# Impact of enriched environment on retinal thickness in zebrafish exposed to valproic acid

## Impacto do ambiente enriquecido na espessura da retina em peixes-zebra expostos ao ácido valproico

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## ABSTRACT

**Objective:** Embryonic exposure to valproic acid is a widely used model for the study of neurodevelopmental disorders such as autism spectrum disorders. In zebrafish, early exposure to valproic acid results in the major behavioral, neurological, and genetic phenotypes of this disorder. Autism spectrum disorders have several associated symptoms, such as changes in sensory processing, sleep problems, and hyper- or hyposensitivity to light. This work investigates the hypothesis of changes at the retinal level due to embryonic exposure to valproic acid in zebrafish. In addition, the possible effects of non-pharmacological treatment with enriched environment are investigated.

**Methods:** Morphometric evaluations of the retina were performed using histological techniques to investigate possible changes in this key structure of the visual system.

**Results:** Our results show that animals exposed to valproic acid during embryogenesis exhibit changes in the thickness of the different layers of the retina in adulthood.

**Conclusions:** These results emphasize the teratogenic effect of valproic acid and suggest that retinal alterations may be related to changes in visual processing in autism spectrum disorders-valproic acid models.

## RESUMO

**Objetivo:** A exposição embrionária ao ácido valproico é um modelo amplamente utilizado para o estudo de distúrbios do desenvolvimento neurológico, como os distúrbios do espectro autista. Em peixes-zebra, a exposição precoce ao ácido valproico resulta nos principais fenótipos comportamentais, neurológicos e genéticos desse distúrbio. Os distúrbios do espectro autista têm vários sintomas associados, como alterações no processamento sensorial, problemas de sono e hiper ou hipossensibilidade à luz. Este trabalho investiga a hipótese de alterações no nível da retina devido à exposição embrionária ao ácido valproico no peixe-zebra. Além disso, são investigados os possíveis efeitos do tratamento não farmacológico com ambiente enriquecido.

**Métodos:** Avaliações morfométricas da retina foram realizadas usando técnicas histológicas para investigar possíveis alterações nessa estrutura fundamental do sistema visual.

**Resultados:** Nossos resultados mostram que animais expostos ao ácido valproico durante a embriogênese apresentam alterações na espessura das diferentes camadas da retina na idade adulta.

**Conclusões:** Esses resultados enfatizam o efeito teratogênico do ácido valproico e sugerem que as alterações na retina podem estar relacionadas a alterações no processamento visual em modelos de transtornos do espectro autista associado ao ácido valproico.

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## INTRODUCTION

Autism spectrum disorder (ASD) is a complex group of neurodevelopmental disorders characterized by impaired socialization, communication difficulties, and restricted or inflexible patterns of behavior and interests.[1] With an estimated worldwide prevalence of 1 in 100 people, it has become one of the focuses of attention of the global scientific community.[2] The classic diagnostic criteria for ASD are usually accompanied by one or more comorbidities, such as anxiety, changes in sleep-wake rhythm, and changes in sensory processing.[3] These differences in sensory processing, which can manifest as hypersensitivity (hyperreactive) or hyposensitivity (hyporeactive), are of particular interest as they may be one of the causes of the atypical response to various sensory stimuli.[4,5] However, more information is needed about these changes and their possible neurobiological substrates.

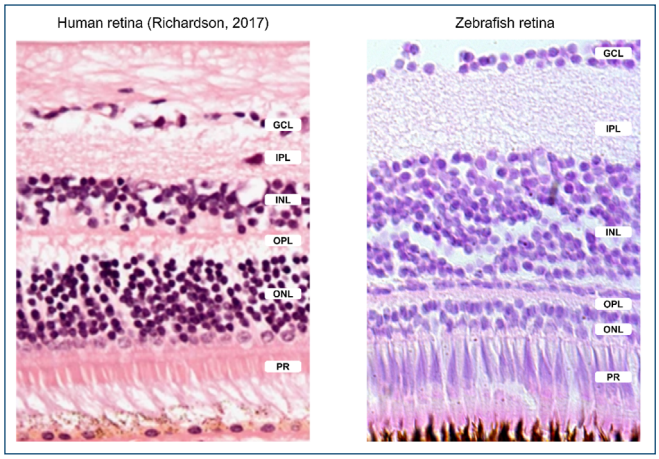
Currently, scientific research on this disorder is supported by various animal models generated from different methodologies. In this sense, one of the classic methods to induce characteristic phenotypes of ASD is embryonic or early exposure to teratogenic drugs, such as valproic acid (VPA). Valproic acid is a widely used drug to stabilize GABAergic activity for the treatment of epilepsy and mood disorders. However, exposure during critical periods of neurodevelopment can alter proper gene expression, as VPA acts as an inhibitor of *histone deacetylase,* which is involved in genetic regulation.[6,7] For this reason, prenatal or early exposure to VPA has been associated with various neurodevelopmental disorders, such as ASD, and it is also significantly associated with a higher incidence of comorbidities.

Recently, the validity of the VPA-induced model in zebrafish was investigated at the behavioral, neurological, and genetic levels. The results are like those observed in humans with ASD and in other species exposed to the same drug.[8-10]

Zebrafish exposed to VPA were found to have deficits in social behavior, locomotor disorders, increased anxiety, disorders of sexual behavior, sensory changes, and sleep behavior.[8,11-14] In addition, fish exposed to VPA exhibit neuroanatomical and cellular changes similar to those of human ASD, including reduced cerebellar and telencephalon cell numbers, reduced cerebellar volume, changes in cell proliferation, and neurochemical changes in the inhibitory/excitatory system.[15-17] Significant changes in gene expression were also observed after prenatal exposure to VPA, particularly in genes associated with CNS formation and synaptic function.[18-20]

The relationship between the visual system and alterations in sensory processing has recently been explored in this model. In this sense, embryonic exposure to VPA led to delays in retinal and optic nerve formation and cell differentiation in 5dpf larvae. These morphological and molecular changes were also accompanied by disturbances in sleep behavior in 15dpf larvae.[11,21] However, the eye morphology of zebrafish at later developmental stages and at different times of VPA exposure to VPA is still poorly understood.

This species has behavioral, neuroanatomical, molecular, and genetic homologies that make it an excellent model for studying various aspects of ASD, including comorbidity related to visual sensory processing. In this sense, fish and humans have a very similar retinal shape and physiology (Figure 1). Both species are diurnal, have the same number of layers that make up the retina, and have a similar density of photoreceptors.[22] The major cell classes found in both human and zebrafish retinas are arranged in a similar layered pattern, with light-sensitive photoreceptors occupying the outermost layer and ganglion cells, the retina's projection neurons, situated in the innermost neuronal layer, closest to the lens. Between the layers of photoreceptors and ganglion cells lie the retinal interneurons, also known as the bipolar and horizontal amacrine cells.[23]




Source: Richardson et al.[22]
GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; PR: photoreceptor.

**Figure 1.** Comparison of human retinal cytoarchitecture and adult zebrafish. The left image shows a histological section of the human retina stained with hematoxylin & eosin obtained from Richardson et al.[22] On the right side, a histological section of the adult zebrafish retina stained with hematoxylin & eosin was obtained from our work.

The genetic and biological homology, as well as the technical and economic advantages of working with

these species, make it a model of what is widely used in the fields of biomedical sciences, neuroscience, and the study of ocular pathologies.[24-26]

On the other hand, an important aspect of basic research into ASD is the question of possible treatments that improve the quality of life of people with this disorder. In this sense, one of the non-pharmacological treatments with promising results at the behavioral, cellular and molecular levels in the treatment of ASD in humans and laboratory animal models is sensory stimulation. In the laboratory, we can study the impact of sensory stimulation through captivity in enriched environments (EE). The concept of EE refers to changes in the captive environments of any species that increase exposure to sensory and social stimuli, which in turn promotes the development of complex behaviors.[24-26] In this sense, EE has been shown to significantly improve behavioral, cellular, and molecular aspects in both normally developing animals and ASD animal models.[15,27-29]

The aim of this study was to investigate the effects of embryonic exposure to VPA on the retina of adult zebrafish. The focus was on analyzing the thickness of the retinal layers using a morphometric approach. We also investigated the hypothesis of the effect of AD treatment from hatching to adulthood on the retina of VPA fish as a protective factor for retinal morphology.

## METHODS

### Animals

Wild-Type *Danio rerio* zebrafish strain bred at the Instituto de Investigaciones Cerebrales (IICE) (Brain Research Institute) were used, according to previously established protocols for use in the zebrafish laboratory.[30] The fish were maintained in clear glass containers with a capacity of 3.5 L at a temperature of 25º to 28ºC and a light-dark photoperiod of 14:10 and fed twice daily with fish food in flake form (Tetra Color®). All protocols and procedures performed in this work were submitted and approved by the Bioethics Committee of the IICE (CICUAL), code 2021-012a approved on April 20, 2022.

### Obtaining fertilized eggs

To obtain fertilized eggs, males and females were grouped in a 2:1 ratio in a double-bottomed glass container with a capacity of 3.5L at a temperature between 25º and 28ºC at 2 p.m., and the next day, at 12:00 a.m., the fertilized eggs were collected.

### Conformation of experimental groups

The collected embryos were divided equally and randomly into two groups for drug exposure and the control group. The 0 hpf embryos were transferred to 6-well cell culture boxes with a capacity of 12 mL. They were evenly distributed to maintain a density of 15 embryos/12 mL for the formation of the experimental groups according to the protocols established in our laboratory.[15]

### Drug exposure

Pharmacological exposure was performed according to previously established protocols and replicated in our laboratory.[14] 12 mL of AVP solution (Sigma-Aldrich, Naucalpan, Mexico) was applied diluted with system water at a concentration of 48 µm, while the same procedures were performed for the control group, but with system water only. After 24 hours, the solution was replaced, and the animals remained under these conditions until 48 hpf. Subsequently, the remains of the AVP were washed with stock water.

### Environmental treatment

After hatching the larvae, the AVP and Control groups were divided equally to form 4 experimental groups: Control Group, EE Group, AVP Group, and EE + VPA Group. Each experimental group consisted of n= 3 for a total of n=12. The environmental treatment for the groups with EE was conducted in 3.5 L clear glass fish tanks and in which structures and accessories such as plastic plants, substrate stones, bubble systems, colored toys, and plastic caves were added and changed once a week to maintain novelty.[12,15] The amount of structural aggregates in EE fish tanks was calculated to occupy one-third of the total volume of the fish tank.[12] In these groups, feeding was also varied twice a week with hatched brine shrimp and microalgae for commercial use. In contrast, in the control groups, the fish were housed in 3.5 L containers covered with a semi-transparent paper to reduce external stimuli, and none of the above materials were added. All experimental groups were kept under these conditions for up to 120dpf.

### Euthanasia and tissue processing

At 120 dpf, all experimental groups were euthanized due to temperature reduction, following protocols previously described and replicated in our laboratory.[31] The zebrafish were placed in a small fish tank containing tap water in a cool box with ice. A thermometer was used to measure the temperature until it dropped to 4ºC. After 15 minutes,

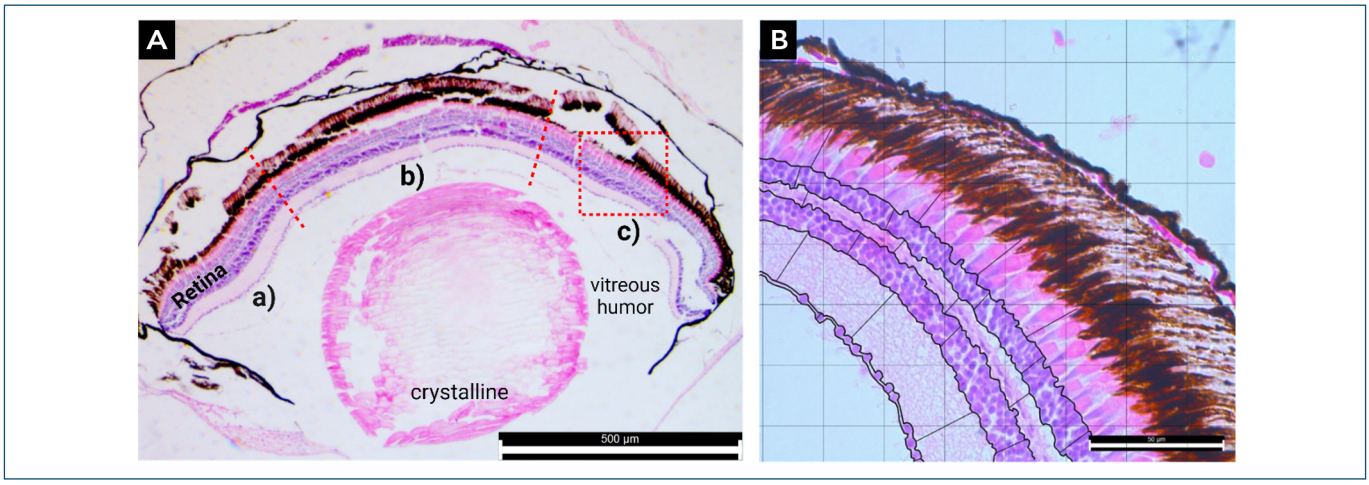



**Figure 2.** (A) Photomicrograph of a sagittal section of the entire eye of an adult zebrafish, stained with hematoxylin and eosin (4X). (a) Distal part of the retina. (b) Central part of the retina. (c) Distal part of the retina (repeated). The red dotted lines show the virtual division of the retina into three sections. The red dotted box delimits the region selected for retinal analysis. (B) Photomicrograph of the zebrafish retina stained with hematoxylin and eosin (40X) with a dissector rack created in ImageJ software superimposed. The black lines show the subdivisions of the different layers that make up the retina.

the eye, tail, and heartbeat reflexes were examined using dissecting forceps. The fish was then decapitated with a diagonal cut between the operculum and the pectoral fin, to facilitate manipulation of the head to remove the eyes. The rest of the organism was preserved for future research. Using a dissecting microscope (Zeiss) and dissecting forceps, the scales around the eye were removed and an incision was made at the level of the posterior eye fold to carefully remove the eyes. The tissue sample was placed in 4% paraformaldehyde (Sigma-Aldrich, 30525-89-4) for 48 hours. The tissue was then treated with ascending concentrations of sucrose (10%, 20% and 30%) for 24 hours and stored in sucrose at 30% and 4°C until processing.

## Retinal evaluation

Zebrafish eyes were soaked in paraffin and sectioned with a rotary microtome (Leica® RM2125) at 8 µM. The sections were spotted on gelatinized slides and stained with the histological technique of hematoxylin and eosin. These histological sections were analyzed to determine the cytoarchitecture of the retina. Photographs were taken using an OLYMPUS microscope with objectives ranging from 10X to 40X. The images were processed, and the dimensions of the retinal layers were calculated using ImageJ software (Fiji).

To measure the retinal layers, the histology of the eye was divided into three sections: the left distal part, the central part, and the right distal part (Figure 2). Bioimages of the central and one distal section were selected. Six layers of the retina were then delineated in each section using Fiji software, a 500 µm² grid was randomly applied and passed through the grid, and the width of each layer in between was measured.

## Statistical analysis

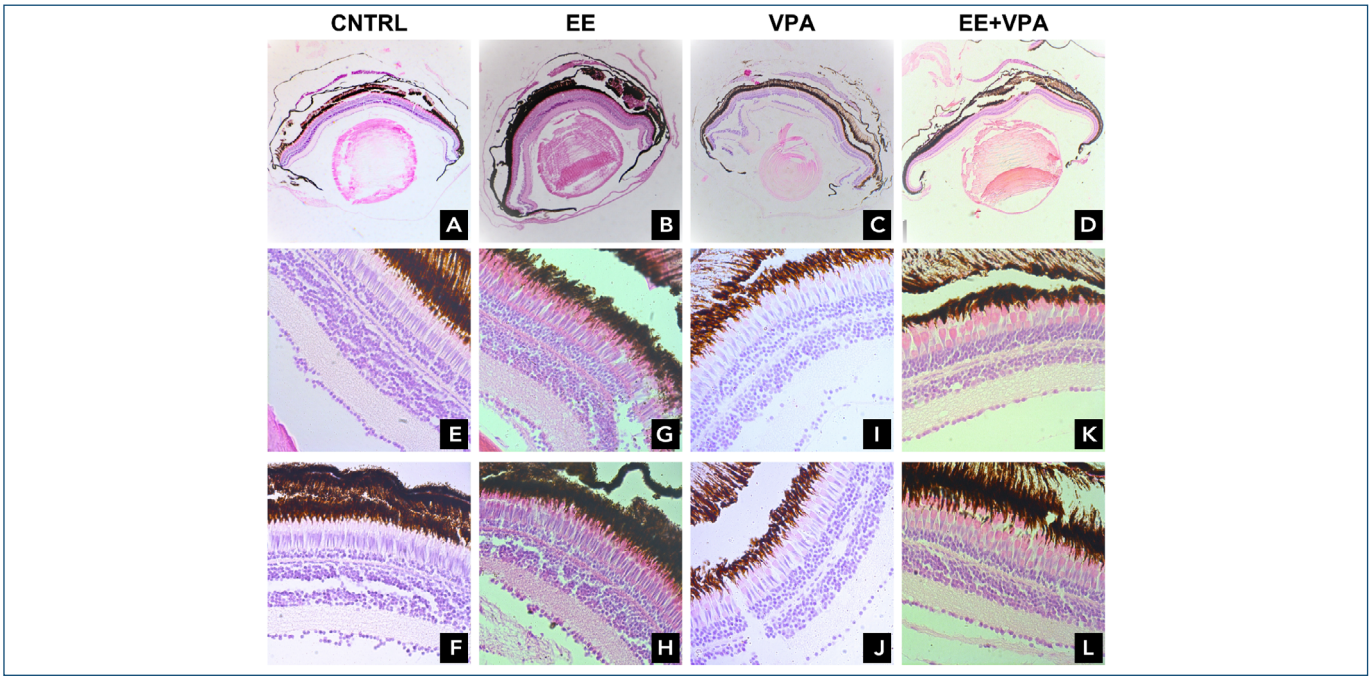
For the statistical analysis, the average values of the individual layers were calculated using Microsoft Excel software and compared with the Prism Graph statistical software (version 8.0.2). Statistical tests of analysis of variance (Anova) and a Tukey multiple comparison test were performed. All values were considered significant if $p < 0.05$.

# RESULTS

The qualitative comparison of retinal cytoarchitecture between the different experimental groups did not show significant differences in the order or composition of the retina under any of the treatments. The different layers that make up the retina were observed in the same order, and no significant absences or changes were shown that would suggest dramatic effects on the cellular composition of either layer. This suggests that none of the treatments (VPA, EE or their combination) has an obvious impact on retinal cytoarchitecture at the macroanatomical histological level (Figure 3).

## Ganglion cell layer statistical analysis results

A two-way Anova was performed to evaluate the effects of VPA, EE, and their combination on central and distal




CTRL: control; EE: enriched environment; VPA: valproic acid.

**Figure 3.** Representative images of histological sections of the retina of zebrafish from the different experimental groups, stained with hematoxylin and eosin. (A) Histological section of the entire eye of a fish with typical development at 10X. (B) Histological section of the complete eye of a fish treated with EE at 10X. (C) Histological section of the complete eye of a fish exposed to valproic acid at 10X. (D) Histological section of the complete eye of a fish exposed to valproic acid and treated with AD at 10X. (E) Histological section of the central retinal region of a fish with typical development (F) Cutting of the distal region of the retina of a fish typically developing at 40X. (G) Cutting of the central region of the retina of a fish treated with AD at 40X. (H) Cutting of the distal region of the retina of a fish treated with AD at 40X. (I) Cutting of the central region of the retina of a fish exposed to valproic acid at 40X.(J) Cutting of the region K) Slice of the central retinal region of a fish exposed to valproic acid at 40X. L) Cut of the distal retinal region of a fish exposed to valproic acid and treated with EA at 40X. L) Cut of the distal region of the retina of fish exposed to valproic acid and treated with EE at 40X.

ganglion cell layer (GCL) thickness of zebrafish. In the central part, the analysis indicated that the treatments had a significant effect on the thickness of this layer, explaining 69.15% of the total variability observed ($F(3,6) = 7.634$; $p = 0.0180$). This suggests that experimental conditions significantly influence the thickness of this retinal layer. On the other hand, variability between individuals did not present a significant effect ($F(2,6) = 2.108$; $p = 0.2026$), indicating that individual differences do not explain the observed differences.

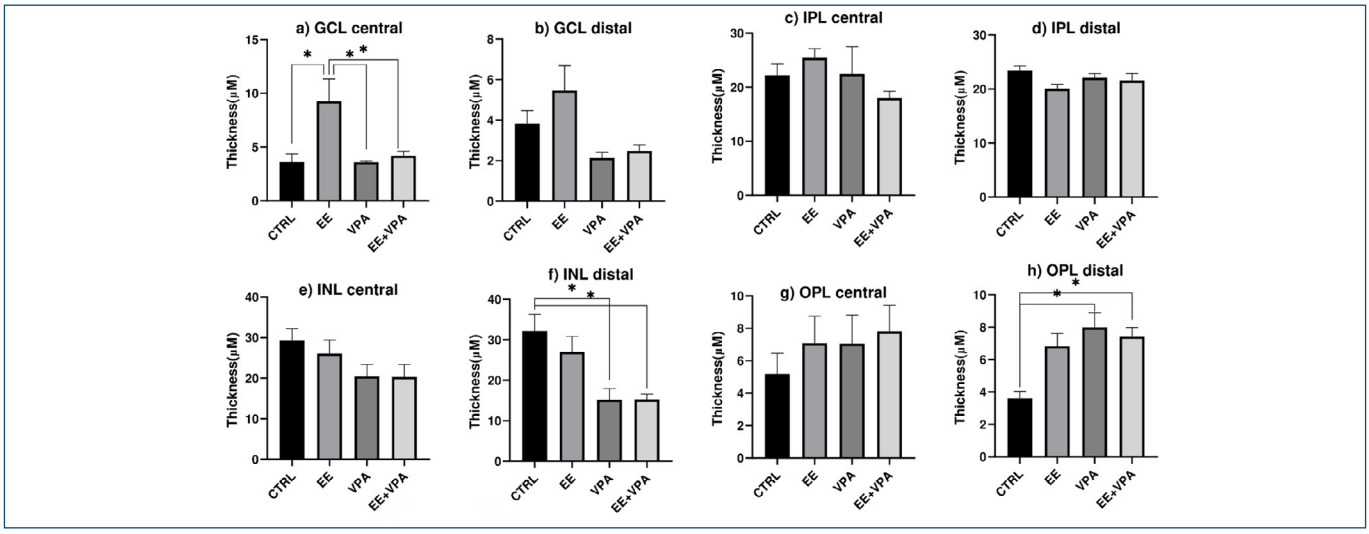
Tukey's post-hoc test revealed a significant decrease in central GCL thickness in the AD group compared to the control group (mean difference = -5.658, 95% CI = -10.53--0.7850; $p = 0.0267$). Similarly, the thickness of the central GCL was greater in the AD group compared to the VPA group (mean difference = 5.685, 95% CI = 0.8120-10.56; $p = 0.0262$). A significant difference was also found between the AD and EE + VPA groups (mean difference = 5.070, 95% CI = 0.1970-9.944; $p = 0.0426$).

Regarding the thickness of the GCL layer in the distal part of zebrafish, the results showed that the treatment factor had a significant effect on thickness, explaining 62.02% of the total variability ($F(3,6) = 4.827$; $p = 0.0485$). This suggests that experimental conditions significantly influence the thickness of this layer. On the other hand, variability between individuals did not present a significant effect ($F(2,6) = 1.434$; $p = 0.3096$), showing that individual differences between fish do not considerably explain the observed variation.

Tukey's post-hoc comparisons revealed no significant differences between the groups evaluated in the distal part of the GLC (Figure 4).

## Results of the statistical analysis of the inner plexiform layer

The results obtained from the two-way Anova analysis on the central IPL layer of zebrafish exposed to VPA and AD show that there were no significant effects of both treatment and individual differences. The analysis revealed that the treatment explains 28.89% of the total variation, with an F value of 1.061 and a p-value of 0.4324. The individual factor effect was responsible for 16.68% of the




* Represents a difference of less than 0.05. The data is displayed as the mean + SEM. CTRL: control; EE: enriched environment; VPA: valproic acid; GCL: ganglionic cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer.

**Figure 4.** Comparison of retinal layer thickness by Tukey's two-way and post-hoc analysis of variance.

total variation, with an F value of 0.9192 and a p-value of 0.4485.

Regarding the distal part of the IPL layer, the results show that there were no significant effects of both treatment and individual differences. The treatment explains 45.13% of the total variation, with an F value of 2.516 and a p-value of 0.1550, while the individual factor was responsible for 19.00% of the total variation, with an F value of 1.589 and a p-value of 0.2794, indicating that neither treatment nor individual differences showed a significant impact on outcomes.

## Results of the statistical analysis of central inner nuclear layer

A two-way Anova was performed to compare the thickness of inner nuclear layer (INL) in the central portion in zebrafish. The analysis showed that the treatment only explains 44.11% of the total variability observed ($F(3,6) = 2.339$; $p = 0.1729$) and that the variability between individuals accounts for 18.18% of the total variability ($F(2,6) = 1,446$; $p = 0.3073$).

These results show that neither VPA treatment nor EE, nor their combination, have a significant effect on central INL thickness in zebrafish.

Regarding the distal part of the INL, the analysis revealed that the treatment had a significant effect on the thickness of the INL, explaining 72.98% of the total variability observed ($F(3,6) = 7.578$; $p = 0.0183$). On the other hand, variability between individuals did not present a significant effect ($F(2,6) = 1.209$; $p = 0.3622$), which shows that the differences are influenced by the different experimental treatments.

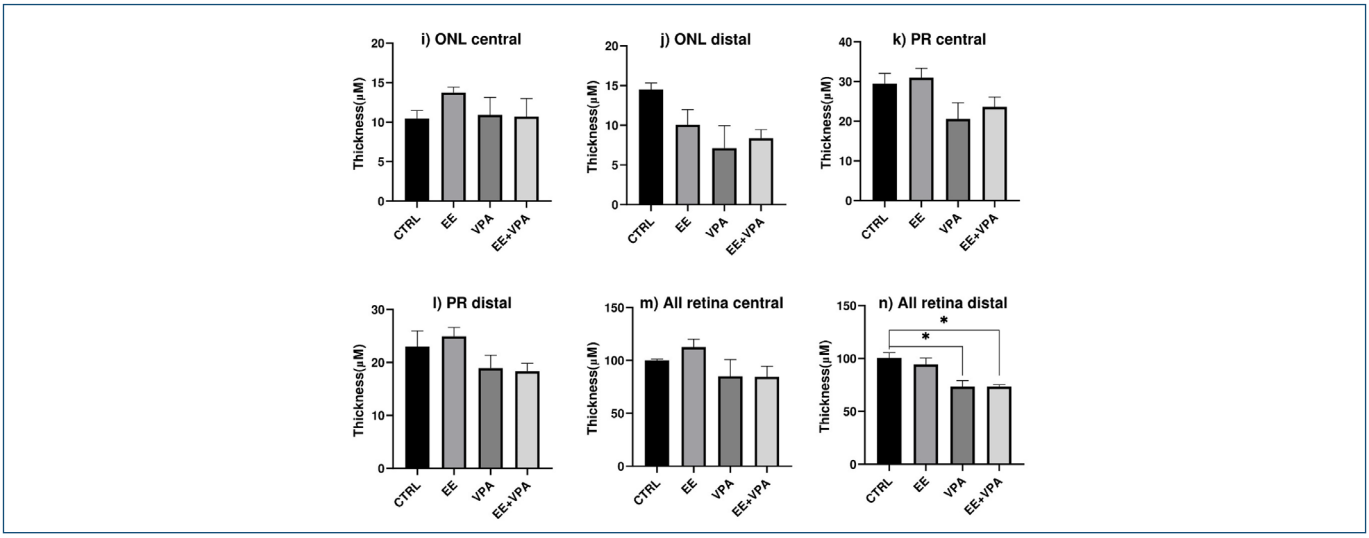
Post-hoc testing shows a significant increase in INL thickness in its distal part in the control group compared to the VPA group (mean difference = 16.99, 95% CI = [1.779, 32.21]; $p = 0.0316$). A significant difference was also found between the control and EE + VPA groups (mean difference = 16.90, 95% CI = [1.687, 32.12]; $p = 0.0324$).

## Outer plexiform layer statistical analysis results

Statistical analysis of the central portion of outer plexiform layer (OPL) revealed that the treatment had no significant effect on thickness, explaining only 15.94% of the total variability observed ($F(3,6) = 0.5557$; $p = 0.6631$). On the other hand, variability between individuals did not present a significant effect either ($F(2,6) = 1.397$; $p = 0.3176$), showing that individual differences between fish do not explain a significant part of the observed variation. Both VPA treatment and AS, either alone or in combination, showed no significant effects on central OPL compared to control.

On the other hand, the analysis of the distal portion of OPL showed that the treatment had a significant effect on thickness, explaining 75.28% of the total variability observed ($F(3,6) = 6.962$; $p = 0.0222$), while the individual factor did not present a significant effect ($F(2,6) = 0.4298$; $p = 0.6692$).

Tukey's post-hoc analysis revealed a significant decrease in distal OPL thickness in the control group compared to the VPA group (mean difference = -4.375, 95% CI = [-8.008, -0.7409]; $p = 0.0228$). A significant difference was also found between the control and EE + VPA groups (mean difference = -3.812, 95% CI = [-7.446, -0.1782]; $p = 0.0412$).




* Represents a difference of less than 0.05. The data is displayed as the mean +SEM.
ONL: outer nuclear layer; PR: photoreceptors layer; CTRL: control; EE: enriched environment; VPA: valproic acid.

**Figure 5.** Comparison of retinal layer thickness by Tukey's two-way and post-hoc Anova.

VPA treatment, either alone or in combination with EE, showed significant effects on distal OPL thickness compared to control.

## Outer Nuclear Layer statistical analysis results

The analysis of the central portion of ONL showed that the treatment did not have a significant effect, explaining only 23.57% of the total variability observed ($F(3,6) = 1.172$; $p = 0.3956$), in the same way the individual factor did not present a significant effect ($F(2,6) = 2,700$; $p = 0.1458$).

Neither VPA treatment nor an enriched environment nor their combination showed significant effects on the thickness of ONL in its central portion of the retina compared to the control group (Figure 5).

Similarly, the analysis of ONL in the distal portion showed that the treatment was not significant, explaining 53.59% of the total variability observed ($F(3,6) = 2.590$; $p = 0.1482$) as well as the individual differences ($F(2,6) = 0.3645$; $p = 0.7089$), indicating that no changes were found in this portion of the layer.

## Results of the statistical analysis of the photoreceptor

Analysis of the central photoreceptor (PR) layer of zebrafish showed that the treatment had a significant effect, explaining 50.74% of the total variability observed ($F(3,6) = 5.203$; $p = 0.0416$). This suggests that experimental conditions significantly influence the thickness of this retinal layer and cannot be explained by individual differences ($F(2,6) = 4.578$; $p = 0.0620$). However, the post hoc analysis did not reveal significant differences when comparing the different experimental groups.

Regarding the distal portion of PR, the results showed that the treatment did not have a significant effect, explaining 43.40% of the total variability observed ($F(3, 6) = 1.540$; $p = 0.2981$) although they are not explained by individual differences since no significance was observed ($F=0.01254$ and $p=0.9876$).

## Results of the statistical analysis of the entire retina

Statistical analysis of the complete retinal thickness in the central portion showed that the treatment had no significant effect, explaining 40.11% of the total variability observed ($F(3, 6) = 3.233$; $p = 0.1030$). Regarding the individual factor, a non-significant effect was also observed, with an F value of 4.241 and a p-value of 0.0711, indicating that individual differences between fish do not contribute significantly to the observed variation in the thickness of the central layer of the retina.

However, analysis of the thickness of the entire retina in its distal part shows that the treatment had a significant effect on retinal thickness, explaining 75.61% of the total variability observed ($F(3, 6) = 8.825$; $p = 0.0128$). In contrast, the individual factor showed a non-significant effect, with an F value of 1.270 and a p-value of 0.3469, showing that individual differences between fish do not contribute significantly to the observed variation in distal retinal thickness.

The post hoc test demonstrates a significant difference between the VPA group and the control group (mean

difference = 27.16, 95% CI = [3.910, 50.42]; p = 0.0260), as well as between the EE + VPA and control groups (mean difference = 27.15, 95% CI = [3.894, 50.40]; p = 0.0261).

## DISCUSSION

Embryonic exposure to VPA is frequently used as a trigger of ASD-like phenotypes at behavioral, cellular and genetic levels in various species. In this study, we investigate the possibility of structural changes in the retina of zebrafish exposed to VPA during early embryogenesis. Analysis of the retina focused on evaluating the thickness of the central and distal zones of each layer that make up the structure, as well as the full thickness considering all layers.

Previous work in mouse models has shown that early exposure to VPA at doses equivalent to those used in this work causes changes in visual behavior as well as in synaptic, glutamatergic, and GABAergic expression in the retina.[32] Similarly, alterations in the GABAergic signal produced by a photonic stimulus have been observed in ASD human individuals.[33] These studies suggest that alterations in ASD sensory processing could be due to substantial differences in retinal neurochemistry. In our work, we did not analyze visual function through behavioral tests, nor the expression of proteins related to retinal functioning, although it would be worthwhile to explore possible explanations for the structural changes identified in different layers of the retina.

Regarding retinal cytoarchitecture, studies conducted with humans have found significant thinning in the nearby ellipsoid zone, as well as in the macula and ONL, by optical coherence tomography (OCT) analysis.[34,35] However, no differences were found in the population of photoreceptor cells in the retina of ASD individuals when compared to typically developing individuals, suggesting that alterations in these retinal layers could be related to alterations in sensory processing at the level of photonic signal transduction that sensitizes photoreceptors. In our work, we did not analyze the population of photoreceptors; however, it would be worthwhile to investigate whether what was observed in human individuals is congruent with our model of pharmacological exposure.

The qualitative analysis of the morphology of the retinal layers did not identify appreciable changes in the conformation of the structure. Therefore, the idea that pharmacological exposure could cause dramatic changes such as the absence of or decomposition of the retinal layers in the typical histological order is discarded.

About changes in retinal thickness, different studies have detected thinning of the retina in autistic individuals in an analogous way to the findings of the present work. OCT analysis of 34 ASD adults detected significant thinning of the ONL layer when compared to typically developing individuals.[35] Our results identified significant thinning linked to GCL and INL drug exposure, as well as of the entire retina, while a thickening of the OPL layer was observed. GCL contains ganglion cells and amacrine cells. Generally, the dendrites of the smaller GCLs are arborized in the IPL, while the dendrites of the larger GCLs are arborized in other layers.[23] In this sense, the thinning of INL is consistent with this result because it is mainly formed from fibers from the retinal ganglion cells. Therefore, changes in the cellularity of this layer could imply changes in the function of transmitting information to the CNS.

The changes observed in the present work are likely due to the disruptive action of VPA during the period of cell proliferation and differentiation of the layers that make up the retina and that come from the same embryonic layer that will give rise to CNS. The impact of VPA on these cellular processes has been linked to the effect it exerts on the histone deacetylase system through the inhibition of histone deacetylase, which is crucial for the correct gene expression prior to the closure of the neural tube.[6,17,36,37]

On the other hand, the impact of AD treatment at the behavioral, cellular, and molecular levels has been described as protective or compensatory to the changes induced by VPA in animal models.[15,25,27,29] However, in our study, we did not see changes related to AD treatment that suggest that it has a positive or blocking effect at the retinal level. It is likely that early eye formation is not sensitive to environmental changes that drive positive or protective changes in compensation for pharmacological exposure due to its poor plastic capacity. However, specific studies are needed to evaluate this hypothesis in depth.

In conclusion, the model of induction of ASD-type phenotypes by embryonic exposure to VPA in zebrafish manages to partially reproduce the retinal alterations reported in ASD individuals and other animal models implemented in species such as rats and mice. The EE had little or no effect on the retina; however, it is not ruled out that its positive effects at the behavioral and CNS levels lead to improvements in visual processing at other levels. The results of the present work provide valuable information for the ASD-VPA model in zebrafish and the changes induced by this drug in the visual system at the retinal level.

## AUTHORS' CONTRIBUTION

Mariana Aguirre-Rebolledo: Conceptualization, Investigation; Writing – original draft.

Maria Rebeca Toledo-Cardenas: Supervision; Writing – review & editing.

Elizabeth Valero-Pacheco: Writing – review & editing; Technical guidance.

Ana María Aguirre-Martínez: Writing – review & editing; Technical guidance.

Bernardo Flores-Prieto: Conceptualization; Supervision; Investigation; Writing – original draft.

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