle according to Table 1), the mm vs. points ratio was not preserved in the upper range of the controls. For this reason, it was decided to amplify the score system up to 6 points (5: > 1.5 circles; 6: > 2

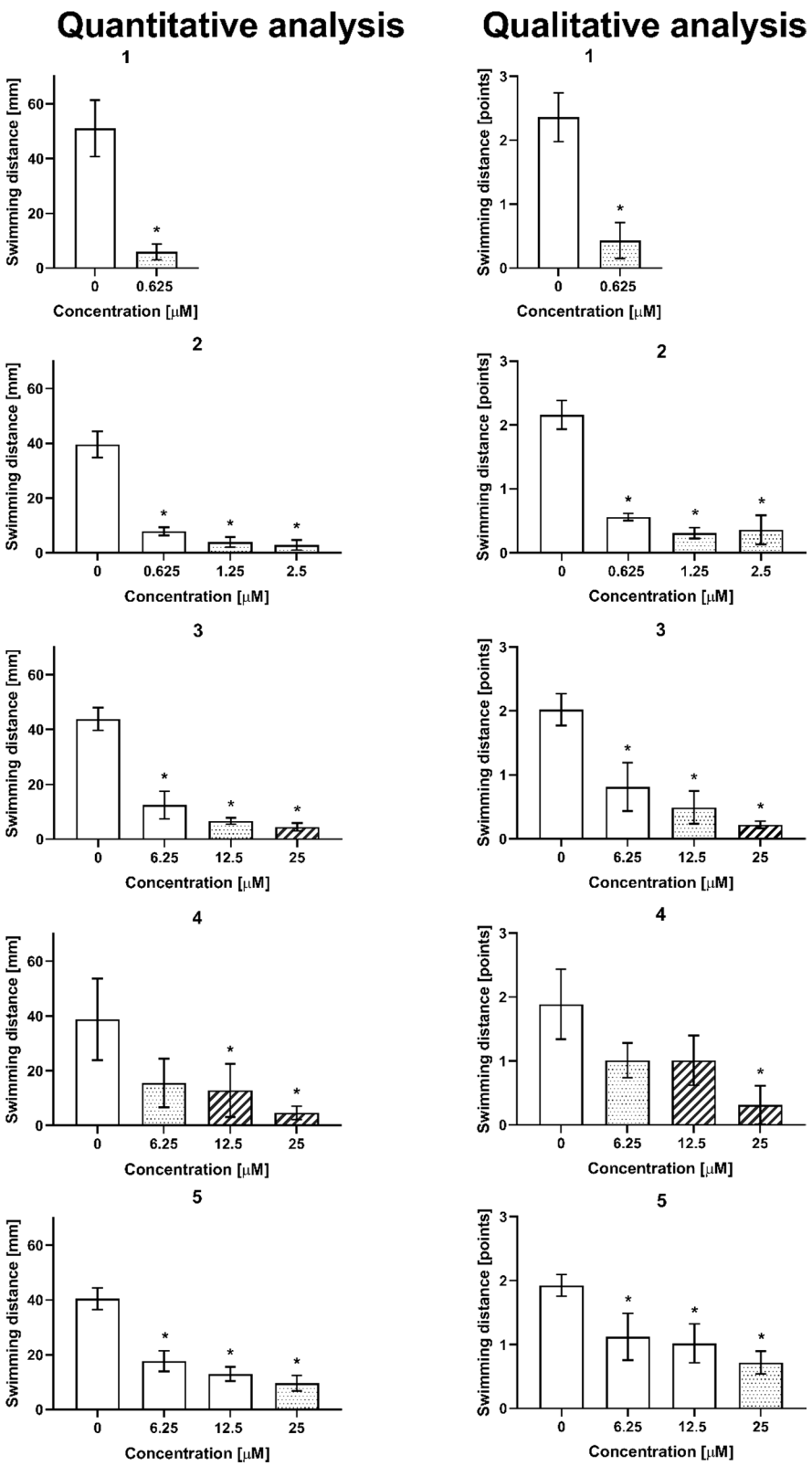

Fig. 3 Evaluation of the TER of embryos exposed to compounds 1, 2, 3, 4 and 5. See Fig. 2 Legend

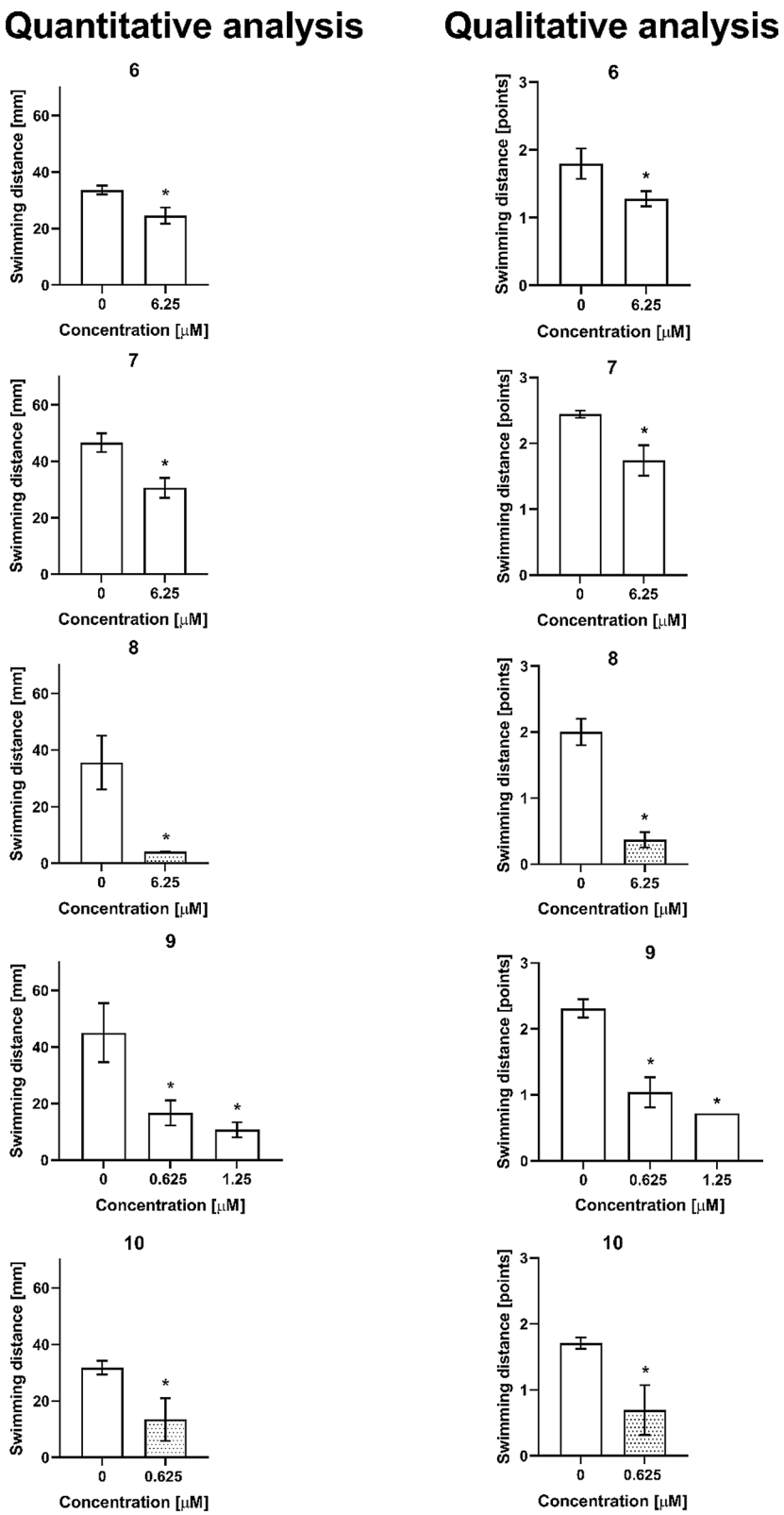

Fig. 4 Evaluation of the TER of embryos exposed to compounds 6, 7, 8, 9 and 10. See Fig. 2 Legend

Table 2 Human AChE (hAChE) inhibitory concentration 50 (IC_{50}) of the tested compounds

Compound	hAChE IC_{50} [nM]
1	4.3 ± 0.4
2	4.8 ± 0.7
3	6.9 ± 0.8
4	11.6 ± 0.9
5	5.9 ± 0.3
6	2.6 ± 0.2
7	16.8 ± 2.2
8	4.9 ± 0.4
9	7.2 ± 0.8
10	2.2 ± 0.5
Huprine Y	1.1 ± 0.1

Paraoxon's IC_{50} cannot be directly determined due to the irreversibility of the inhibition. According to Rosenfeld and Sultatos [30], paraoxon's inhibition constant k_i in human recombinant acetylcholinesterase is 0.125–0.180 $nM^{-1}\ h^{-1}$ (24–37 °C)

circles). The values of all embryos were recalculated with these new scores and the correlation analysis was repeated. With this change, the correlation of both methods was improved ($R^2 = 0.84$; Fig. 5d), but the variability within the controls significantly increased ($SD_{0\text{-}4} = 0.3$; $SD_{0\text{-}6} = 0.73$; $p < 0.0001$; Additional file 1: Figure S4). According to that, we recommend the use of the score system up to 6 points, and we present a comparative table to use it with different dishes/well-plates formats to help to improve inter-laboratory comparability (Table 3).

Discussion

Zebrafish embryos develop very quickly and their behaviour also changes rapidly during the first 120 h of development. Therefore, it is important to select an appropriate time-window to perform a behavioural test. Due to the embryonic development, the TER assay cannot be evaluated before 26 hpf [14]. However, around 26 hpf measurements can be confusing because zebrafish can still present some spontaneous coilings, which start

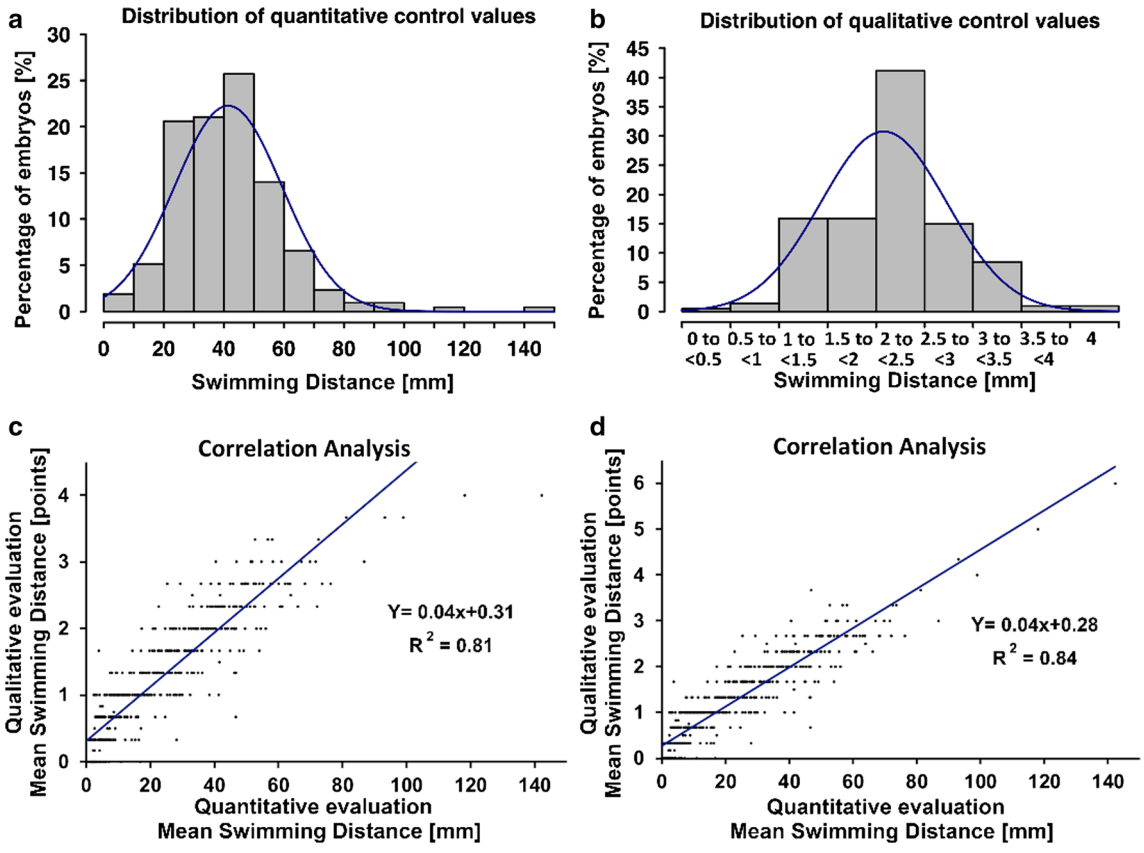

Fig. 5 Global comparison of the quantitative and qualitative evaluations at individual embryo level. **a**, **b** Normal distribution of zebrafish embryos control groups of all compounds tested and evaluated quantitatively (**a**) or qualitatively (**b**). Histograms represent the percentage of embryos whose mean swimming distance of 3 tactile stimuli fits into each interval group. **c**, **d** Correlation analysis between the quantitative and qualitative evaluation using the score 0 to 4 (**c**) or 0 to 6 (**d**) of all embryos corresponding to all concentrations of all tested compounds in this study ($n = 599$). Each dot represents the mean of 3 tactile stimuli of a single embryo

Table 3 Equivalence of qualitative scoring scale for the evaluation of TER in Petri dishes and 24-well plates

Points	mm	Petri dish D = 29 mm (In circles)	24 Well plate D = 15 mm (In circles)
0	0	No movement	No movement
1	< 23	< ¼	< ½
2	23-47	≥ ¼ to ≤ ½	≥ ½ to ≤ 1
3	48-94	> ½ to ≤ 1	> 1 to ≤ 2
4	95-118	> 1 to ≤ 1 + ¼	> 2 to ≤ 2 + ½
5	118- 141	> 1 + ¼ ≤ 1 + ½	> 2 + ½ to ≤ 3
6	142-187	> 1 + ½ ≤ 2	> 3 to ≤ 4

Adaptation of the score system for 24-well plates proposed by Haigis et al. [28] to small Petri dishes of 29 mm of diameter used in this study, and the corresponding millimetre range

at 17 hpf and start decreasing at around 21 hpf at 28 degrees [31]. On the other hand, it has been described that the general motor activity of zebrafish at around 96 hpf may be inherently more variable than at any other age, because at this age they are making the transition from an inactive swim bladder to a fully inflated one [32]. Our own experience agrees well with literature describing that, despite using standardized protocols, control embryos display larger behavioural variability at 96 hpf in this assay [32]. In our case, we observed that it is possible to perform the assay at 96 hpf, but the variability is significantly higher than at 72 hpf either using the 0–4 ($SD_{72hpf}=0.3$; $SD_{96hpf}=0.85$; $p<0.0001$) or the 0–6 scoring ($SD_{72hpf}=0.73$; $SD_{96hpf}=0.96$; $p=0.0014$ Additional file 1: Figure S4). With this information, the optimal time-window to perform the TER assay seems to be approximately between 48 and 72 hpf. As AChE activity increases during development in zebrafish [27, 33], the evaluation time of 72 hpf has been selected to have the opportunity of longer exposure periods and to have a broader dynamic test range.

Our results proved that with this experimental set-up it is possible to detect specific behavioural effects "on-target", which will be useful for testing future pharmaceutical drugs or testing organophosphorus compounds including pesticides or chemical warfare nerve agents. A limitation of the study is that the inclusion of embryos with structural dysmorphogenesis could not be completely avoided in the highest concentrations, but was documented and kept to the minimum possible by choosing 6 out of the 10 embryos in each group. Nevertheless, the effect observed in swimming distance was in all cases clearly larger than the percentage of dysmorphogenesis.

Our results are in good agreement with previous studies testing organophosphorus compounds in zebrafish showing that paraoxon, chlorpyrifos and diazinon exposure decrease overall swimming activity [34, 35] and that paraoxon reduces light-induced startle response. However, touch-response was not measured in these studies, and some of them were performed in larvae older than 120 hpf, which is already a protected life stage by animal welfare regulations [9]. Why organophosphates consistently induce hypoactivity in these cases, and not hyperactivity as they induce in the spontaneous tail-coiling assay performed at 24 hpf, has already been discussed before as a matter of the interaction of exposure duration and concentration, and probably arises from the capability of the spontaneous tail-coiling test to measure basic responses of primary motor neurons instead of secondary ones [11]. Interestingly, Kokel and colleagues used a different behavioural test, the photomotor response assay (PMR), to successfully identify new AChE inhibitors [36]. Among the different behavioural studies performed in zebrafish embryos exposed to organophosphates, the most comparable to our experimental protocol detects a decrease in swimming distance after chlorpyrifos 48 h exposure when the TER is performed at 72 hpf [37]. Therefore, the proposed evaluation day seems to be a good time-point to detect the effect of these compounds also in other laboratories. Besides recommending this time-point, in case several compounds need to be tested at once and high-throughput is needed we recommend the use of the qualitative score system with the 0 to 6 scale, as it correlates better with the quantitative analysis.

It is important to remark that with both scoring systems an embryo that responds to the touch by moving the head or tail but not moving forward, cannot be distinguished from an embryo that doesn't respond at all. A parallel score registering response/no response would be recommended in case the test compound requires to distinguish between both effects, but the use of this score alone is not recommended as there is a considerable loss of information, and only gross effects could be detected.

Although the method proposed has been optimized for the testing of AChE inhibitor compounds as new drug candidates for the treatment of Alzheimer's disease, the method can be easily adapted for ecological use. Considering that AChE inhibition has been identified as one of the most dominant molecular initiating event among

the neurotoxic chemicals detected in samples from 3 European rivers (Danube, Rhine, Mulde/Saale), it is very relevant to have easy methods capable of identifying neurotoxic effects in organisms inhabiting contaminated ecosystems [10]. Our optimization of a non-animal based method to detect neurotoxicity could also help in future strategies to assess eco-neurotoxicity of single new chemicals or real samples containing chemical mixtures. As previously described by Legradi et al. [10], effect-directed analysis using behavioural tests like the one proposed here, could be a powerful tool for the detection of chemicals causing deleterious effects to aquatic organisms.

Conclusions

In summary, based on the principle that AChE is critical for the correct development and function of neuromuscular synapse, we present a methodology that is useful to evaluate the behavioural effects of AChE inhibitors in zebrafish. We propose 2 different ways to assess the outcomes, and we show that they provide comparable results. This way of performing and evaluating the TER assay can have applications in neuropharmacology, for the discovery of new drugs, and in neurotoxicology and eco-neurotoxicology for insecticide hazard assessment, waste water monitoring or environmental samples screening.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12302-020-00421-7.

Additional file 1: Figure S1. Chemical structure of huprine Y and general structure of the huprine derivatives 1-10. **Figure S2.** Evaluation of the TER in controls with (1%) and without DMSO. **Figure S3.** Quantitative evaluation of the touch-evoked response assay. **Figure S4.** Comparison of control qualitative values of the touch-evoked response assay performed at 72 or 96 hpf and evaluated using the 0–4 or 0–6 scoring system (n_{72hpf}=214; n_{96hpf}=90). **Table S1.** Calculated molecular properties of the tested compounds[a]. **Table S2.** Detailed description of the procedure followed to quantitatively evaluate the TER in zebrafish embryos using ImageJ. **Table S3.** Structural dysmorphogenesis (in % ±SD) of zebrafish embryos exposed to the tested compounds in the embryotoxicity assay. **Table S4.** Percentage of mortality (±SD) of zebrafish embryos exposed to the tested compounds.

Abbreviations

ACh: Acetylcholine; AChE: Acetylcholinesterase; D: Dysmorphogenesis; DNT: Developmental neurotoxic; hAChE: Human AChE; hpf: Hours post-fertilization; IC_{50}: Inhibitory concentration 50; L: Lethality; Paraoxon: Paraoxon-methyl; SD: Standard deviation; TER: Touch-evoked response.

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Authors' contributions

LGuzman analysed all TER videos for the qualitative response, prepared graphs and contributed to the writing of the manuscript. GB analysed the dysmorphogenesis and lethality data. DL performed the zebrafish embryotoxicity assay and the TER and evaluated the quantitative response. LGonzález performed the zebrafish embryotoxicity assay and the TER and evaluated the quantitative response. CP synthesized the test compounds and provided study material. MBartolini performed the hAChE assay and analysed the results, ACH performed the 96 hpf TER assay and analysed the results, JL participated in the conception and design of the study, and critically reviewed the manuscript, DMT critically reviewed the manuscript, provided study material and provided financial support, JGC participated in the conception and design of the study, critically reviewed the manuscript, contributed to the statistical analysis, and provided financial support. MBarenys participated in the conception and design of the study, analysed the quantitative response of the TER assay, contributed to the statistical analysis, contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Experiments were conducted in accordance with the Ethics Committee for Animal Experimentation of the University of Barcelona (CCEA). The maintenance of an adult zebrafish colony was accepted by the Department of Environmental and Housing of the Generalitat de Catalunya with license number 334/18.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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