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

Andrea Messina[1*], Greta Baratti[1*], Alessia Musa[1], Alice Adiletta[1], Paola Sgadò[1#§] and Valeria Anna Sovrano[1#]

[1]Center for Mind/Brain Sciences, University of Trento, Piazza della Manifattura 1, 38068 Rovereto (TN), Italy

*****These first authors contributed equally

[#]These senior authors contributed equally

[§]Correspondence to:

Paola Sgadò, PhD,
Center for Mind/Brain Sciences
University of Trento
Piazza della Manifattura 1, 38068 Rovereto (TN), Italy
Phone: +39 0464 808691
paola.sgado@unitn.it

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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 reduced functional and structural cerebral asymmetry. 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, a compound used to model the core signs of ASD in many vertebrate species, zebrafish exhibit social deficits and alterations in lateralized social responses to their own reflected image. Valproic acid exposure also induced changes in the asymmetric gene expression of the thalamic marker *kctd12.1/leftover* and had a significant effect on 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 altered 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, that is 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 is 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, decreased lateralized responses associated with language processing have been linked to autism symptoms severity (Jouravlev et al., 2020). A difference in the prevalence of left-handedness has 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 have described 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 behaviour (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 a 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 same administration regimen, zebrafish exhibit, later in life, social deficits, and alterations in lateralized social responses to their own reflected image. We also found loss of lateralized gene expression in the thalamus and a significant effect of VPA treatment on 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 $CaCl_2$, 0.33 mM $MgSO_4$ 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 centre 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 centre 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 anaesthetized 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 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 (Liu et al., 2016), referred to as social preference index, 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 preference for the social (proximal) zone) to 0% (full preference 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 the first change of zone and the spontaneous alternations was evaluated by multifactorial analysis of variance (ANOVA), and the effect of treatment on the left visual field index was evaluated by one-way ANOVA. Statistical evaluation of the expression levels was performed on the log2 gene expression levels (dCt), and 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 a random factor. A Left Lateralization Index (LI) was calculated using the linear expression levels for the two hemispheres 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 social preference tests at one month of age. To account for the 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 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 a 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 animals 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 a 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 have described lateralized responses 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 assessed the behavioural 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 the survival of zebrafish larvae at 5 dpf and have confirmed VPA deleterious impact on the expression of several neurodevelopmental genes, recapitulating what was previously reported (Jacob et al., 2014; Baronio et al., 2018). We extended our previous studies and demonstrated significant changes in social interaction induced by the 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 in the adult brain 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 lateralized epithalamic gene expression in zebrafish, opening new perspectives to investigate 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-hour 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), which 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). Similar to the facial recognition responses towards familiar conspecifics shown by other fish species using visual cues in the face region (Kohda et al., 2015; Satoh et al., 2016; Hotta et al., 2017, 2019; Wang and Takeuchi, 2017; Kawasaka et al., 2019), zebrafish larvae and other fish species 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 et al., 1999, 2001; De Santi et al., 2001; Sovrano, 2004; Sovrano and Andrew, 2006). Moreover, zebrafish larvae show 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-hour VPA treatment groups show a reduction of the left visual field index, and decrease it to chance levels, indicating the neutralization of the behavioural lateralization in response to social cues.

In addition to the reduction of functional lateralization, we also demonstrate that embryonic VPA exposure induces changes in epithalamic genetic pathways (*lov*, *dex* and *ron*) associated with 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 strains, artificial selection or genetic manipulation (Barth et al., 2005; Facchin et al., 2009, 2015; Dadda et al., 2010). Interestingly, 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's behavioural 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. Further targeted behavioural evaluation should 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 sign 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, is highly expressed on the (left) side closely opposed to the parapineal (Gamse et al., 2003) and continues to be highly expressed on the left epithalamus into adulthood. 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 the right side having expression levels of *lov* similar to the left side (Figure 3B).

We also analyzed the expression of pallial genes associated with ASD and 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 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 compared to previous reports, one potential confound 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 effects 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). The differences in the asymmetrical distribution of the epithalamic markers upon VPA treatment may reflect neuroanatomical differences at the levels of neuronal subpopulations in combination to direct alterations of asymmetric gene expression. As for the pallial gene expression, a similar effect of VPA on neuroanatomically defined cell subpopulation may be taking place. Previous studies have shown changes in the 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 should clarify the effect of VPA on the development and the neuroanatomy of the epithalamus as well as of 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 reduced structural and functional cerebral asymmetry in ASD. This study analyzes the effect of VPA exposure, a compound used to model the core signs of ASD 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 suggest that one-micromolar doses of VPA are sufficient to neutralize both the functional lateralization and the genetic asymmetry, opening new perspectives to investigate altered 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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Corresponding author

Correspondence to Paola Sgadò.

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 begins at 5 hpf and lasts for 24 or 48 hours. The social preference test starts 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 (#) indicates 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 relative to the closest mirror. Bottom, scheme of the experimental timeline, VPA treatment begins at 5 hpf and lasts for 24 or 48 hours. The mirror test starts at 21 dpf. (B) Box and whisker plot (median, min to max) showing the left visual field index. The number sign (#) indicates 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 begins at 5 hpf and lasts for 24 hours. Gene expression is 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 (#) indicates 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 begins at 5 hpf and lasts for 24 hours. Gene expression is 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 (#) indicates significant departures of the left lateralization index from chance level (0.5), marked by the red line. $^{\#}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

References

Aizawa H, Goto M, Sato T, Okamoto H (2007) Temporally Regulated Asymmetric Neurogenesis Causes Left-Right Difference in the Zebrafish Habenular Structures. Developmental Cell 12:87–98.

Annett M (2002) Handedness and brain asymmetry: The right shift theory. New York, NY, US: Psychology Press.

Baronio D, Puttonen HAJ, Sundvik M, Semenova S, Lehtonen E, Panula P (2018) Embryonic exposure to valproic acid affects the histaminergic system and the social behaviour of adult zebrafish (Danio rerio). British journal of pharmacology 175:797–809 Available at: https://www.ncbi.nlm.nih.gov/pubmed/29235100.

Barth KA, Miklosi A, Watkins J, Bianco IH, Wilson SW, Andrew RJ (2005) fsi Zebrafish Show Concordant Reversal of Laterality of Viscera, Neuroanatomy, and a Subset of Behavioral Responses. Current Biology 15:844–850.

Burt DM, Perrett DI (1997) Perceptual asymmetries in judgements of facial attractiveness, age, gender, speech and expression. Neuropsychologia 35:685–693.

Concha ML, Burdine RD, Russell C, Schier AF, Wilson SW (2000) A Nodal Signaling Pathway Regulates the Laterality of Neuroanatomical Asymmetries in the Zebrafish Forebrain. Neuron 28:399–409.

Concha ML, Russell C, Regan JC, Tawk M, Sidi S, Gilmour DT, Kapsimali M, Sumoy L, Goldstone K, Amaya E, Kimelman D, Nicolson T, Gründer S, Gomperts M, Clarke JDW, Wilson SW (2003) Local Tissue Interactions across the Dorsal Midline of the Forebrain Establish CNS Laterality. Neuron 39:423–438.

Concha ML, Wilson SW (2001) Asymmetry in the epithalamus of vertebrates. Journal of Anatomy 199:63–84.

Dadda M, Domenichini A, Piffer L, Argenton F, Bisazza A (2010) Early differences in epithalamic left–right asymmetry influence lateralization and personality of adult zebrafish. Behavioural Brain Research 206:208–215.

Dawson G, Finley C, Phillips S, Lewy A (1989) A comparison of hemispheric asymmetries in speech-related brain potentials of autistic and dysphasic children. Brain and Language 37:26–41.

De Santi A, Sovrano VA, Bisazza A, Vallortigara G (2001) Mosquitofish display differential left- and right-eye use during mirror image scrutiny and predator inspection responses. Animal Behaviour 61:305–310.

Deemyad T, Puig S, Papale AE, Qi H, LaRocca GM, Aravind D, LaNoce E, Urban NN (2021) Lateralized Decrease of Parvalbumin+ Cells in the Somatosensory Cortex of ASD Models Is Correlated with Unilateral Tactile Hypersensitivity. Cerebral Cortex 32:554–568.

Demaree HA, Everhart DE, Youngstrom EA, Harrison DW (2005) Brain Lateralization of Emotional Processing: Historical Roots and a Future Incorporating “Dominance.” Behavioral and Cognitive Neuroscience Reviews 4:3–20.

Desjardins JK, Fernald RD (2010) What do fish make of mirror images? Biology Letters 6:744–747.

Domenichini A, Dadda M, Facchin L, Bisazza A, Argenton F (2011) Isolation and Genetic Characterization of Mother-of-Snow-White, a Maternal Effect Allele Affecting Laterality and Lateralized Behaviors in Zebrafish. PLoS ONE 6:e25972.

Donati G, Davis R, Forrester GS (2020) Gaze behaviour to lateral face stimuli in infants who do and do not receive an ASD diagnosis. Scientific Reports 10:13185.

Dreosti E, Lopes G, Kampff AR, Wilson SW (2015) Development of social behavior in young zebrafish. Frontiers in Neural Circuits 9:39.

Dundas E, Gastgeb H, Strauss MS (2012a) Left Visual Field Biases when Infants Process Faces: A Comparison of Infants at High- and Low-Risk for Autism Spectrum Disorder. Journal of Autism and Developmental Disorders 42:2659–2668.

Dundas EM, Best CA, Minshew NJ, Strauss MS (2012b) A Lack of Left Visual Field Bias When Individuals with Autism Process Faces. Journal of Autism and Developmental Disorders 42:1104–1111.

Dwivedi S, Medishetti R, Rani R, Sevilimedu A, Kulkarni P, Yogeeswari P (2019) Larval zebrafish model for studying the effects of valproic acid on neurodevelopment: An approach towards modeling autism. Journal of Pharmacological and Toxicological Methods 95:56–65.

Facchin L, Bisazza A, Vallortigara G (1999) What causes lateralization of detour behavior in fish? evidence for asymmetries in eye use. Behavioural Brain Research 103:229–234.

Facchin L, Burgess HA, Siddiqi M, Granato M, Halpern ME (2009) Determining the function of zebrafish epithalamic asymmetry. Philosophical Transactions of the Royal Society B: Biological Sciences 364:1021–1032.

Facchin L, Duboué ER, Halpern ME (2015) Disruption of Epithalamic Left–Right Asymmetry Increases Anxiety in Zebrafish. The Journal of Neuroscience 35:15847–15859.

Forrester GS, Davis R, Malatesta G, Todd BK (2020) Evolutionary motor biases and cognition in children with and without autism. Scientific Reports 10:17385.

Gamse JT, Kuan Y-S, Macurak M, Brösamle C, Thisse B, Thisse C, Halpern ME (2005) Directional asymmetry of the zebrafish epithalamus guides dorsoventral innervation of the midbrain target. Development 132:4869–4881.

Gamse JT, Shen Y-C, Thisse C, Thisse B, Raymond PA, Halpern ME, Liang JO (2002) Otx5 regulates genes that show circadian expression in the zebrafish pineal complex. Nature Genetics 30:117–121.

Gamse JT, Thisse C, Thisse B, Halpern ME (2003) The parapineal mediates left-right asymmetry in the zebrafish diencephalon. Development 130:1059–1068.

Gogolla N, Leblanc J, Quast K, Südhof T, Fagiolini M, Hensch T (2009) Common circuit defect of excitatory-inhibitory balance in mouse models of autism. J Neurodev Disord 1:172–181.

Grabrucker S, Haderspeck JC, Sauer AK, Kittelberger N, Asoglu H, Abaei A, Rasche V, Schön M, Boeckers TM, Grabrucker AM (2018) Brain Lateralization in Mice Is Associated with Zinc Signaling and Altered in Prenatal Zinc Deficient Mice That Display Features of Autism Spectrum Disorder. Frontiers in Molecular Neuroscience 10:450.

Halpern ME, Liang JO, Gamse JT (2003) Leaning to the left: laterality in the zebrafish forebrain. Trends in Neurosciences 26:308–313.

Hotta T, Kawasaka K, Satoh S, Kohda M (2019) Fish focus primarily on the faces of other fish. Scientific Reports 9:8377.

Hotta T, Satoh S, Kosaka N, Kohda M (2017) Face recognition in the Tanganyikan cichlid Julidochromis transcriptus. Animal Behaviour 127:1–5.

Jacob J, Ribes V, Moore S, Constable SC, Sasai N, Gerety SS, Martin DJ, Sergeant CP, Wilkinson DG, Briscoe J (2014) Valproic acid silencing of ascl1b/Ascl1 results in the failure of serotonergic differentiation in a zebrafish model of fetal valproate syndrome. Disease Models & Mechanisms 7:107–117 Available at: https://www.ncbi.nlm.nih.gov/pubmed/24135485.

Jones EJH, Venema K, Earl R, Lowy R, Barnes K, Estes A, Dawson G, Webb SJ (2016) Reduced engagement with social stimuli in 6-month-old infants with later autism spectrum disorder: a longitudinal prospective study of infants at high familial risk. Journal of Neurodevelopmental Disorders 8:7.

Jouravlev O, Kell AJE, Mineroff Z, Haskins AJ, Ayyash D, Kanwisher N, Fedorenko E (2020) Reduced Language Lateralization in Autism and the Broader Autism Phenotype as Assessed with Robust Individual-Subjects Analyses. Autism Research 13:1746–1761.

Karplus I, Algom D (1981) Visual Cues for Predator Face Recognition by Reef Fishes. Zeitschrift für Tierpsychologie 55:343–364.

Kawasaka K, Hotta T, Kohda M (2019) Does a cichlid fish process face holistically? Evidence of the face inversion effect. Animal Cognition 22:153–162.

Keehn B, Vogel-Farley V, Tager-Flusberg H, Nelson CA (2015) Atypical Hemispheric Specialization for Faces in Infants at Risk for Autism Spectrum Disorder. Autism Research 8:187–198.

Kjelgaard MM, Tager-Flusberg H (2001) An investigation of language impairment in autism: Implications for genetic subgroups. Language and Cognitive Processes 16:287–308.

Kohda M, Jordan LA, Hotta T, Kosaka N, Karino K, Tanaka H, Taniyama M, Takeyama T (2015) Facial Recognition in a Group-Living Cichlid Fish. PLoS ONE 10:e0142552.

Liu X, Zhang Y, Lin J, Xia Q, Guo N, Li Q (2016) Social Preference Deficits in Juvenile Zebrafish Induced by Early Chronic Exposure to Sodium Valproate. Frontiers in behavioral neuroscience 10:201.

Lombardo MV, Pierce K, Eyler LT, Carter Barnes C, Ahrens-Barbeau C, Solso S, Campbell K, Courchesne E (2015) Different Functional Neural Substrates for Good and Poor Language Outcome in Autism. Neuron 86:567–577.

MacNeilage PF, Rogers LJ, Vallortigara G (2009) Origins of the Left & Right Brain. Scientific American 301:60–67.

Markou P, Ahtam B, Papadatou-Pastou M (2017) Elevated Levels of Atypical Handedness in Autism: Meta-Analyses. Neuropsychology Review 27:258–283.

Masulli P, Galazka M, Eberhard D, Johnels JÅ, Gillberg C, Billstedt E, Hadjikhani N, Andersen TS (2022) Data-driven analysis of gaze patterns in face perception: Methodological and clinical contributions. Cortex 147:9–23.

McCleery JP, Akshoomoff N, Dobkins KR, Carver LJ (2009) Atypical Face Versus Object Processing and Hemispheric Asymmetries in 10-Month-Old Infants at Risk for Autism. Biological Psychiatry 66:950–957.

Messina A, Boiti A, Sovrano V, Sgadò P (2020) Micromolar Valproic Acid Doses Preserve Survival and Induce Molecular Alterations in Neurodevelopmental Genes in Two Strains of Zebrafish Larvae. Biomolecules 10.

Messina A, Boiti A, Vallortigara G (2021) Asymmetric distribution of pallial-expressed genes in zebrafish (Danio rerio). European Journal of Neuroscience 53:362–375.

Miletto Petrazzini ME, Sovrano VA, Vallortigara G, Messina A (2020) Brain and Behavioral Asymmetry: A Lesson From Fish. Frontiers in Neuroanatomy 14:11.

Nunes AR, Carreira L, Anbalagan S, Blechman J, Levkowitz G, Oliveira RF (2020) Perceptual mechanisms of social affiliation in zebrafish. Scientific Reports 10:3642.

Pickles A, Anderson DK, Lord C (2014) Heterogeneity and plasticity in the development of language: a 17-year follow-up of children referred early for possible autism. Journal of Child Psychology and Psychiatry 55:1354–1362.

Rogers LJ, Vallortigara G, Andrew RJ (2013) Divided Brains: The Biology and Behaviour of Brain Asymmetries. Cambridge University Press.

Satoh S, Tanaka H, Kohda M (2016) Facial Recognition in a Discus Fish (Cichlidae): Experimental Approach Using Digital Models. PLoS ONE 11:e0154543.

Shephard E, Milosavljevic B, Mason L, Elsabbagh M, Tye C, Gliga T, Jones E, Charman T, Johnson M, BASIS T (2020) Neural and behavioural indices of face processing in siblings of children with autism spectrum disorder (ASD): A longitudinal study from infancy to mid-childhood. Cortex 127:162–179.

Sovrano VA (2004) Visual lateralization in response to familiar and unfamiliar stimuli in fish. Behavioural Brain Research 152:385–391.

Sovrano VA, Andrew RJ (2006) Eye use during viewing a reflection: Behavioural lateralisation in zebrafish larvae. Behavioural Brain Research 167:226–231.

Sovrano VA, Bertolucci C, Frigato E, Foà A, Rogers LJ (2016) Influence of exposure in ovo to different light wavelengths on the lateralization of social response in zebrafish larvae. Physiology & Behavior 157:258–264.

Sovrano VA, Bisazza A, Vallortigara G (2001) Lateralization of response to social stimuli in fishes: A comparison between different methods and species. Physiology & Behavior 74:237–244.

Sovrano VA, Rainoldi C, Bisazza A, Vallortigara G (1999) Roots of brain specializations: preferential left-eye use during mirror-image inspection in six species of teleost fish. Behavioural Brain Research 106:175–180.

Stancher G, Sovrano VA, Vallortigara G (2018) Motor asymmetries in fishes, amphibians, and reptiles. Progress in brain research 238:33–56.

Sun T, Patoine C, Abu-Khalil A, Visvader J, Sum E, Cherry TJ, Orkin SH, Geschwind DH, Walsh CA (2005) Early Asymmetry of Gene Transcription in Embryonic Human Left and Right Cerebral Cortex. Science 308:1794–1798.

Tager-Flusberg H, Kasari C (2013) Minimally Verbal School-Aged Children with Autism Spectrum Disorder: The Neglected End of the Spectrum. Autism Research 6:468–478.

Toga AW, Thompson PM (2003) Mapping brain asymmetry. Nature Reviews Neuroscience 4:37–48.

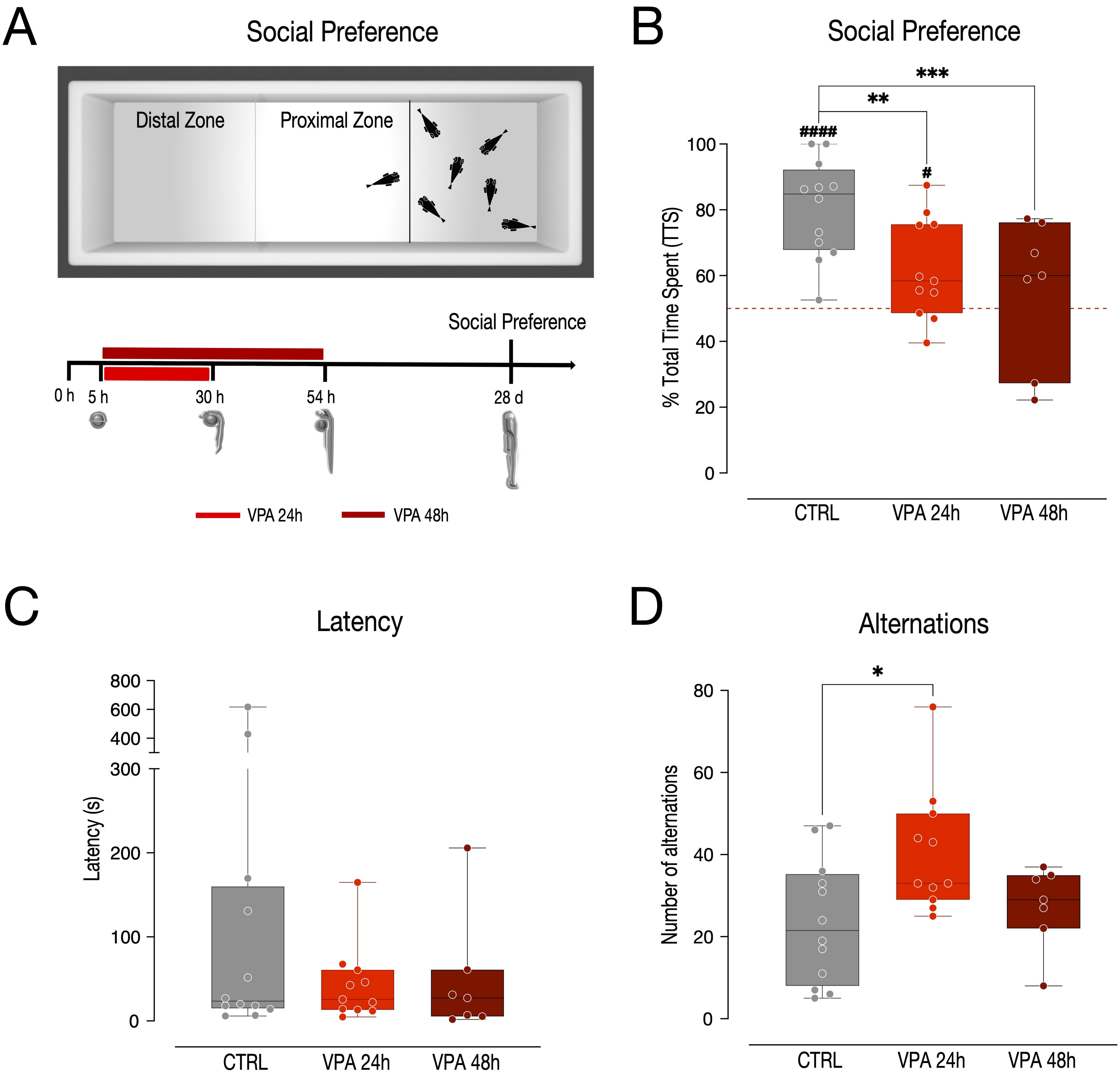
Wang M-Y, Takeuchi H (2017) Individual recognition and the ‘face inversion effect’ in medaka fish (Oryzias latipes). eLife 6:e24728.

Webb SJ, Dawson G, Bernier R, Panagiotides H (2006) ERP Evidence of Atypical Face Processing in Young Children with Autism. Journal of autism and developmental disorders 36:881–890 Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2989721/.

Webb SJ, Neuhaus E, Faja S (2017) Face perception and learning in autism spectrum disorders. Quarterly journal of experimental psychology (2006) 70:970–986.

Zimmermann FF, Gaspary KV, Leite CE, Cognato GDP, Bonan CD (2015) Embryological exposure to valproic acid induces social interaction deficits in zebrafish (Danio rerio):

A developmental behavior analysis. Neurotoxicology and Teratology 52:36–41
Available at: https://www.ncbi.nlm.nih.gov/pubmed/26477937.


A
Social Preference
Distal Zone
Proximal Zone
Social Preference
0 h
5 h
30 h
54 h
28 d
VPA 24h
VPA 48h
B
Social Preference
% Total Time Spent (TTS)
0
20
40
60
80
100
***
**
####
#
CTRL
VPA 24h
VPA 48h
C
Latency
Latency (s)
0
100
200
300
400
600
800
CTRL
VPA 24h
VPA 48h
D
Alternations
Number of alternations
0
20
40
60
80
*
CTRL
VPA 24h
VPA 48h

A
Mirror Test
Right eye
Left eye
Mirror Test
0 h
5 h
30 h
54 h
21 d
VPA 24h
VPA 48h
B
Left Visual Field Bias
Left Visual Field Index
1.0
0.8
0.6
0.4
0.2
0.0
####
****
****
CTRL
VPA 24h
VPA 48h

A
Lateral view
Thalamus
CTRL
VPA 24h
Thalamus
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)
4
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0
-2
-4
-6
-8
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*
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Left Lateralization Index
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Left
Right
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leftover
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right-on

A
Telencephalon
Lateral view
CTRL
VPA 24h
Telencephalon
0 h
5 h
30 h
Adult
VPA 24h
B
Expression levels
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CTRL Left
VPA 24h Right
VPA 24h Left
Expression level relative to housekeeping (log2)
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arrb2
fez
gap43
nipa1
nipa2
robo1
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Left Lateralization Index
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Right
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arrb2
fez
gap43
nipa1
nipa2
robo1