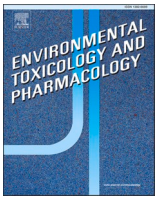
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Sodium valproate effects on the morphological and neurobehavioral phenotype of zebrafish

Giulia Caioni [a,b], Carmine Merola [b], Monia Perugini [b,*], Giovanni Angelozzi [b], Michele Amorena [b], Elisabetta Benedetti [a], Tyrone Lucon-Xiccato [c], Cristiano Bertolucci [c]

[a] Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila 67100, Italy
[b] Department of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Italy
[c] Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

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ABSTRACT

The anticonvulsant sodium valproate (SV) is frequently administered as a medicament but bears several negative effects in case of exposure during development. We analyzed extensively these early development effects of using the zebrafish model. Zebrafish embryos were exposed as eggs to two sublethal concentrations of SV, 10 and 25 mg/L. A general embryo toxicity analysis revealed extended anomalies in the cardiovascular system, and in the craniofacial and the spinal skeleton, as well as high mortality, in the embryos exposed to SV. The teratogenic potential of SV was confirmed in hacthed larvae by morphometric and cartilage profile analysis. Last, neurobehavioral impairments due to SV were highlighted in subjects' activity, anxiety, response to stimulations, habituation learning, and daily synchronization of locomotor activity, overall mirroring typical phenotypes associated with autistic spectrum disorders. In conclusion, our results confirmed the presence of extended and multifaced impacts of exposure to SV during development.

1. Introduction

Antiepileptic medication sodium valproate (SV) is frequently prescribed to treat migraine headaches, bipolar disorders, and epilepsy. However, evidence suggests that it can harm the growing fetus if used during pregnancy, raising concerns over the cost/benefit ratio of its administration (Macfarlane and Greenhalgh, 2018). Prospective and retrospective research has demonstrated that the teratogenic impacts of valproic acid are broad and complex, but can ultimately be ascribed to three principal components: an increase in major morphological abnormalities, a significant intellectual disability, and developmental problems impacting cognitive functions and behavior (Ornoy, 2009). The most common morphological alterations involve the heart, craniofacial and limp skeleton, and urinary tracts (Christensen et al., 2021; Koren et al., 2006; Reynolds and Green, 2020; Wu et al., 2010). Regarding the nervous system, morphological alterations have been reported at the level of the spinal cord, while functional abnormalities are mostly recognizable in terms of cognitive impairments (Bromley et al., 2010; Shallcross et al., 2011). Valproic acid possesses a multi-point action mechanism, including histone deacetylases inhibition, which modulates the gene transcription and has the strongest impact on the fetus (Fan et al., 2020; Pontiki and Hadjipavlou-Litina, 2012) and disruption in the protein synthesis, which has the strongest associations with major congenital malformations (Lloyd, 2013).

This study aims to exploit the advantages of the zebrafish, *Danio rerio*, embryonic model to conduct an exhaustive evaluation of the teratogenic effects induced by SV exposure in terms of morphological profile, behavior, and cognition. Due to its many genetic and developmental parallels to humans, the zebrafish is considered an effective model organism to study SV's impact on development. For instance, in zebrafish embryos, SV determines several developmental anomalies, including reduction of cell proliferation (Lee et al., 2014), aberrant gene expression patterns (Torres-Hernández et al., 2016), and delayed neuronal specification (Dixon et al., 2023). The zebrafish model might also provide additional information on the impact of SV during development. The transparency of zebrafish embryos allows to easily observe the developmental processes and morphological alterations. Furthermore, the zebrafish exhibits complex behaviors and cognitive abilities relevant to human health and disease, such as learning, memory, social attraction, anxiety, and aggression (Levin and Cerutti, 2009), making it

* Correspondence to: Department of Bioscience and Agro-Food and Environmental Technology, University of Teramo, Teramo, Italy.
E-mail address: mperugini@unite.it (M. Perugini).

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an excellent tool to study the drug's impact on behavior.

In the first part of the study, the acute toxicity of SV was determined using Fish Embryo Toxicity tests (FET), focusing on relevant parameters such as morphological anomalies at the level of the circulatory system and skeleton. In the second part of the study, zebrafish embryos were exposed to two concentrations of SV (10 and 25 mg/L) from 0 to 96 hours post fertilization (hpf) to evaluate the effects on larval morphological (e.g., body length, swimming bladder and yolk sac areas, head cartilagins, ocular dimensions) and neurobehavioral phenotype (open-field test, light startle test, habituation test, circadian rytmicity test). The neurobehavioural assays were expected to permit the identification of eventual autism-like alterations (Chen et al., 2018).

2. Materials and methods

2.1. Chemicals

Valproic acid sodium salt (CAS number 1069–66–5, purity ≥ 98 %) was purchased from Merck Life Science, Milano, Italy. Dilution water (DW) was prepared according to OECD TG 203, Annex 2 (OECD, 1992). SV was dissolved in DW obtaining a stock solution of 15 g/L. Formalin 37 %, isopropanol absolute, and ethanol absolute were obtained from Sigma-Aldrich as well as the following chemicals: trypsin (SM-2002-C Sigma-Aldrich), ethanol 99 % (51976 Sigma-Aldrich, purity ≥ 99.8 %), glacial acetic acid (A6283 Sigma-Aldrich, purity ≥ 99 %), hydrogen peroxide (H1009 Sigma-Aldrich), Alcian Blue (A5268 Sigma-Aldrich), and agarose (A9539 Sigma-Aldrich).

2.2. Zebrafish maintenance and egg collection

Zebrafish at early-life stages used in the experiments were obtained from the spawn of AB adult wild-type zebrafish maintened at the University of Teramo facility (protocol number n. 4236). These adult fish were bred in 3.5 L ZebTec tanks (Tecniplast S.p.a., Buguggiate, Italy) in a recirculating water system. The temperature was maintained at 28 °C, the pH at 7 ± 0.2, the conductivity at 500 ± 100 μS/cm, and the dissolved O_2 at 6.1 mg/L. The photoperiod was 14 h light, and 10 h dark, and water chemical parameters were kept as follows: ammonia 0.02 mg/L, nitrite 0.02 mg/L, nitrate 21.3 mg/L. Animals were fed twice a day with live food (*Artemia salina*) and supplemented with ZEBRAFEED (Sparos, Olhão, Portugal).

To obtain the experimental subjects, five groups of females and males (sex ratio: 1:1) were selected as spawners and introduced into 1.7 L sloping breeding tanks (Tecniplast S.p.a., Buguggiate, Italy). Spawners were free from any externally visible diseases. Immediately after spawning, which was initiated by morning light, fertilized eggs were collected with a sieve and rinsed thoroughly with deionized water and DW. Eggs were transferred to Petri dishes with DW and unfertilized eggs or injured embryos were eliminated.

2.3. Experimental design

The first phase included FET tests performed following OECD guideline n°236 (OECD, 2013). The standardization of toxicological investigations produced using FET tests is relevant to study SV toxicity since the experimental procedure, such as the pH range, may alter SV uptake in the zebrafish early life stages. The second phase of this research involved exposing zebrafish embryos to two sublethal amounts of SV (10 mg/L and 25 mg/L) for 96 hpf. In human the adverse effects following chronic exposure, in particular neurotoxicity appeared from the lowest dosage and to confirm this hypothesis in zebrafish we tested these 2 concentrations focusing on the SV developmental effects. After the larvae' hatching, morphometric and etological characteristics were examined. The morphological phenotype was assessed via analysis of the following parameters: body length, swimming bladder and yolk sac areas, head, and ocular dimensions. Furthermore, the presence of head cartilaginous abnormalities was assessed by performing Alcian Blue staining. The neurobehavioral phenotype was analyzed using a battery of four tests. The first one was the open-field test, which measures the behavioral response to a novel environment. It is widely used to quantify activity and anxiety/boldness differences in zebrafish larvae (Ahmad and Richardson, 2013). The second test, the light startle test, measures the immediate response to an unexpected stimulation. The larvae kept in the dark were exposed to a sudden turning on the light, which was expected to cause a decrease in activity (Merola et al., 2021) similar to that observed when the larvae perceive predation cues (Lucon-Xiccato et al., 2020). In the third test, we measured habituation learning abilities (Faria et al., 2019). The zebrafish were exposed to repeated mechanical, innocuous stimulations to record their capacity to reduce the associated response because of habituation learning (Best et al., 2008). Last, we conducted an assay of daily behavioral activity because zebrafish larvae normally show the capacity to entrain their activity to the photoperiod (Di Rosa et al., 2015). Our test recorded this capacity by exposing the fish to a 12 h dark / 12 h light photoperiod.

2.4. Fish embryo acute toxicity tests

For the FET tests the following SV concentrations were tested: 10 mg/L, 25 mg/L, 40 mg/L, 55 mg/L, 70 mg/L, 85 mg/L, and 100 mg/L. Concentrations were chosen based on the results of an earlier range-finding test. Embryos at 1–3 hpf, were examined under a dissecting microscope and inserted individually in the wells of 24-well plates (TPP, Trasadingen, Switzerland) with 2 mL of test solution. The test plates had been pre-incubated (saturated) for at least 24 hours with the test solutions. Following that, self-adhesive foil (SealPlateTM by EXCEL Scientific, Dunn, Asbach, Germany) was used to seal the well plates. Twenty embryos were exposed to seven different concentrations of the substance of interest in each treatment. Test solutions were freshly prepared in dilution water according to annex 2 of OECD TG 203 (OECD, 1992). Before each experiment, the pH of the DW was adjusted to around 7.75.

A negative control (DW) and a positive control (4 % 3,4 dichloroaniline) were also tested. Embryos were incubated for 96 hours at 26 ± 1 °C and photoperiod (14 h light:10 h dark) conditions. The inverted optical microscope (CKX 41, Olympus, Japan) was used to record the four apical observations as markers of lethality: coagulation of fertilized eggs, lack of somite development, lack of separation of the tail-bud from the yolk sac, and absence of heartbeat. FET tests were run in three biological replicates and the percentage of mortality was calculated for each treatment. Percentages were used to establish dose-mortality curves and calculate lethal concentration 10, 20, and 50 (LC 10, 20, 50) values using TOXRAT v. 3.3 software (ToxRat Solutions GmbH, Germany).

According to von Hellfeld et al., in addition to the lethal end-points prescribed by the OECD TG 236, any other observation was recorded as a further lethal or sublethal end-point (von Hellfeld et al., 2020). Examples include decreased heartbeat or blood flow, inhibited or missing pigmentation, delayed or altered development, modified movement(s), spine deformity, and the production of various types of edemata. The developmental period after the exposure did not fall under the regulatory frameworks governing animal experimentation, and all experiments were carried out following EU Regulation 2010/63/EU.

2.5. Morphometric analyses

For each condition, ten larvae at 96 hpf were collected and fixed overnight at 4 °C in phosphate-buffered saline 1X solution (PBS) with 4 % formalin. Following washing with 1X PBS, they were mounted on a microscope slide with 1 % agar solution in bi-distilled water. Specimens were viewed and photos were captured using a stereomicroscope Leica S8 Apo coupled with an EC3 camera (Leica Microsystem, Milano, Italy). Body length (BL), eye length (EL), eye width (EW), inter-ocular distance (IOD), head length (HL), head width (HW), swimming bladder area

(SBA), and yolk sac area (YSA) (Martínez et al., 2019) were all measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. Alcian blue staining

Alcian Blue staining was carried out on 96 hpf larvae as described by Raldùa and colleagues (2008). Larvae were fixed overnight in 4 % formalin at 4 °C; the next day, following multiple washing with PBS/0.1 % Tween 20, they were incubated overnight in 0.1 % Alcian Blue in a solution of ethanol (80 %) and glacial acetic acid (20 %). The samples were then rinsed in 80 % ethanol and gradually rehydrated in PBS before being incubated in 0.05 % trypsin solution for 50 minutes and re-fixed in 4 % formalin for 20 minutes. After numerous washes in PBS, the larvae were bleached in 3 % hydrogen peroxide in 1 % KOH for 30 minutes (Raldúa et al., 2008). The samples were observed using a Leica S8 Apo stereo microscope, and the photos were captured using an EC3 camera (Leica Microsystem, Wetzlar, Germany). All following cartilage analysis (Staal et al., 2018) was performed on ten specimens for each condition using ImageJ software. Meckel's-palatoquadrate (M-PQ) angle, PQ length, ceratohyal (CH) angle, M angle, the distance between the left and right M structures (M-M distance), PQ-PQ distance, M-PQ, and PQ-CH angles are the metrics examined.

2.7. Behavioral tests

2.7.1. Open-field test

We conducted the open-field test and the other behavioral tests in the DanioVision system (Noldus Information Technology, The Netherlands). The system consists of a recording chamber that isolated the subjects from the external environment and a computer running a software (EthoVision; Noldus Information Technology, The Netherlands) that tracked the behavior of the subjects and controlled experimental parameters such the illumination. To perform the open-field test, we collected each subject at the age of 4 dpf from the treatment plate and moved it into a 2 mL round well of a 24-wells culture plate. These wells represented the novel environment, i.e., the open-field arena, for the fish. After placing the subjects in the wells, we immediately moved the plate into the DanioVision system and started a pre-set program of the software. The software turned on the illumination system (a LED plate placed above the wells) and tracked the behavior of the subjects for 60 min. We chose this time interval because in prior experiments we found that it was sufficient to observe the various phases of the novelty response of zebrafish larvae, such as initial hyperactivity and a subsequent activity decrease (Merola et al., 2021). The software provided as output the distance moved by the subjects (a proxy of locomotor activity) and a measure of anxiety consisting of the time spent close to the edge of the arena with a 1-body size threshold (thigmotaxis; (Schnörr et al., 2012)). Both these variables were saved in 10-min time bins to include the temporal variability of behavior in our analysis. Each plate contained subjects of the three experimental groups. We tested overall 5 well plates, for a total of 120 subjects equally divided between the three experimental groups (N = 40 subjects per group).

2.7.2. Light startle test

The light startle test was performed immediately after the open-field test, exploiting the DanioVision system. The subjects were the same as the open-field test (N = 120 overall; N = 40 per group). We followed a procedure of earlier studies (Merola et al., 2021; Peng et al., 2016). First, the system turned off the LED plate, exposing the subjects to no light for 10 min. Thereafter, the system suddenly turned on the light, for an exposure of 10 min. This dark/light alternation was repeated twice to obtain two measures of startle behavior. The tracking software recorded the distance moved by the subjects as described in the previous experiment. With this variable, we calculated an index of response to the light change for each individual as [(distance moved in the light phase – distance moved in the dark phase) / distance moved in the dark phase]. Because zebrafish larvae on average decrease their activity after being presented with the light, the index was expected to have negative values.

2.7.3. Habituation learning test

The habituation learning test was performed immediately after the light startle test, with the same 120 subjects (N = 40 subjects per experimental group). The EthoVision software activated a solenoid placed at the base of the DanioVision (Tapping Device add-on; Noldus Information Technology, The Netherlands). The solenoid delivered mechanical stimulation to the subjects, which was repeated 50 times with a 1-s interval (Faria et al., 2019; Lucon-Xiccato et al., 2023). The software recorded the activity (distance moved) of the subjects in the second after each stimulation. To analyze habituation learning, we calculated a index that represented the change in response in any given stimulation as compared to the first stimulation: [(distance moved after the first stimulation – distance moved after a given stimulation) / distance moved after the first stimulation]. This resulted in a repeated measures variable with 49 observations per fish. Considering that the typical response to the mechanical stimulation is a sudden activity increase and that habituation learning causes a reduction in the response after repeated stimulation, we expected that the learning index had negative values in case the habituation learning occurred.

2.7.4. Daily behavioural activity

We followed the general procedure exploited in former studies (e.g., Zarantoniello et al., 2020). After completing the aforementioned three behavioral tests, we placed the well plates with the subjects in an incubator (28 °C) overnight. The following day, we collected a subset of 96 subjects (N = 32 per treatment) and we individually moved them into the wells of a 96-wells plate. The well plate was placed in the DanioVision system, where the subjects were exposed to a 12 h light / 12 h dark (LD) photoperiod for approximately 3 days. The light was on between 7:30 and 19:30. On the second day of the experiment, the recording was paused between 14:30–15:00 to allow the experimenter to sobstitute 50 % water in the wells and provide a small amount of food to the fish (Micron, Sera, Heinsberg, Germany). The system recorded the activity of the subjects (distance moved) in 10-min time bins. Because 4 subjects died during the test, the final sample size was as follows: N = 31 control subjects; N = 31 subjects treated with SV 10 mg/L; N = 30 subjects treated with SV 25 mg/L.

2.8. Statistical analysis

A Bayesian technique was used for the statistical analysis of sublethal changes detected during FET tests. The beta distribution was generated to compare the 95 % confidence intervals for variations in the proportion of anomalies and hatching rate in different groups (concentrations and period of exposure). The threshold for significance was set at $p<0.05$.

The results of morphometric analysis and cartilage measures were expressed as the mean SEM. GraphPad Prism 8 software was used for statistical analysis (GraphPad software). The statistical difference between the exposed and control groups was determined using one-way ANOVA. The post-hoc test was Dunnett's test. If the p values were $p<0.05$ the difference was considered statistically significant.

Statistical analyses of behavior data were run in R Studio (https://www.rstudio.com) if not stated otherwise. The main dependent variables of the open-field test (activity and thigmotaxis), the light startle test (response index), and the habituation learning test (learning index) had a repeated measure structure. We therefore analyzed them with repeated measures models run with the 'lme' function of the 'nlme' R package. All these models were fitted with treatment (3 levels: control; SV 10 mg/L; and SV 25 mg/L) as fixed effect and subject ID as random effect (to deal with the repeated measurements). Additionally, each model included the fixed effect of the variable describing the levels of

the repeated measurements (open-field test: 6 10-min time blocks; light startle test: repetition 1 and repetition 2; habituation learning test: index from 1 to 49). In case of significant main effect of treatment in the initial model, we run Tuckey post-hoc tests to compare each level of the factor (i.e., control versus SV 10 mg/L, control versus SV 25 mg/L, and SV 10 mg/L versus SV 25 mg/L). In case of significant interaction between treatment and the repeated measures factor, we run post-hoc models divided for the latter variable to analyze the effect of treatment in each phase of the experiment. To deal with right-skewed distributions, the variable 'distance moved' (activity) was log transformed before the analysis in the open-field test and before computing the indexes. In the daily behavioural activity test, we assessed the presence of significant rhythmicity in each subject using the Cosinor analysis implemented in the function DiscoRhythm 1.2.1 (https://mcarlucci.shinyapps.io/discorhythm/). We then compared the occurrence of individuals with significant daily rhythmicity between the experimental groups using Chi-squared test in R.

3. Results

3.1. Fish embryo acute toxicity tests

The toxicity of SV was calculated at 96 hpf as LC_{10}, LC_{20}, and LC_{50}, and values were shown in Table 1. SV exposure did not increase mortality at 24 and 48 hpf, and zebrafish in early-life stages started to display mortality at 72 hpf (Table 1).

n.d.: not determined

The main lethal end-points detected in exposed early-life stages in both doses were coagulation and the lack of heartbeat. In addition, zebrafish showed noteworthy sublethal abnormalities such as decreased blood flow, pericardial edemata, decreased heartbeat, blood congestion, yolk-sac deformation, craniofacial deformity, lordosis, and scoliosis. The percentage of zebrafish embryos with developmental alterations increased during the observation period (from 48 hpf to 72 hpf and from 72 hpf to 96 hpf) for the SV concentration of 25 mg/L ($p < 0.05$).

Furthermore, zebrafish embryos exposed to the SV highest concentrations (55 mg/L, 70 mg/L, 85 mg/L, and 100 mg/L) showed a 72 hpf hatching rate delay compared to the negative control ($p<0.05$).

3.2. Morphometric measurements

At 96 hpf, morphometric measurements of the following larval phenotypic parameters were taken: BL, SBA, YSA, EL, EW, IOD, HL, and HW (Figs. 1–2). After exposure to both concentrations, effects were seen in the form of an increase in YSA and a decrease in IOD, HW, and HL (Figs. 1C, 2C). SBA, EL, and EW are all significantly reduced at the concentration of 25 mg/L (Figs. 1C, 2C).

3.3. Alcian blue staining

At 96 hpf, cartilage staining revealed some alterations in treated larvae (Fig. 3). At both concentrations, the M-PQ angle and CH-angle dramatically increased while the M-CH distance and PQ-PQ length decreased. At 25 mg/L, the PQ-PQ distance is significantly shortened. ($p<0.005$) (Fig. 3C).

Table 1
Lethal concentration (LC) and confidence intervals (CL) of SV derived from FET tests.

Time (hpf)	LC_{10} (95 % CL) mg/L	LC_{20} (95 % CL) mg/L	LC_{50} (95 % CL) mg/L
24	n.d.	n.d.	n.d.
48	n.d.	n.d.	n.d.
72	66. 3(54.8–74.7)	84.7 (75.2–99.5)	135.2 (111.1–213.9)
96	55.2 (46.3–61.5)	67.3 (60.3–73.4)	98.5 (89.2–115.2)

3.4. Behavioral tests

3.4.1. Open-field test: thigmotaxis

The analysis on thigmotaxis (i.e., the time spent close to the edge of the arena) showed a significant main effect of treatment ($F_{2117} = 4.755$, $P = 0.010$; Fig. 4A) and a significant interaction between treatment and time ($F_{10,585} = 2.749$, $P = 0.003$), but not significant main effect of time ($F_{5585} = 0.564$, $P = 0.728$).

The interaction was due to a significant effect of treatment in the min blocks of the second half of the test (min 1–10, 11–20, and 21–30: all $P > 0.07$; blocks 31–60 min: all $P < 0.008$). Post-hoc tests conducted in the first half of the test, in which the effect of treatment was significant, indicated that subjects exposed to both SV 10 mg/L (min 1–10: $t = 2.979$, $P = 0.010$; min 11–20: $t = 2.580$, $P = 0.030$; min 21–30: $t = 3.017$, $P = 0.009$) and SV 25 mg/L (min 1–10: $t = 2.426$, $P = 0.044$; min 11–20: $t = 3.434$, $P = 0.002$; min 21–30: $t = 3.512$, $P = 0.002$) displayed greater thigmotaxis, compared to control subjects (Fig. 4A). There was no difference in thigmotaxis between the two groups of subjects exposed to SV (all $P > 0.5$).

3.5. Open-field test: activity

The analysis activity revealed a significant main effect of treatment ($F_{2117} = 4.274$, $P = 0.016$; Fig. 4B) and a significant main effect of time ($F_{5585} = 15.996$, $P < 0.001$). Additionally, the interaction between treatment and time was significant ($F_{10,585} = 4.524$, $P < 0.001$; Fig. 4B).

The significant interaction was due to the fact that the effect of treatment was significant from min 20 onwards (min 1–10, and 11–20: all $P >0.4$;, min 21–30, 31–30, 41–50, and 51–60:all $P < 0.009$). A post-hoc analysis on the significant time intervals found that subjects exposed to SV 25 mg/L displayed higher activity compared to control subjects in all the relevant intervals (all $P > 0.007$; Fig. 4B). In two intervals, the activity of subjects exposed to SV 25 mg/L was greater compared to that of subjects exposed to SV 10 mg/L (min 31–40: $t = 32.529$, $P = 0.034$; min 41–50: $t = 2.766$, $P = 0.018$). The distance moved by subjects exposed to SV 10 mg/L was not different from that of the control subjects in any of the time intervals considered (all $P > 0.3$).

3.6. Light startle test

The analysis of the response index of the startle test indicated a significant main effect of treatment ($F_{2117} = 43.666$, $p< 0.001$; Fig. 5A) and a significant main effect of startle repetition ($F_{1117} = 7.200$, $p= 0.008$). There was no significant interaction between these two terms ($F_{2117} = 0.229$, $p= 0.796$). A post-hoc test conducted on the response index found that subjects exposed to SV 25 mg/L displayed lower responsiveness compared to subjects exposed to SV 10 mg/L ($t = 5.639$, $p< 0.001$) and to control subjects ($t = 6.052$, $p< 0.001$; Fig. 5A). There was no significant difference between subjects exposed to SV 10 mg/L and control subjects ($t = 0.413$, $p= 0.910$).

3.7. Habituation learning test: learning index

In the learning index analysis, we found a significant main effect of treatment ($F_{2,65} = 5.419$, $P = 0.007$; Fig. 5B). The effect of the other factor in the model, the repetition, was not significant ($F_{2117} = 2.927$, $P = 0.087$). Critically, there was a significant treatment x repetition interaction ($F_{2117} = 5.427$, $P = 0.004$; Fig. 5B). This indicated that subjects exposed to the SV 25 mg/L treatment displayed a smaller reduction of the response to the startles, and therefore reduced habituation learning, compared to the other groups, and that this effect was mainly visible in the last stimulations of the series (Fig. 5B).

3.8. Daily behavioral activity test

Fig. 6 represents the data of the daily behavioral activity test.

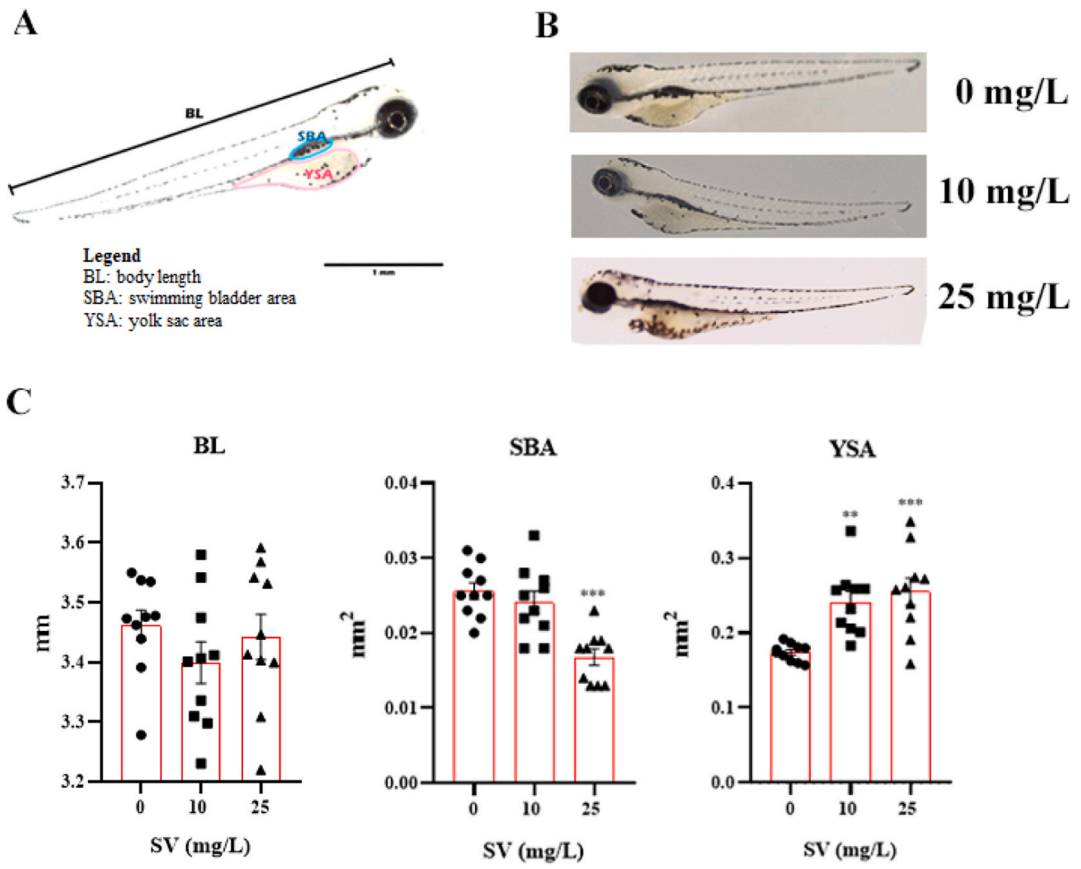


Fig. 1. Morphometric measurements in 96 hpf larvae. A) Lateral view showing body length (BL), swimming bladder area (SBA), yolk sac area (YSA). Scale bar, 1 mm. B) Representative images of larvae in lateral view. Scale bar, 0.2 mm. C) Morphometric parameters (BL, SBA, and YSA) presented by their mean value ± SEM. The symbol * indicates a significant difference between the tested samples and the control (**p < 0.005; ***p < 0.0005).

Graphical inspection suggests that initially, only the control subjects displayed visible behavioral rhythmicity. The analysis of the daily activity as distance moved across the three days of recording was performed individually for each subject. The analysis was divided into two phases, before and after the water change that occurred on day 2, respectively. Results indicated that 27 out of 31 (87.10 %) control subjects exhibited significant daily rhythmicity in phase 1, and all of them did so in phase 2. For the group exposed to SV 10 mg/L, significant rhythmicity was detected for 9 out of 31 subjects (29.03 %) in phase 1 and 28 out of 31 subjects (90.32 %) in phase 2. For the group exposed to SV 25 mg/L, significant rhythmicity was detected for 12 out of 30 subjects (40.00 %) in phase 1 and for 22 out of 30 subjects (73.33 %) in phase 2.

A statistical comparison of the number of subjects with significant rhythmicity between the experimental groups indicated that at the beginning of the test (phase 1), SV at both concentrations reduced entrainment compared to control (Table 2). In the second part of the test (phase 2), only SV 25 mg/L treatment reduced entrainment compared to the control treatment (Table 2).

4. Discussion

We performed a multidisciplinary analysis of SV effects on zebrafish development to detect the presence of the main neurotoxic alterations described in other animal models and humans. First, we performed a FET toxicological test following OECD guidelines (OECD, 2013). The FET test highlighted an increase in mortality in the late stages of development and a delay in hatching, suggesting a time and dose-dependent mortality. The subsequent increase in late-stage mortality can potentially be attributed to the emergence of pronounced alterations incompatible with viability.

Moreover, in the embryonic measurements, we detected several sublethal issues related to the cardiocirculatory system, including decreased blood flow, pericardial edema, decreased heartbeat, and blood congestion. Despite manifesting as nonspecific endpoints, the role of SV in inhibiting the histone-deacetylase activity cannot be disregarded, as it has been implicated in the induction of cardiac malformation, which could be correlated with hatching delay as reported by Wu and colleagues (2010) (Wu et al., 2010). Disruptions in hatching chronology and frequency have been previously documented in the literature by Lee and colleagues (2014) (Lee et al., 2014). This preliminary study allowed us to choose two sublethal concentrations 10 and 25 mg/L for the second part of our study on the embryos and larvae skeleton morphological abnormalities.

Regarding morphological alterations, our results demonstrated an altered shape of the jaw in treated embryos. In detail, the ceratohyal cartilages in the treated specimens had a distinct inclination, and at 25 mg/L, they appeared flatter than control fish. As a result, the palatoquadrates that articulate on the ceratohyal cartilages of the treated subjects were wider and slightly curved. These changes determined the rise in the ceratohyal angle and distance between the palatoquadrates in the treated fish. The articulation between Meckel's cartilage and the palatoquadrates was also less visible, and the palatoquadrates length reduced, as result of SV exposure. Moreover, the events leading to the right jaw conformation necessitate signaling pathways that include the cellular matrix, which has an impact on the end output due to its composition and spatial structure (Reeck and Oxford, 2022). As a result, in addition to the direct effects on chondrocytes, SV may influence the surrounding matrix, as previously reported in investigations on mouse mesenchymal cells (Hatakeyama et al., 2011). Finally, the shortening of

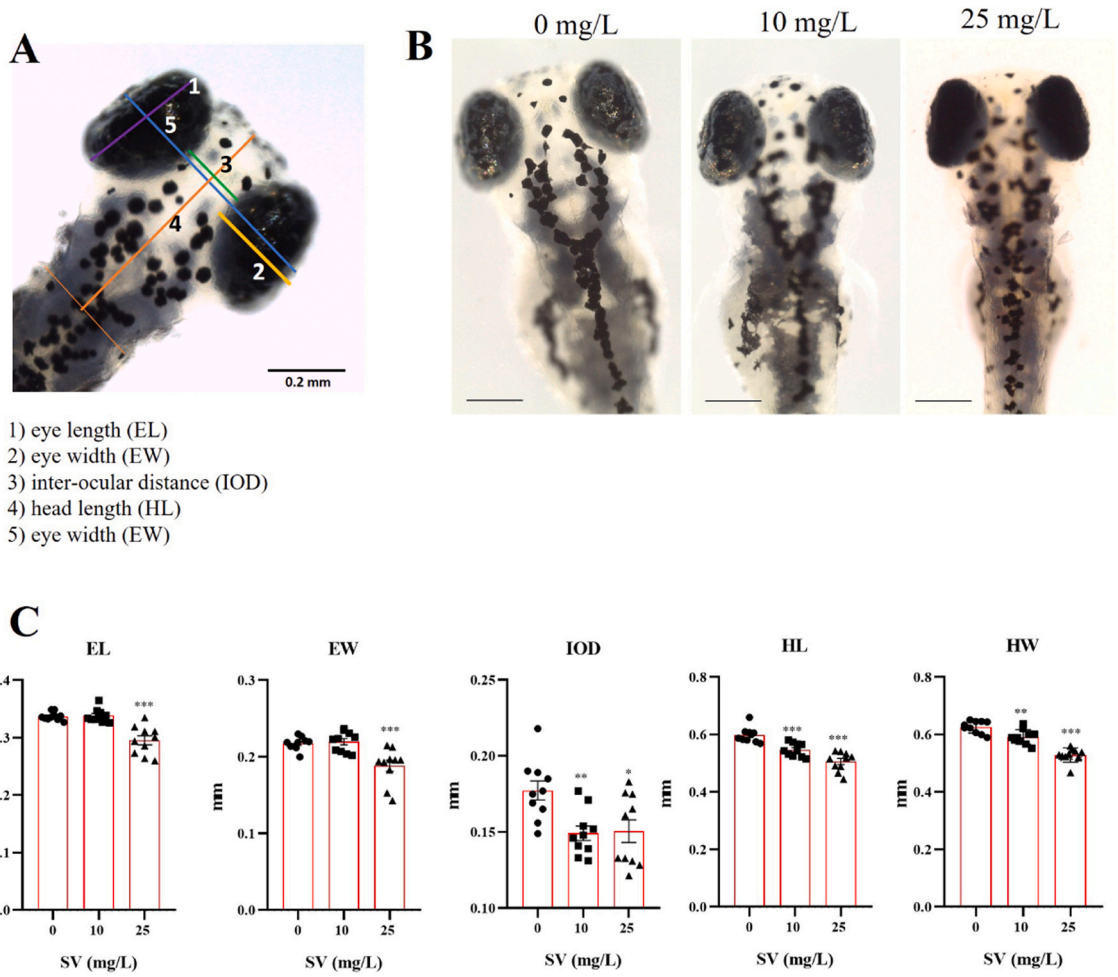


Fig. 2. Morphometric measurements in 96 hpf larvae. A) Dorsal view showing eye length (EL), eye width (EW), inter-ocular distance (IOD), head length (HL), head width (EW), Scale bar, 0.2 mm. B) Representative images of larvae in dorsal view. Scale bar, 0.2 mm. C) Morphometric measures are shown by their mean value ± SEM. The symbol * indicates a significant difference between treated larvae and control (*p<0.05; **p < 0.005; ***p < 0.0005).

the mandibular structures corresponds to the reported small head measurements. Our data on cartilaginous structural abnormalities reflects the research conducted by Gebuijis and colleagues, albeit with different concentrations and exposure methods (Gebuijs et al., 2020).

We also analysed other morphological parameters. At the end of the exposure, we found a reduction in ocular dimensions, an increase in the yolk sac, and a reduction in the swim bladder, which may have a non-canonical morphology due to increased lipid storage. This increase in the YSA measure indicates that SV can impact lipid metabolism. In this situation, too, we find support in the literature. Treatment with SV, for example, is known to promote hepatic steatosis, the causes of which (albeit not fully understood) allude to increased fat uptake in hepatic cells (Grünig et al., 2020). Moreover, as previously reported, SV is able to increase nuclear receptor peroxisome proliferator-activated receptor-gamma (Ezhilarasan and Mani, 2022; Merola et al., 2023) an important transcription factor implicated in lipid storage and metabolism.

We also found a significant decrease in head length and width in treated larvae at both SV concentrations. The reduction in head dimensions stands out among the variety of morphological factors that demand closer examination, but it is possibly related to the observed cartilaginous structural abnormalities. The study of microcephaly in embryonic or larval zebrafish embryos can offer insights into the potential consequences of different conditions. Microcephaly leads to a reduction in brain size, which affects the overall structure and functioning of the brain (Rea and Van Raay, 2020). Zebrafish with microcephaly might exhibit altered behaviors compared to their normal counterparts. Depending on the underlying cause, microcephaly in zebrafish may be associated with other neurological problems, including abnormal neural circuit formation or functioning. Zebrafish research on microcephaly could offer insights into human health, revealing potential genetic factors, molecular pathways, and interventions.

Also motivated by these considerations, the behavioral research conducted enabled an extensive functional characterization of the impact of SV on the development of the nervous system. The open-field test revealed the action of SV in increasing thigmotaxis (an index of anxiety), and activity, but the latter only at the higher concentration. We cannot exclude that the change in activity was somehow linked to the altered morphology of the treated fish, althgou in this case one would expect activity do decrease after the SV treatment due to difficulty to swim. The change in thigmotaxis, a paramter measured as spatial preference, was less likely subjected to this problem. All toghether, these behavioural results might indicate a heightened reaction to the novel environment due to SV exposure, and the findings are consistent with previous research on the consequences of embryological exposure to valproate in zebrafish (Zimmermann et al., 2015). Increased anxiety is also characteristic of rodent models in which the autistic phenotype was induced using valproate (Servadio et al., 2018). For instance, in a test functionally analogous to that used in our zebrafish, VPA-exposed rats spent considerably less time in the open arms of the raised plus maze, indicating higher levels of anxiety (Servadio et al., 2018). Overall, the alignment of data from various vertebrate models (zebrafish and rats)

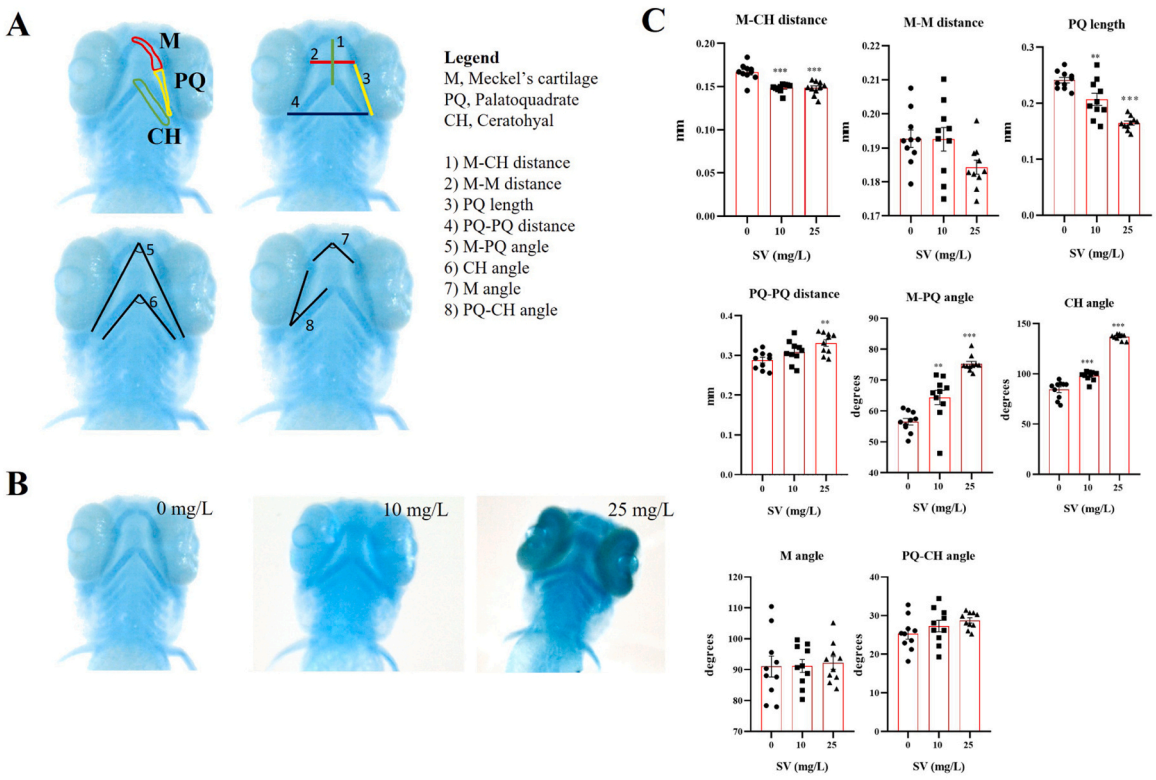


Fig. 3. A) Ventral view of Alcian Blue Staining in 96 hpf larvae, showing the examined parameters, as reported in the legend. Scale bar, 0.2 mm. B) Representative images of 96 hpf larvae in ventral view. Scale bar, 0.2 mm. C) The measures are shown by their mean value ± SEM. The symbol * indicates a significant difference between treated larvae and control (*p<0.05; **p < 0.005; ***p < 0.0005).

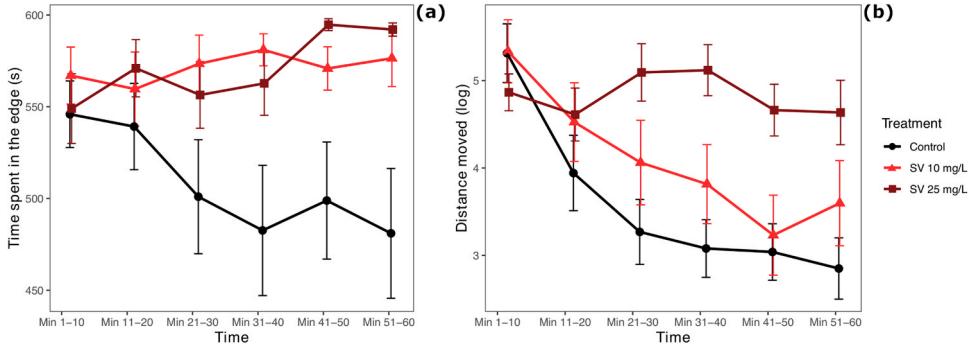


Fig. 4. Results of the open-field test. (A) Thigmotaxis measured as time spent within 1 body-length from the edge of the arena and (B) activity measured as time spent moving. Datapoints represent means; error bars represent standard errors.

strengthens the idea of severe effects of SV on anxiety and similar behaviors.

The diminished reactivity to a possible danger in the light startle test and the reduced capacity to learn in the habituation learning test observed in subjects exposed to the highest SV dose employed are likely attributable to cognitive deficiencies. The diminished reactivity might be associated with reduced capacity to perceive external stimulation and perform the appropriate behavioral response. A study carried out in children with autism revealed similar hyporesponsiveness (Baranek et al., 2013). It is worth noting that the reduced reactivity might be, as previsouly discussed for the activity in the open-field test, directly affected by the morphological alterations of the treated larvae. More research is therefore needed on this aspect. Regarding the habituation test, it measures a simple form of non-associative learning that is essential for life because it allows animals to disregard repetitive stimuli and concentrate on important ones (Jijie et al., 2021). As we analysed habituation learning with an index corrected for the first response of the subject, this trait was not expected to be passively affected by the morphological issues observed in the treated larvae. The fact that this cognitive ability was disrupted by SV administration suggests that the very bases of zebrafish cognition were affected. While autism is not considered a learning disorder, it can have an impact on learning abilities. Therefore, also on this aspect, our findings on zebrafish align with the clinical effects of SV known for humans. For future research, the tests used in our study might provide a starting point for determining the level at which drugs like SV act. Furthermore, the zebrafish model might allow us to analyze the neural circuits impacted (Renninger and Orger, 2013).

The last behavioral test attempted to investigate the capacity of larvae to entrain their behavioral activity with the light-dark (LD) cycle. It is known that an endogenous system (i.e., the circadian clock) regulates daily rhythms in behavior. The clock operates on a 24-hour cycle

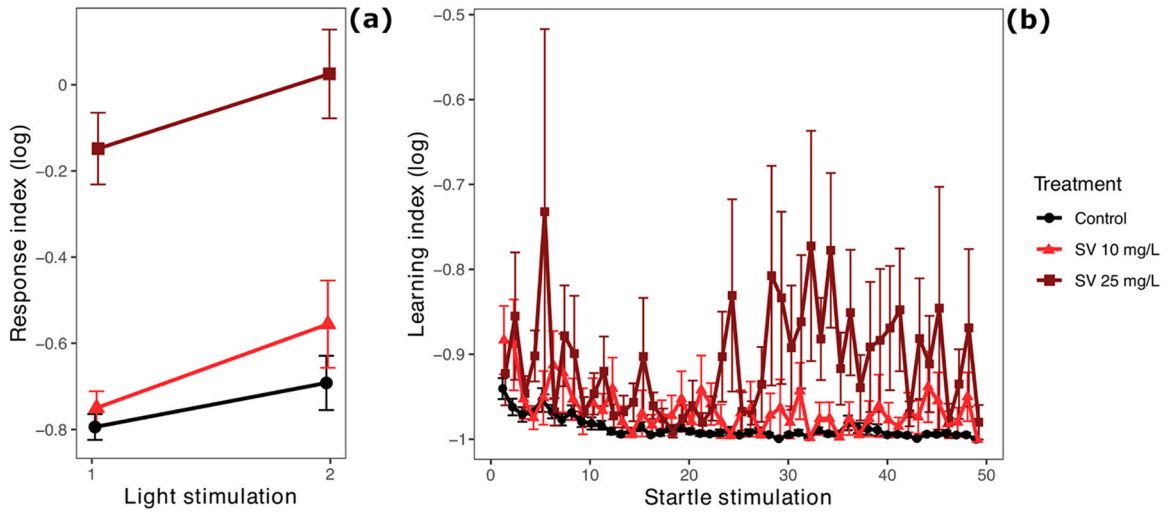


Fig. 5. Results of the (A) light startle and the (B) habituation learning test. Datapoints represent means; error bars represent standard errors.

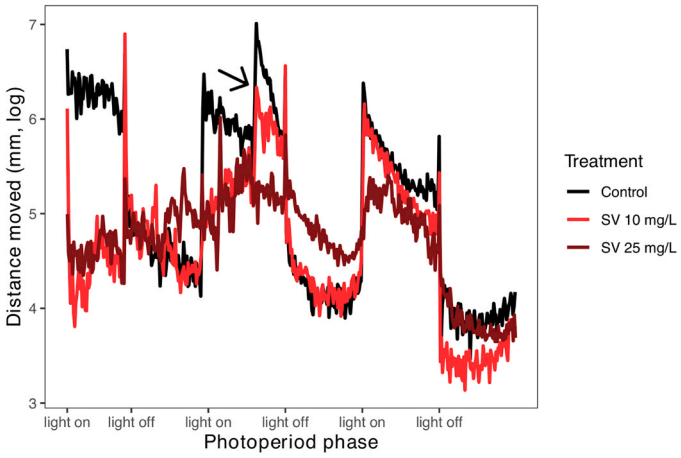


Fig. 6. Results of the daily behavioral activity test. Lines connect means of activity organized in 10-min time bins; the arrow represents the recording interruption performed to change the water and feed the fish.

Table 2
Results of Chi-squared tests to compare the number of subjects with significant circadian rhythmicity in activity (distance moved) across the three treatments and the two experimental phases. Bold indicates significant differences.

		Control	SV 10 mg/L
SV 10 mg/L	Phase 1	$X^2_1 = 21.462$, $P < 0.001$	-
SV 10 mg/L	Phase 2	$X^2_1 = 3.153$, $P = 0.076$	-
SV 25 mg/L	Phase 1	$X^2_1 = 14.666$, $P < 0.001$	$X^2_1 = 0.812$, $P = 0.367$
SV 25 mg/L	Phase 2	$X^2_1 = 9.514$, $P = 0.002$	$X^2_1 = 2.977$, $P = 0.084$

and can be entrained by environmental stimuli, which advance or delay the clock's phase and influencing human physiology and health. Valproic acid has been implicated in interrupting the circadian transcriptional feedback cycle in *in vitro* systems (Griggs et al., 2018). In our study, the SV-treated fish were initially not entrained with the light phase, but after approximately 1 day they began to show the same entrainment observed in the controls. This can be interpreted in two ways. Either SV reduces the capacity to entrain the circadian clock to the LD cycle or slows down the early development of the circadian clock in zebrafish larvae. Autism is usually associated with changes in the circadian sleep-wake rhythm (Tordjman et al., 2015). Individuals with autism spectrum disorder (ASD) frequently have reduced total sleep and greater sleep latency, as well as nocturnal and early morning awakenings (Honomichl et al., 2002). Hence, also on this point, our data seems to indicate that SV administration in zebrafish might produce phenotypes analogous to the autism symptoms observed in humans under similar circumstances. However, numerous possibilities could explain the changes in photic entrainment in this context. It would be particularly interesting to determine whether the observed variances have anything to do with the detected microphthalmia and with the delayed retinal development showed after SV or other chemical exposure (Caioni et al., 2023; DeOliveira-Mello et al., 2023).

5. Conclusions

Overall, our results showed the influence of SV on the morphology, behavior, and cognition of zebrafish during early ontogenesis, confirming that this model may be used to study the action of this compound on neurodevelopmental disorders such as microcephaly and Autism Spectrum Disorder. Importantly, the observed neurobehavioral alterations align with a ASD-like phenotype. Clearly, this is only the first step, as it is required to define the observed changes and identify the genetic processes and other mechanisms involved. A greater understanding of the modifications caused by the SV is necessary both to further identify the hazards associated with its exposure (particularly at the embryological stage) and to allow for a better understanding of the mechanisms behind the onset of cognitive processes.

CRediT authorship contribution statement

Tyrone Lucon-Xiccato: Software, Formal analysis. **Elisabetta Benedetti:** Writing – review & editing, Supervision. **Cristiano Bertolucci:** Writing – review & editing, Visualization. **Monia Perugini:** Supervision, Funding acquisition, Data curation. **Carmine Merola:** Writing – original draft, Formal analysis, Conceptualization. **Michele Amorena:** Supervision. **Giovanni Angelozzi:** Formal analysis. **Giulia Caioni:** Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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