Valproic acid exposure alters social visual lateralization and asymmetric gene expression in zebrafish larvae

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Background

Cerebral asymmetry is a fundamental aspect of brain organization. Abnormal language hemispheric activation and differences in the prevalence of handedness have been observed in individuals with ASD, suggesting atypicality in cerebral structure and lateralization. Zebrafish are increasingly emerging among the key model species to study brain lateralization, with asymmetric development of the epithalamus as a model to investigate the relationship between brain asymmetry and behavior.

We exposed zebrafish embryos at 5 hour post-fertilization to one micromolar dosage of valproic acid for 24 and 48 hours, assessed social interaction and visual lateralization in a social task, the mirror test, and measured gene expression changes in the thalamus and the telencephalon. We show that after exposure to valproic acid, zebrafish exhibit social deficits and alterations in lateralized social responses to their own reflected image. Valproic acid also induced changes in the asymmetric gene expression of the thalamic marker *kctd12.1/leftover* and had a significant effect on the expression of telencephalic genes known to be asymmetrically expressed in adult fish. Our data indicate that one micromolar doses of VPA are sufficient to neutralize both the visual field bias and the asymmetric epithalamic gene expression, opening new perspectives to investigate brain lateralization and its link to ASD in a zebrafish model.

Introduction

Functional lateralization has been documented in several vertebrate species (Rogers et al., 2013), including fish (for reviews, see Stancher et al., 2018; Miletto Petrazzini et al., 2020). Human and animal model studies suggest a general pattern of specialization of the right hemisphere for social behavioural control and response to danger and novelty, associated with social-emotional-processing, and a left specialization for categorization, attention, and fine motor skills, that underly the left hemisphere language dominance and the population-level right-handedness in humans and non-humans (Annett, 2002; MacNeilage et al., 2009). More importantly, hemispheric dominance and functional lateralization seem to be critical for typical cognitive development (Toga and Thompson, 2003), while loss of cerebral lateralization (either weaker or absent asymmetry) underlies poorer cognitive abilities, and in some cases are associated with brain disorders, including autism (Forrester et al., 2020).

In vertebrates with front-facing eyes and binocular vision, such as humans, lateralized processing can be documented and measured through the observation of visual field biases, as, for example, the strong left visual field bias demonstrated in humans in face detection (Burt and Perrett, 1997) and emotional processing (Demaree et al., 2005). A large body of literature suggests alterations in hemispheric functional asymmetry associated with Autism Spectrum disorder (ASD), emerging since early development (Webb et al., 2017).

ASD comprise a heterogeneous group of conditions characterized by atypical social interaction and communication, restricted interests and repetitive behaviour, and sensory processing abnormalities. Deficits in language processing have also been consistently described in ASD (Kjelgaard and Tager-Flusberg, 2001; Tager-Flusberg and Kasari, 2013; Pickles et al., 2014; Lombardo et al., 2015) with associated abnormal hemispheric activation in response to speech stimuli (Dawson et al., 1989). Interestingly, a decreased lateralized responses associated with language processing have also been linked to autism symptoms severity (Jouravlev et al., 2020). A difference in the prevalence of handedness have also been observed in individuals with ASD, suggesting atypicality in cerebral structure and language processing lateralization (Markou et al., 2017). In addition to aberrant lateralized responses to speech and language processing, lack of left visual field bias in face and emotional processing (Dundas et al., 2012b, 2012a; Masulli et al., 2022) have been extensively reported in ASD, suggesting altered functional lateralization already at early developmental stages (Donati et al., 2020). Moreover, neurophysiological and neuroimaging studies also indicate an altered pattern of lateralized activation in cortical brain areas (e.g., fusiform face area) associated with configurational information and categorization of faces

(Webb et al., 2006; McCleery et al., 2009; Keehn et al., 2015; Jones et al., 2016; Shephard et al., 2020).

In recent years, studies in vertebrate species displaying functional lateralization have significantly contributed to widening the knowledge about the mechanisms underlying brain asymmetry and its role in cognitive functions (Rogers et al., 2013). Zebrafish are increasingly emerging among the key model species to study functional and anatomical aspects of brain asymmetry (Miletto Petrazzini et al., 2020). Thanks to the bilateral positioning of the eyes and an almost complete decussation of the optic chiasm, the perception and processing of stimuli in zebrafish can be inferred on the basis of the simple observation of the visual field use during spontaneous behaviour, already at early developmental stages, similar to the visual field biases measured in humans. Studies in zebrafish larvae suggest that social stimuli are processed by the right hemisphere, as revealed by a left visual field bias the larvae show while observing their image reflected in a mirror (Sovrano and Andrew, 2006).

Several studies focused on the asymmetric development of the zebrafish epithalamus as a model to study the relationship between brain asymmetry and behavior (Facchin et al., 2009, 2015). In the zebrafish epithalamic region the parapineal organ is located on the left side in most of the embryos, asymmetrically influencing the development of the habenular nuclei. As a consequence the two dorsal habenula nuclei show differences in size, connectivity and gene expression (Concha et al., 2000, 2003; Concha and Wilson, 2001; Gamse et al., 2003, 2005). The *kctd12.1/leftover* gene is typically upregulated in the left habenula compared to the right (Gamse et al., 2003), while two other members of the same gene family, *ktcd12.2/right-on* and *ktcd8/dexter* have an opposite expression pattern (Gamse et al., 2005).

Previous studies have established a detrimental effect of VPA on social interaction, locomotor activity and anxiety in zebrafish larvae. In a previous report, we have shown that minimal dosage of VPA (1µM VPA for 24 hours) can induce alterations in the expression of several genes previously described to be linked to the pharmacological effect of VPA, supporting high larvae survival. Here, we show that after being exposed to 1µM VPA for 24 and 48 hours in embryo, zebrafish exhibit, later in life, social deficits, and alterations in lateralized social responses to their own reflected image. We also found a lack of lateralized gene expression in the thalamus and a significant effect of VPA treatment on the expression of telencephalic genes known to be asymmetrically expressed in adult fish.

Materials and Methods

Ethical Regulations. All husbandry and experimental procedures complied with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Scientific Committee on Animal Health and Animal Welfare (Organismo Preposto al Benessere Animale, OPBA) of the University of Trento and by the Italian Ministry of Health (Protocol n. 333/2021-PR).

*Animals***.** Adult AB wild-type zebrafish were moved into breeding tanks overnight separated by a transparent barrier. The day after, the barrier was removed, and fish were left to breed. Embryos were collected in E3 medium (5.00 mM NaCl, 0.44 mM CaCl2, 0.33 mM MgSO4 and 0.17 mM KCl). At 5 hpf embryos were placed into 10 cm Petri dishes containing E3 medium (control) and E3 medium with 1 μM VPA (Sigma-Aldrich, P4543; Merck Life Science Srl, Milan, Italy) for 24 or 48 h. At the end of the treatment, the medium was replaced by E3 medium and zebrafish larvae were grown at 28.5°C until the right stage useful for experimental procedures (Messina et al., 2020).

Social Preference. Thirty larvae of the AB strain were examined, each experimental group was composed of 12 controls (CTRL), 11 and 7 larvae treated with 1μM VPA for 24 and 48 hours, respectively. Each group was observed only once. The apparatus consisted of a circular amaranth tank (diameter × height: 175 × 27 cm), surrounded by a circular black curtain fixed on a wood-and-metal frame in which the social preference apparatus was placed. The social preference apparatus was made of white plastic walls (11.2 × 4.2 × 8 cm) and divided into two chambers by a transparent barrier so that the experimental larva could see the companions. One of the chambers (the social chamber, 7 × 4.2 × 8 cm) hosted the experimental larva under test, while the other (4.2 × 4.2 × 8 cm) hosted 6 conspecifics (Figure 1A). To analyze the social preference, the social chamber was divided into two zones (3.5 × 4.2 × 8 cm), one proximal to the conspecifics and one distal from them. The water was 2.5 cm deep and was kept at 26 ± 1°C. The apparatus was lit from above (height: 30 cm) through a set of LED lights (~ 1700 lumen). Each larva was placed in turn in the center of the social chamber and video-recorded using a high-resolution camera (FLIR Systems, BFLY-PGE-12A2M-CS monochromatic, cs mount; Fujinon varifocal LENS-30F2-V80CS) for 5 min. The video recordings were coded offline, and the time spent by the larvae in the two zones was scored manually.

Mirror test. One hundred twenty-four larvae of the AB strain were observed. Each experimental group was composed of 39 vehicle-treated controls (CTRL), 46 and 39 larvae treated with 1μM VPA for 24 and 48 hours, respectively. Each group was observed only once. The apparatus consisted of a circular amaranth tank (diameter × height: 175 × 27 cm), surrounded by a circular black curtain fixed on a wood-and-metal frame. The mirror test apparatus was placed inside the bigger tank and was composed of white plastic walls (20 × 5 × 8 cm), with mirrors on the long walls (one per side) and lit from above (height: 100 cm) through a 24-watt fluorescent white light tube (Lumilux, Osram GmbH, D) (Figure 2A). The water was 2.5 cm deep and its temperature was maintained at 26 ± 1°C by using a 50-watt heater (NEWA Therm®, NEWA). Each larva was placed in turn in the center of the test apparatus and video-recorded from above through a webcam (LifeCam Studio, Microsoft) for 5 min. The positions of each larva were manually scored offline every 2 s, by superimposition on the computer screen of a cursor on the long axis of the body, using the video recording. Body angle was taken relative to the closest of the two mirrors. All the positions where the larva was in a central 4 mm wide area were discarded. Positions in which the body was aligned parallel to the nearest mirror ("parallel observations") and at an angle to the mirror ("angled observations": 1°-179° towards the left or right eye use) were scored jointly.

Microdissection and RNA extraction. Three months-old AB zebrafish (six controls and six treated with 1µM VPA for 24 hrs at 5 hpf) were anesthetized in an ice-cold water bath and sacrificed by decapitation; their brains were removed and dissected in ice-cold phosphate-buffered saline solution (PBS; Fisher Bioreagents, USA). The two hemispheres of telencephalon (including the pallium and the subpallium) and of the thalamus were collected separately from each animal and used for total RNA extractions using the RNeasy Mini Kit (QIAGEN; Milan, Italy). Briefly, tissues were homogenized in lysis buffer, run onto RNeasy spin columns, treated with DNase (RNase-Free DNase Set, QIAGEN; Milan, Italy) and eluted in RNase/DNase-free water. Total RNAs were quantified using NanoDrop™ (Thermo Fisher Scientific; Monza, Italy), and reverse transcribed using the SuperScript VILO™ cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific; Monza, Italy) according to the manufacturer's instructions.

Quantitative real-time PCR. RT-qPCR experiments were performed using specific, commercially synthesized primer pairs (Merck Life Science Srl, Milan, Italy) as previously reported (Messina et al., 2020). The triplicate reactions/samples were performed using the PowerUp™ SYBR™ Green Master Mix and a CFX96™ Real-Time System (Bio-Rad, Milan, Italy). The dCt method was used for expression quantification, raw expression data were normalized on the expression of the 18S reference gene. The complete protocols and the lists of primers are reported in Messina et al. (2021).

Statistical analysis. To evaluate the social preference of zebrafish larvae, the following variables were measured: the percentage [%] of total time spent (TTS) in the proximal zone (social preference index, Liu et al., 2016), the latency [s] to the first change of zone, and the number of alternations between the two zones (proximal-to-distal; distal-to-proximal) during the social preference test. Values of social preference index (%) were calculated as ((TTS in proximal zone/TTS proximal + TTS distal) x100), the values range from 100% (full choice for the social (proximal) zone) to 0% (full choice for the distal zone), where 50% represents the absence of preference. For the mirror test, the left visual field index was calculated as (frequency of left eye use)/(frequency of right eye use + frequency of left eye use). Values significantly higher than 0.5 indicate a preference for left eye use, while values significantly lower than 0.5 indicate a preference for right eye use. The effect of treatment and time on the social preference index, the latency to first change of zone and the spontaneous alternations was evaluated by multifactorial analysis of variance (ANOVA), the effect of treatment on the left visual field index was evaluated by one-way ANOVA. Statistical evaluation on the expression levels was performed on the log2 gene expression levels (dCt), the effect of treatment, brain side and transcript type was estimated using a linear mixed model, considering treatment, brain side and transcript as fixed factors and the experiment (experimental unit) as random factor. A Left Lateralization Index (LI) was using the transformed expression levels for the two hemisphere calculated as previously reported (Messina et al., 2021): LI = (left expression – right expression)/(left expression + right expression). For all the tests, significant departures of the social preference index/left visual field index/LI from chance level (50%, 0.5 and 0, respectively) were estimated by one-sample two-tailed t-tests. All statistical analyses were performed with GraphPad Prism 9 and Rstudio, using the nlme package for the linear mixed models and the emmeans package for Tukey pairwise comparison tests. Alpha was set to 0.05 for all tests.

Results

Social preference. Thirty larvae of the AB strain treated with vehicle (12), 1µM VPA for 24 hrs (11) or 48 hrs (7) underwent to social preference tests at one month of age. To account for potential habituation of the fish to the environment, we evaluated the effect of time (three and five minutes) on the social preference and its interaction with VPA treatment.

We did not detect any effect of time ($F_{(1,54)} = 0.283$, $p = 0.597$) nor of the interaction between time and treatment ($F_{(2,54)} = 0.107$, $p = 0.899$) but a significant effect of treatment ($F_{(2,54)} = 11.813$, $p < 0.0001$). Twenty-four and 48 hours of treatment with VPA significantly decreased the preference of the fish to spend time in the chamber close to the conspecifics (Figure 1B;

pairwise comparisons: CTRL vs VPA 24h $t_{(54)}$= 3.791, p = 0.0011; CTRL vs VPA 48h $t_{(54)}$= 4.352, p = 0.0002; VPA 24h vs VPA 48h $t_{(54)}$= 1.008, p = 0.5750). Moreover, while fish treated with vehicle or with VPA for 24 hours displayed a significant preference for the proximal chamber, the group of animals treated with VPA for 48 hours did not, indicating a detrimental effect of VPA on the social preference (Figure 1B; one-sample t-test: CTRL $t_{(11)}$ = 7.083, p < 0.0001; VPA24h $t_{(10)}$ = 2.592, p = 0.0269; VPA48h $t_{(6)}$ = 0.659, p = 0.5343; group mean: CTRL 80.43% [95% C.I. 70.97% - 89.88%]; VPA24h 61.93% [95% C.I. 51.67% - 72.18%]; VPA48h 55.54% [95% C.I. 34.96% - 76.12%]). We then analyzed the latency to the first change of zone and the number of alternations between the two zones during the social preference test. We found no significant effect of treatment on the latency (Figure 1C; $F_{(2,27)}$ = 1.320, p = 0.2839) and a significant effect of treatment on the number of spontaneous alternations (Figure 1D; $F_{(2,27)}$ = 4.385, p = 0.0224). Larvae treated with 1 µM VPA for 24 hours, but not for 48 hours, displayed a significant increase in the number of alternations between the proximal and the distal zone (Figure 1D; pairwise comparisons: CTRL vs VPA 24h $t_{(27)}$ = -2.881, p = 0.0203; CTRL vs VPA 48h $t_{(27)}$ = -0.586, p = 0.8288; VPA 24h vs VPA 48h $t_{(27)}$ = 1.911, p = 0.1550).

Mirror test. Given the effect of VPA on social behaviour, we tested the lateralized social response of the larvae to their image reflected in the mirror. One hundred twenty-four larvae of the AB strain, treated with vehicle (39), 1µM VPA for 24 hrs (46) or 48 hrs (39), were subjected to the mirror test according to previous reports (Sovrano and Andrew, 2006). Since the strongest responses were shown at three weeks post-fertilization (Sovrano and Andrew, 2006), we chose to perform the test at this stage. We assessed the effect of treatment on the left visual field bias, expressed as the ratio of left eye use when the fish were observing their reflection close to the mirror. We found a significant effect of treatment (Figure 2B; $F_{(2,121)}$ = 27.76, p <0.0001) on the left visual field index, with a remarkable reduction of the use of the left eyes during the test in both the VPA 24 and 48 hours treatment group (Figure 2B; pairwise comparisons: CTRL vs VPA 24h $t_{(121)}$= 5.980, p < 0.0001; CTRL vs VPA 48h $t_{(121)}$= 6.908, p < 0.0001; VPA 24h vs VPA 48h $t_{(121)}$= 1.206, p = 0.4518). In line with previous reports, the vehicle-treated larvae displayed a significant preference for left eye use, comparable to what was shown previously, while both treatment groups show no preferential visual field use during the test (Figure 2B; one-sample t-test: CTRL $t_{(38)}$ = 10.317, p < 0.0001; VPA24h $t_{(45)}$ = 1.4861, p = 0.1442; VPA48h $t_{(38)}$ = -0.2105, p = 0.8344; group mean: CTRL 0.5960 [95% C.I. 0.5772 – 0.6148]; VPA24h 0.5143 [95% C.I. 0.4949 – 0.5337]; VPA48h 0.4978 [95% C.I. 0.4771 – 0.5186]). Overall, the mirror test results suggest a deleterious effect of VPA on the lateralized behavioural responses of the larvae to the social stimuli.

Asymmetric gene expression analysis. We micro-dissected the left and right thalamus of 3 mpf zebrafish and then evaluated the expression levels of the thalamic markers *kctd12.1/leftover* (*lov*) and *ktcd8/dexter* (*dex*), *ktcd12.2/right-on* (*ron*) (Figure 3A; n = 6 animals per treatment group, 6 independent experiments). To assess the effect of treatment, brain side and transcript type on the expression levels, we used a linear mixed model, considering treatment, brain side and transcript as fixed factors and the experimental unit (experiment) as random factor. We compared a model with random-intercepts-only to one with random slopes and intercepts and found that the second model fitted the data significantly better. The statistical analysis indicated a significant difference in asymmetric gene expression in the treatment groups (interaction treatment * brain side $F_{(1,55)} = 13.8159$, $p = 0.0005$). We also found a significant difference in the expression levels of the three transcripts analyzed (main effect of transcript $F_{(2,55)} = 1332.2072$, $p < 0.0001$) and no other significant main effects or interactions (main effect of treatment $F_{(1,55)} = 0.2256$, $p = 0.6367$; main effect of brain side $F_{(1,55)} = 0.6442$, $p = 0.4257$; treatment * transcript interaction $F_{(2,55)} = 2.6251$, $p = 0.0815$; brain side * transcript interaction $F_{(2,55)} = 2.6440$, $p = 0.0801$; treatment * brain side * transcript interaction $F_{(2,55)} = 1.7248$, $p = 0.1877$). The pairwise comparison between the levels of expression in the treatment groups indicated a difference in the asymmetric expression of *ktcd8/dexter* in the VPA treated samples, with a decreased expression in the left hemisphere of VPA treated zebrafish and no significant changes in the CTRL samples (Figure 3B; *dexter* CTRL right vs left: $t_{(55)} = -1.619$, $p = 0.1112$; *dexter* VPA 24 hrs right vs left: $t_{(55)} = 2.470$, $p = 0.0166$). In line with previous reports (Messina et al., 2021), we observed an asymmetric expression of *kctd12.1/leftover*, with a higher expression on the left hemisphere compared to right one in the control samples, that was not anymore present upon treatment with VPA (Figure 3B; *leftover* CTRL right vs left: $t_{(55)} = -2.930$, $p = 0.0049$; *leftover* VPA 24 hrs right vs left: $t_{(55)} = 1.195$, $p = 0.2374$). No changes in the asymmetric expression of *ktcd12.2/right-on* was detected (Figure 3B; *right-on* CTRL right vs left: $t_{(55)} = 0.980$, $p = 0.3313$; *right-on* VPA 24 hrs right vs left: $t_{(55)} = 1.871$, $p = 0.0667$). As suggested by the gene expression analysis and previously reported (Gamse et al., 2005; Aizawa et al., 2007; Messina et al., 2021), *kctd12.1/leftover* expression was predominant on the left side of the thalamus in the control group at this stage, as shown by the significant departure from chance of the lateralization index, the lateralized expression was however neutralized by VPA treatment (Figure 3C; one sample t-test of left lateralization index *leftover* CTRL $t_{(5)} = 11.73$, $p < 0.0001$; *leftover* VPA 24h $t_{(5)} = 1.235$, $p = 0.2716$; group mean: CTRL 0.2455 [95% C.I. 0.1918 – 0.2993]; VPA 24h -0.0977 [95% C.I. -0.3010 – 0.1056]). Differently from previous reports describing the asymmetric distribution of *ktcd8/dexter* and *ktcd12.2/right-on* primarily on the right hemisphere (Gamse et al., 2005; Aizawa et al., 2007; Messina et al., 2021), these thalamic markers were not clearly asymmetrically distributed in the thalamus at this stage as shown by the lateralization indexes (Figure 3C; one sample t-test of left lateralization index *dexter* CTRL $t_{(5)} = 2.216$, p = 0.0775; *dexter* VPA 24h $t_{(5)} = 2.326$, p = 0.0675; group mean: *dexter* CTRL 0.1358 [95% C.I. -0.0217 – 0.2993]; *dexter* VPA 24h -0.1998 [95% C.I. -0.4205 – 0.0210]; one sample t-test of left lateralization index *right-on* CTRL $t_{(5)} = 0.7083$, p = 0.5104; *right-on* VPA 24h $t_{(5)} = 1.587$, p = 0.1773; group mean: *right-on* CTRL -0.0761 [95% C.I. -0.3525 – 0.2002]; *right-on* VPA 24h -0.1533 [95% C.I. -0.4015 – 0.0949]). We also evaluated the expression of genes previously shown to be asymmetrically distributed in the adult telencephalon (Messina et al., 2021). We micro-dissected the left and right telencephalon and then measured the expression of *arrb2, fez1, gap43, nipa1, nipa2 and robo1* in the two hemispheres (Figure 4A; n = 4 animal per treatment group, 4 independent experiments) of zebrafish at 3 mpf, treated with vehicle or with 1µM VPA for 24 hrs at 5 hpf. To assess the effect of treatment, brain side and transcript type on the expression levels, we used a linear mixed model, considering treatment, brain side and transcript as fixed factors and the experiment (experimental unit) as random factor. Using a random slopes and intercepts model, we found a significant difference in asymmetric gene expression in the treatment groups (Figure 4B; interaction treatment * brain side $F_{(1,69)} = 8.5682$, p = 0.0046). We also found a significant difference in the expression levels of the transcripts analyzed (main effect of transcript $F_{(5,69)} = 1125.7494$, p < 0.0001) and in the brain side (main effect of brain side $F_{(1,69)} = 17.3302$, p = 0.0001) with no other significant main effects or interactions (main effect of treatment $F_{(1,69)} = 0.0695$, p = 0.7928; transcript * brain side interaction $F_{(5,69)} = 1.0046$, p = 0.4217; treatment * transcript interaction $F_{(5,69)} = 0.1690$, p = 0.9732; treatment * brain side * transcript interaction $F_{(5,66)} = 0.4750$, p = 0.7937). The pairwise comparison between the levels of expression in the treatment groups indicated no difference in the asymmetric expression in the control group in the gene analyzed, differently from what previously described, while a significant difference was observed in the expression of *gap43*, *nipa2* and *robo1* in the VPA treatment group (Figure 4B; *arrb2* CTRL right vs left: $t_{(69)} = -0.400$, p = 0.906; *arrb2* VPA 24 hrs right vs left: $t_{(69)} = -0.993$, p = 0.3242; *fez* CTRL right vs left: $t_{(69)} = 0.021$, p = 0.9836; *fez* VPA 24 hrs right vs left: $t_{(69)} = -1.504$, p = 0.1374; *gap43* CTRL right vs left: $t_{(69)} = -0.981$, p = 0.3302; *gap43* VPA 24 hrs right vs left: $t_{(69)} = -2.279$, p = 0.0258; *nipa1* CTRL right vs left: $t_{(69)} = 0.175$, p = 0.8615; *nipa1* VPA 24 hrs right vs left: $t_{(69)} = -0.977$, p = 0.3322; *nipa2* CTRL right vs left: $t_{(69)} = 0.214$, p = 0.8310; *nipa2* VPA 24 hrs right vs left: $t_{(69)} = -3.152$, p = 0.0024; *robo1* CTRL right vs left: $t_{(69)} = -1.170$, p = 0.2460; *robo1* VPA 24 hrs right vs left: $t_{(69)} = -3.377$, p = 0.0012). Differently from previous reports analyzing the asymmetric distribution of these genes (Messina et al., 2021) we did not detect a significant departure from chance of the Left Lateralization Index in the control groups, however in some cases a significant asymmetry in the distribution of the expression was observed in the VPA-treated group, as for example in the case of *arrb2*, *fez* and *nipa2* (Figure 4C; one sample t-test of left lateralization index *arrb2* CTRL $t_{(3)}$ = 0.5855, p = 0.5993; *arrb2* VPA 24h $t_{(3)}$ = 5.336, p = 0.0129; group mean: *arrb2* CTRL 0.0277 [95% C.I. -0.1230 – 0.1785]; *arrb2* VPA 24h -0.0694 [95% C.I. 0.0280 – 0.1109]; one sample t-test of left lateralization index *fez* CTRL $t_{(3)}$ = 0.704, p = 0.9483; *fez* VPA 24h $t_{(3)}$ = 3.272, p = 0.0336; group mean: *fez* CTRL -0.0014 [95% C.I. -0.0668 – 0.0639]; *fez* VPA 24h 0.1048 [95% C.I. 0.0153 – 0.1943]; one sample t-test of left lateralization index *gap43* CTRL $t_{(3)}$ = 0.852, p = 0.4571; *gap43* VPA 24h $t_{(3)}$ = 3.116, p = 0.0526; group mean: *gap43* CTRL 0.0673 [95% C.I. -0.1842 – 0.3188]; *gap43* VPA 24h 0.1571 [95% C.I. -0.0034 – 0.3175]; one sample t-test of left lateralization index *nipa1* CTRL $t_{(3)}$ = 0.247, p = 0.8212; *nipa1* VPA 24h $t_{(3)}$ = 1.596, p = 0.2088; group mean: *nipa1* CTRL -0.0123 [95% C.I. -0.1706 – 0.1461]; *nipa1* VPA 24h 0.0681 [95% C.I. -0.0677 – 0.2039]; one sample t-test of left lateralization index *nipa2* CTRL $t_{(3)}$ = 0.191, p = 0.8605; *nipa2* VPA 24h $t_{(3)}$ = 5.818, p = 0.010; group mean: *nipa2* CTRL -0.0148 [95% C.I. -0.2609 – 0.2312]; *nipa2* VPA 24h 0.2165 [95% C.I. -0.0981 – 0.3349]; one sample t-test of left lateralization index *robo1* CTRL $t_{(3)}$ = 0.899, p = 0.4349; *robo1* VPA 24h $t_{(3)}$ = 2.904, p = 0.0623; group mean: *robo1* CTRL 0.0800 [95% C.I. --0.2033 – 0.3634]; *robo1* VPA 24h 0.2280 [95% C.I. -0.0219 – 0.4778]).

Discussion

Several studies suggest alterations in brain lateralization in ASD. Neuroimaging and neurophysiological studies mainly focused on lateralized response to speech and face processing, demonstrating changes in ASD patients already at early developmental stages (Dundas et al., 2012b, 2012a; Donati et al., 2020; Masulli et al., 2022).

Here we have tested behavioral and biological lateralization in a model ASD in zebrafish based on embryonic administration of VPA. In Messina et al. (2020) we have described the effect of micromolar doses of VPA on survival of zebrafish larvae at 5 dpf and have confirmed VPA deleterious impact on the expression of several neurodevelopmental genes, recapitulating what previously reported (Jacob et al., 2014; Baronio et al., 2018). We extended our previous studies and demonstrated significant changes in social interaction induced by administration of 1µM VPA, for 24 and 48 hrs. Furthermore, we observed deficits in left visual field bias in a social task, the mirror test, based on the animals' response to their reflection in a mirror. We also found that the deficits in behavioural lateralization were accompanied by changes in asymmetrically distributed biological pathways linked to developmental brain lateralization, including the expression of the thalamic marker *kctd12.1/leftover*. Our data indicate that one micromolar doses of VPA induce social preference deficits and are sufficient to neutralize both the visual field bias and the asymmetric epithalamic gene expression in zebrafish, opening new perspectives on the study of brain lateralization and its link to ASD in animal models of the human disorders.

Previous studies have established a detrimental effect of VPA on social interaction, locomotor activity and anxiety in zebrafish larvae. Zimmerman and colleagues (2015) reported alterations in locomotor activity, increased anxiety and social interaction deficits in zebrafish larvae treated with VPA (48 µM). In contrast, Liu and colleagues (2016) described altered locomotor activity and social preference deficits in zebrafish chronically treated with 20µM VPA at one month of age. A recent study by Dwivedi and colleagues (2019) reported increased anxiety, high circling behaviour and reduced social interaction in zebrafish larvae treated with VPA (75µM) already at 21 dpf. In our study we tested the effect of 1 µM doses of VPA using the same setup as Liu and colleagues (2016). We found that VPA exposure for 24 and 48 hours decreases substantially the time spent in the proximal zone, even though only exposure to VPA for 48 hours induced a conspicuous reduction of the social preference index to chance levels (Figure 1B). The significant changes in spontaneous alternations between zones in the twenty-four-hours treatment group (Figure 1 D) may indicate elevated anxiety levels or be related to increased locomotor activity. In addition to the evaluation of the latency to change zone (Figure 1C), that shows no differences in treatment groups, the data suggest no effect of VPA on locomotor activity, however further directed behavioural tests will be necessary to properly evaluate the effect of 1µM VPA on anxiety.

Similar to many other vertebrate species, also fish rely on visual information for social interaction that emerges starting from the third postnatal week (Dreosti et al., 2015), using mainly cues present in the head and face regions to drive their social affiliative responses (Karplus and Algom, 1981; Wang and Takeuchi, 2017; Nunes et al., 2020). Facial recognition of familiar conspecifics has also been documented in different fish species that decrease their gazing to inverted conspecific faces but not to inverted objects (Kohda et al., 2015; Satoh et al., 2016; Hotta et al., 2017, 2019; Wang and Takeuchi, 2017; Kawasaka et al., 2019). Similar to the social approach response shown by other fish species towards visual cues in the face region, zebrafish larvae exhibit spontaneous approach responses to their image reflected in the mirror, responding to the mirror image as a social stimulus representing a conspecific (Desjardins and Fernald, 2010) and displaying a stable left visual field bias (Sovrano and Andrew, 2006). Interestingly, this ability is shared with other fish species (Sovrano et al., 1999, 2001; De Santi et al., 2001; Sovrano, 2004). Moreover, the left eye bias during the inspection of self-image in the mirror is sensitive to ambient lighting: zebrafish larvae showed a preferential left eye-use only when reared from fertilization under light-dark conditions, but not when exposed to complete darkness or monochromatic light, as if exposure to different wavelengths affected the neural network, generating visual asymmetries or not (Sovrano et al., 2016).

This study analyzes for the first time the effect of VPA exposure on this social behavioural lateralization, revealing a dramatic effect of VPA on the left visual field bias displayed by zebrafish larvae when observing their image reflection. Both the 24- and 48-hours VPA treatment groups show a reduction of the left visual field index, decreased to chance levels, indicating the neutralization of the behavioural lateralization in response to social cues.

In addition to the loss of functional lateralization, we also demonstrate that embryonic VPA exposure induces changes in epithalamic genetic pathways (*lov*, *dex* and *ron*) associated to habenular asymmetry. The epithalamus is one region of the zebrafish brain found to have prominent brain asymmetry, hence the neuroanatomical features of the parapineal organ, the positioning, or the asymmetric gene expression of the habenula are considered evidence of the left or right bias of the entire brain. Several studies have examined the functional relevance of loss or reversals of left epithalamic asymmetry in zebrafish induced by perturbation of habenular identity or disruption of the parapineal organ using genetic and pharmacological approaches or physical ablation (Facchin et al., 1999, 2009, 2015; Barth et al., 2005; Dadda et al., 2010; Domenichini et al., 2011). The predominant view is that a reversal of the epithalamic asymmetry induces inverted or absent behavioural lateralization, with substantial differences given by different zebrafish strain, artificial selection and genetic manipulation (Barth et al., 2005; Facchin et al., 2009, 2015; Dadda et al., 2010). Facchin et al. (2015) also suggested an increase in anxiety behaviour in zebrafish larvae displaying reversal of epithalamic left-right asymmetry either spontaneous or mediated by physical ablation of the parapineal organ. Our data is in line with the idea that perturbations of epithalamic asymmetry mediated by VPA exposure affect the larvae behavioral lateralization, with a complete neutralization of the left visual field bias shown by the zebrafish larvae at three weeks postfertilization. Interestingly, the evaluation of the spontaneous alternations between zones and of the latency to change zone in the social preference tests seem to suggest increased anxiety, however further targeted behavioural evaluation will determine whether anxiety levels are increased in VPA treated larvae and the neuroanatomical correlates of this phenotype.

As for the molecular pathways involved in left-right asymmetry, the earliest signs of brain asymmetry in zebrafish is the activation of the Nodal pathways starting at about 18 hpf in the left epithalamus (Halpern et al., 2003). Shortly after the activation of the Nodal pathway, the parapineal appears on the left side of the pineal anlage and induces the left lateralization of the habenular nuclei (Gamse et al., 2002, 2003). The habenular nuclei show left-right differences in their size, their neuropil density and innervation pattern and the expression of the *lov* gene as early as 2 dpf (Gamse et al., 2003). Expression of the *lov* gene is originally asymmetric, being highly expressed on the (left) side closely opposed to the parapineal (Gamse et al., 2003) and continue to be highly expressed on the left epithalamus into adulthood. Wnt signaling also seems to be necessary for the communication between the parapineal organ and the left habenular nuclei to establish asymmetric activation of Nodal pathway genes in the brain (Carl et al., 2007). In case of an overactivation of the canonical Wnt/β-catenin pathways, as in the *masterblind* (*mbl*) mutants, the habenular nuclei develop symmetrically, with double right-sided habenulas and a symmetrical expression of *lov* at right-side (low) expression levels (Carl et al., 2007). Interestingly, in a small proportion of larvae with disrupted left-right asymmetry that develop bilateral parapineal organs, both habenular nuclei show high expression of the *lov* gene (Gamse et al., 2003). VPA seem to induce a similar scenario, with an inversion in the expression levels of *lov*, with the right side having a higher expression of the left side (Figure 3B).

We also analyzed the expression of pallial genes associated to ASD known to have a left-right asymmetric distribution in humans (Sun et al., 2005), mice (Grabrucker et al., 2018) and zebrafish (Messina et al., 2021). We found no asymmetric expression of the analyzed genes in vehicle-treated zebrafish, while VPA treatment induced a significant left lateralization in the expression of *arrb2*, *fez* and *nipa2*. As for the lack of correspondence between the left-right distribution of these pallial markers in control animals, one potential confounds between the present study and the one from Messina et al. (2021) is the inclusion in our samples of both pallial and subpallial regions.

Taken together the gene expression data suggest a complex set of effect of VPA on the molecular pathways governing the epithalamic left-right asymmetry with potential neuroanatomical differences underlying some of the results. In the zebrafish epithalamus the expression of the gene *lov* is restricted to the lateral habenula cells, on the other hand *ron* is found in the medial cell population (Aizawa et al., 2007). As for the pallial gene expression a similar effect of VPA on neuroanatomically defined cell subpopulation may be taking place. Previous studies have shown asymmetric distribution of GABAergic interneurons in the sensory cortex of mouse models for ASD (Deemyad et al., 2021), including in animals exposed to VPA (Gogolla et al., 2009). Further studies will clarify the neuroanatomical effect of VPA on the development of the epithalamus as well as the telencephalic structures.

Conclusions

Cerebral asymmetry is a fundamental aspect of brain organization and it is also associated with neuropsychiatric diseases, including autism. Patients with ASD show reduced language lateralization and a higher frequency of left-handedness, accompanied by asymmetric functional changes in different brain areas, suggesting a general loss of cerebral asymmetry in ASD. This study analyzes the effect of VPA exposure, a compound used to induce ASD-like behavioral deficits in many vertebrate species, on social behavioural lateralization, revealing a dramatic effect of VPA on the left visual field bias displayed by zebrafish larvae when observing their image reflection. One micromolar VPA also induced changes in the asymmetric gene expression including that of the thalamic marker *kctd12.1/leftover*. Our data show for the first time that one micromolar doses of VPA are sufficient to neutralize both the visual field bias as well as the asymmetric expression of the epithalamic marker *lov*, opening new perspectives to investigate brain lateralization and its link to ASD in a zebrafish model.

Ethics approval

The animal study was reviewed and approved by the Ethical Committee of the University of Trento and the Italian Health Ministry (permit number 333/2021-PR).

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

AM, PS and VAS contributed to conceptualization; AM and GB contributed to methodology; AM and PS contributed to formal analysis; AM, GB, AM and AA contributed to investigation; PS contributed to writing and original draft preparation; AM, PS and VAS contributed to writing, review and editing. All authors have read and agreed to the published version of the manuscript.

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Figure Legends

Figure 1. Social preference test. (A) Top, apparatus used for the social preference test, showing the conspecifics chamber and the two areas, the proximal and distal zone. Bottom, scheme of the experimental timeline, VPA treatment begin at 5 hpf and last for 24 or 48 hours. The social preference test started at 28 dpf. (B, C, D) Box and whisker plot (median, min to max) showing (B) the % of time spent in the proximal zone (social preference index), (C) the latency to change zone and (D) the number of alternations between proximal and distal zones. The number sign (#) indicate significant departures of the social preference index from chance level (50%), marked by the red line. $^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$; $^{*}p < 0.05$; $^{**}p < 0.01$.

Figure 2. Mirror test. (A) Top, apparatus used for the mirror test, showing the position of the mirrors and the angles of viewing that defined monocular vision with the right or left eye. Data were discarded when the fish was perpendicular to the mirror (binocular stimulation, transparent fish) or when it formed an angle larger than 90º with respect to the closest mirror. Bottom, scheme of the experimental timeline, VPA treatment begin at 5 hpf and last for 24 or 48 hours. The mirror test started at 21 dpf. (B) Box and whisker plot (median, min to max) showing the left visual field index. The number sign (#) indicate significant departures of the left visual field index from chance level (0.5), marked by the red line. $^{\#\#\#\#}p < 0.0001$; $^{****}p < 0.0001$.

Figure 3. Gene expression in the left and right thalamus of zebrafish. (A) Top, schematic representation of thalamic regions selected for the analyses. Bottom, scheme of the experimental timeline, VPA treatment begin at 5 hpf and last for 24 hours. Gene expression was analyzed in adult zebrafish. (B) Box and whisker plot (median, min to max) of relative expression (dCt, log2) values for each treatment group for *kctd12.1/lov*, *kctd8/dex* and *kctd12.2/ron* in the left and right thalamus of zebrafish. (C) Left lateralization index for *kctd12.1/lov*, *kctd8/ dex* and *kctd12.2/ron*. The number sign (#) indicate significant

departures of the left lateralization index from chance level (0.5), marked by the red line. $^{\#\#\#\#}p < 0.0001$; $^{*}p < 0.05$; $^{**}p < 0.01$.

Figure 4. Gene expression in the left and right telencephalon of zebrafish. (A) Top, schematic representation of telencephalic (pallial and subpallial) regions selected for the analyses. Bottom, scheme of the experimental timeline, VPA treatment begin at 5 hpf and last for 24 hours. Gene expression was analyzed in adult zebrafish. (B) Box and whisker plot (median, min to max) of relative expression (dCt, log2) values for each treatment group for *arrb2*, *fez, gap43, nipa1, nipa2* and *robo1* in the left and right telencephalon of zebrafish. (C) Left lateralization index for *arrb2*, *fez, gap43, nipa1, nipa2* and *robo1*. The number sign (#) indicate significant departures of the left lateralization index from chance level (0.5), marked by the red line. $^{\#}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

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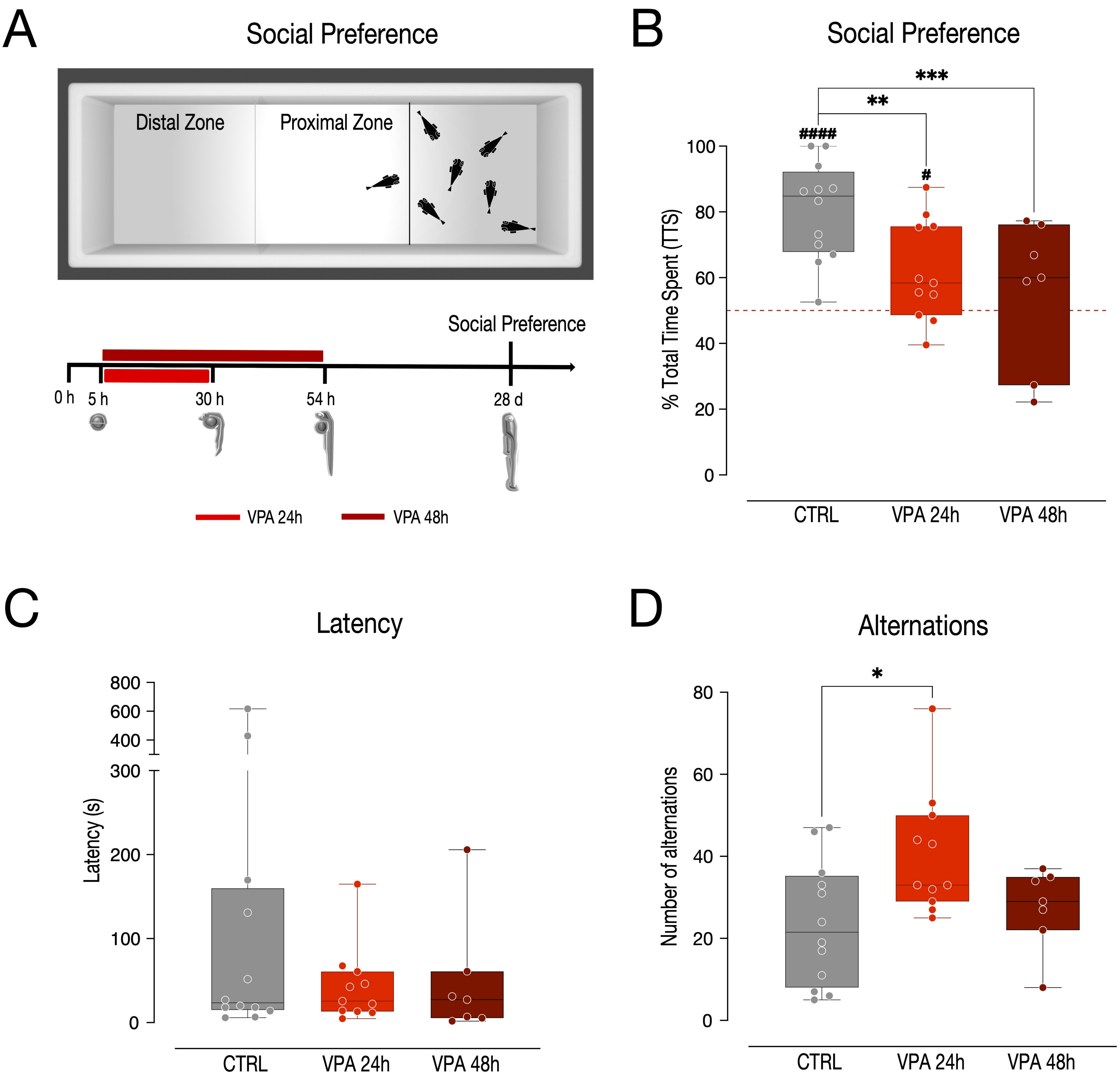
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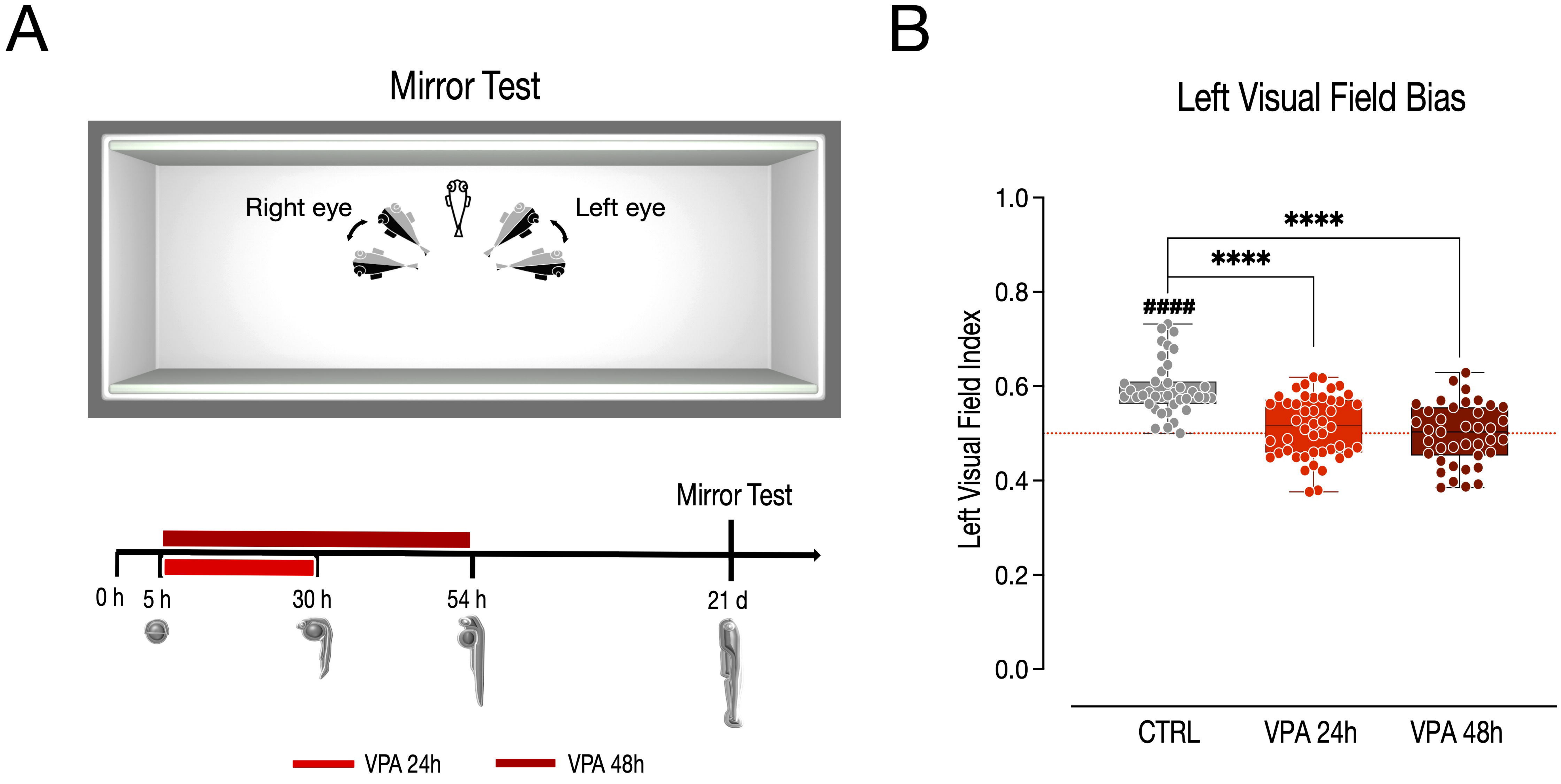
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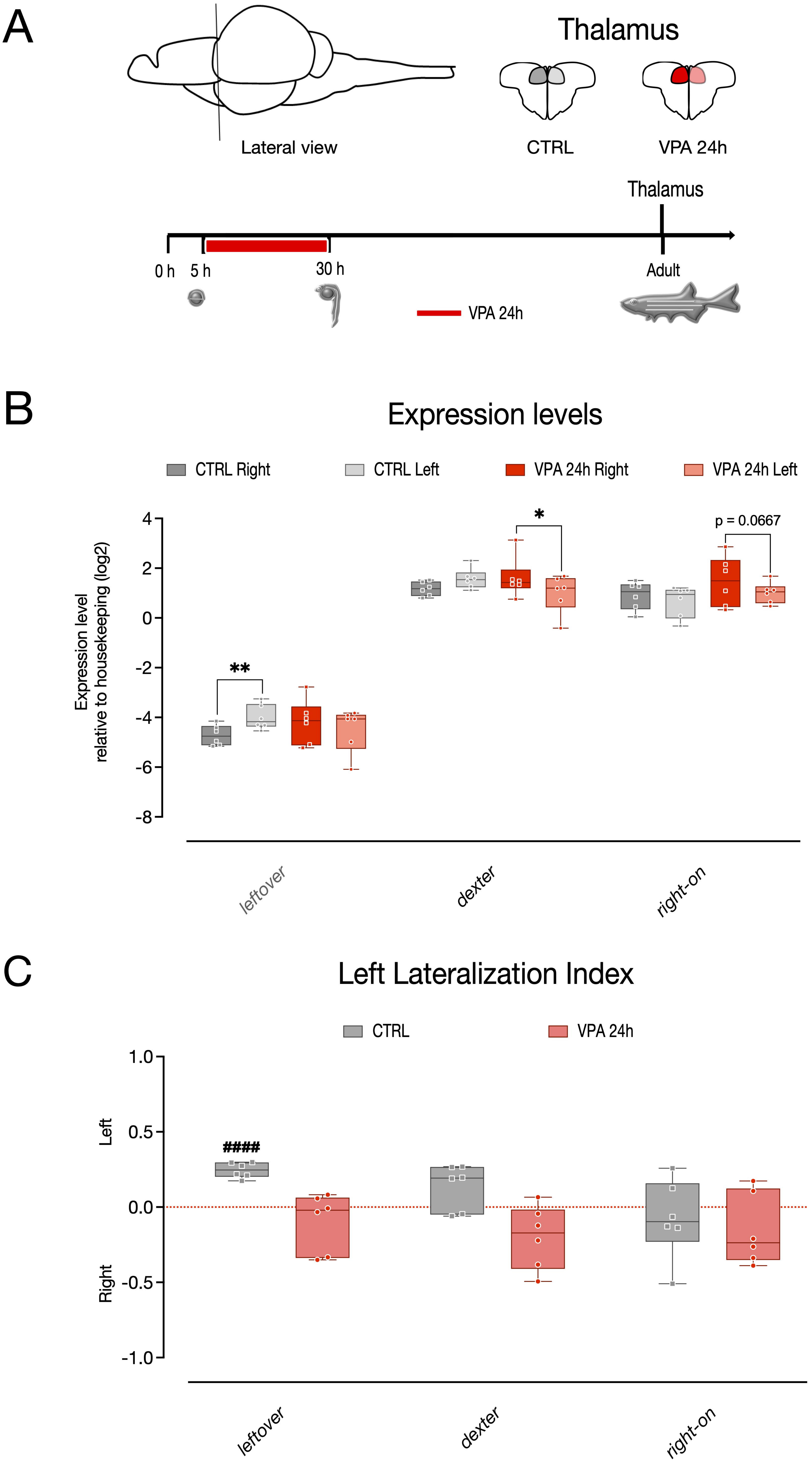
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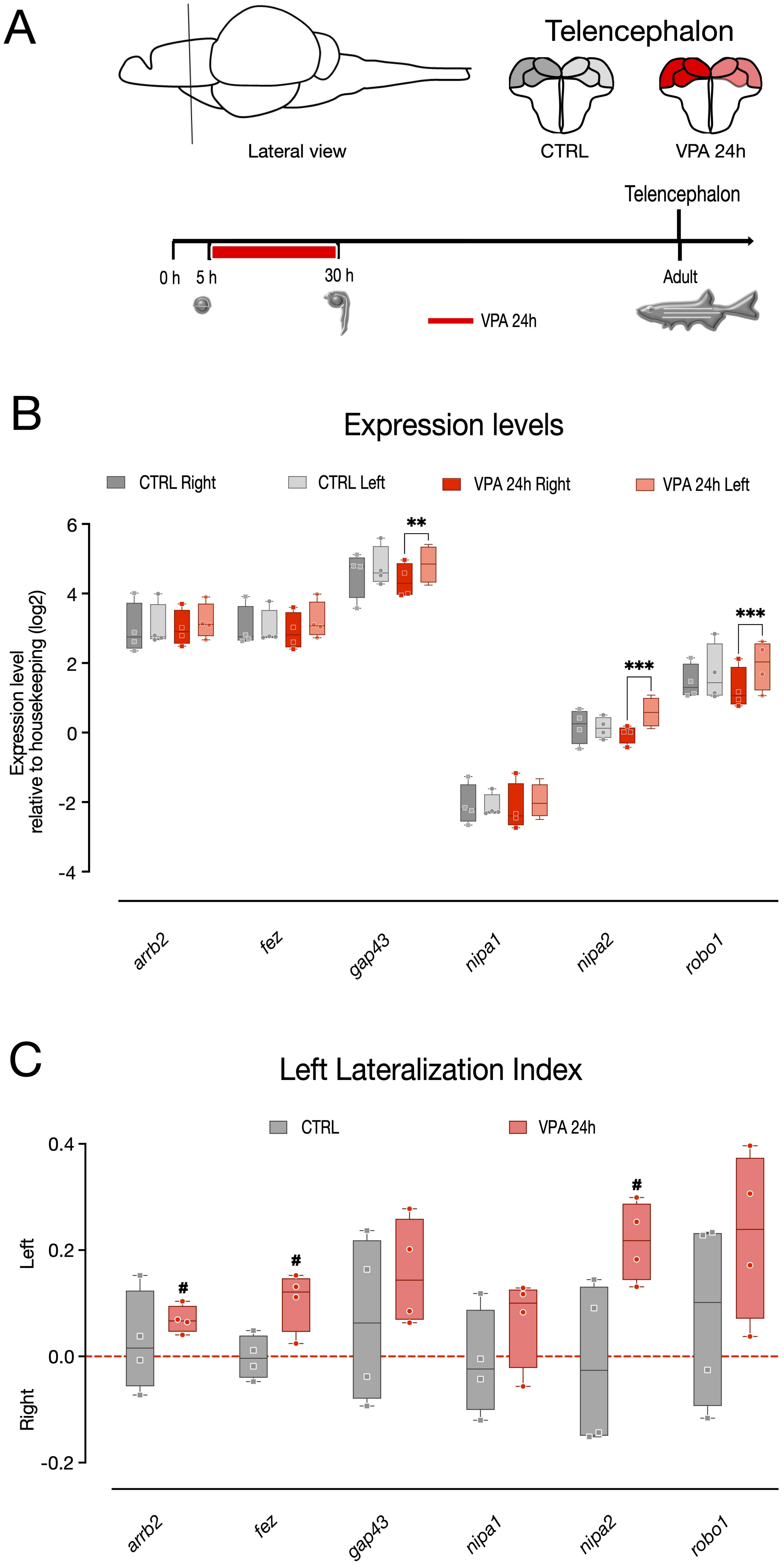
**
*
p = 0.0667
leftover
dexter
right-on
C
Left Lateralization Index
CTRL
VPA 24h
Left
Right
1.0
0.5
0.0
-0.5
-1.0
####
leftover
dexter
right-on


A
Telencephalon
Lateral view
CTRL
VPA 24h
Telencephalon
0 h
5 h
30 h
Adult
VPA 24h
B
Expression levels
CTRL Right
CTRL Left
VPA 24h Right
VPA 24h Left
Expression level relative to housekeeping (log2)
6
4
2
0
-2
-4
**
***
***
arrb2
fez
gap43
nipa1
nipa2
robo1
C
Left Lateralization Index
CTRL
VPA 24h
Left
Right
0.4
0.2
0.0
-0.2
#
#
#
arrb2
fez
gap43
nipa1
nipa2
robo1