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Estrogenic regulation of *claudin 5* and *tight junction protein 1* gene expression in zebrafish: A role on blood–brain barrier?

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Abstract

The blood–brain barrier (BBB) is a physical interface between the blood and the brain parenchyma, playing key roles in brain homeostasis. In mammals, the BBB is established thanks to tight junctions between cerebral endothelial cells, involving claudin, occludin, and zonula occludens proteins. Estrogens have been documented to modulate BBB permeability. Interestingly, in the brain of zebrafish, the estrogen-synthesizing activity is strong due to the high expression of Aromatase B protein, encoded by the *cyp19a1b* gene, in radial glial cells (neural stem cells). Given the roles of estrogens in BBB function, we investigated their impact on the expression of genes involved in BBB tight junctions. We treated zebrafish embryos and adult males with 17β-estradiol and observed an increased cerebral expression of *tight junction* and *claudin 5* genes in adult males only. In females, treatment with the nuclear estrogen receptor antagonist ($ICI_{182,780}$) had no impact. Interestingly, telencephalic injuries performed in males decreased *tight junction* gene expression that was partially reversed with 17β-estradiol. This was further confirmed by extravasation experiments of Evans blue showing that estrogenic treatment limits BBB leakage. We also highlighted the intimate links between endothelial cells and neural stem cells, suggesting that cholesterol and peripheral steroids could be taken up by endothelial cells and used as precursors for estrogen synthesis by neural stem cells. Together, our results show that zebrafish provides an alternative model to further investigate the role of steroids on the expression of genes involved in BBB integrity, both in constitutive and regenerative physiological conditions. The link we described between capillaries endothelial cells and steroidogenic neural cells encourages the use of this model in understanding the mechanisms by which peripheral steroids get into neural tissue and modulate neurogenic activity.

KEYWORDS
blood–brain barrier, claudin, estradiol, injury, regeneration zebrafish, tight junction protein

1 | INTRODUCTION

The blood–brain barrier (BBB) is a physical interface between the blood and the brain parenchyma. It plays a crucial role in maintaining brain homeostasis and normal neuronal activities within the central nervous system. The main characteristic of the BBB is its highly selective and regulated permeability that controls the entrance of many potentially harmful molecules, cells, and/or pathogens traveling through the bloodstream. In mammals, the BBB is anatomically composed of multiple cell types (mainly endothelial cells, pericytes, and astrocyte endfeet) organized in an interdependent and functional network, isolating the nervous tissue from systemic blood circulation (Abbott et al., 2010; O'Brown et al., 2018).

Tight junctions localized at the luminal side of endothelial cells are anchoring molecular networks including transmembrane (claudins, occludins, and adherent junction molecules), cytoplasmic (zonula occludens or tight junction protein -tjp-), and cytoskeleton proteins (actin). Claudins are a family of transmembrane proteins composed of more than 27 members. One of them, Claudin 5 (encoded by the *cdln5* gene), is of major importance in the sealing properties of tight junctions (Furuse et al., 1998; Tsukita & Furuse, 1999; Turksen & Troy, 2004; Wolburg & Lippoldt, 2002). In addition, several cytoplasmic proteins such as zonula occludens (tight junction protein) accumulate close to the membrane of cerebral endothelial cells (Itoh et al., 1999; Mitic & Anderson, 1998; Stevenson et al., 1986). For instance, zonula occludens 1 encoded by *tjp1* gene serve as scaffold molecules connecting transmembrane proteins such as Claudin 5 to actin from the cytoskeleton. Gain and loss of function studies have demonstrated the major role played by these proteins in BBB integrity. Overexpression of Claudin 5 leads to a decrease in paracellular permeability (Ohtsuki et al., 2007). In contrast, decreasing Claudin 5 expression using siRNA approach in a model of human brain endothelial cell culture resulted in increased BBB permeability (Luissint et al., 2012), also observed in Claudin 5 deficient mice (Nitta et al., 2003). Similarly, Tjp1 knock-out caused severe defects in vascular development leading to embryonic lethal mice severe defects in vascular development leading to embryonic lethal mice (Katsuno et al., 2008).

Numerous reports have highlighted the regulatory effects of hormones on the brain, particularly on BBB permeability and integrity. Steroid receptors (membrane and intracellular receptors) have been described at the level of brain endothelial cells and have been shown to regulate the expression of tight junction proteins by nongenomic and genomic mechanisms (Katsuno et al., 2008). The effects of estrogen signaling were reported in in vitro and in vivo models. Thus, murine endothelial cell lines treated with 17β-estradiol (E2) exhibit a stronger transendothelial electric resistance mediated due to increased Claudin 5 expression and so barrier tightness (Burek et al., 2014). As well, the role of endogenous estrogen in BBB function is highlighted by studies bringing out a close relationship between BBB integrity failure with age and circulating estrogen decline (Burek et al., 2014). Furthermore, increased BBB leakage induced by ischemia in brain endothelial cell cultures and rodent animal models (Na et al., 2015; Shin et al., 2016; Xiao et al., 2018) is abolished by E2 treatment that exerts neuroprotective effects by rescuing tight junction protein expression (Lu et al., 2016; Shin et al., 2013, 2016).

Interestingly, several studies suggest that sex steroids, including E2, could be incorporated into high-density lipoprotein (HDL) particles, suggesting the capacity for these lipoproteins to transport and deliver hormones to the tissue (Höckerstedt et al., 2004; Leszczynski & Schafer, 1991; Tikkanen et al., 2002). HDLs represent a class of lipoproteins whose primary function is to ensure the reverse transport of cholesterol from peripheral tissues to the liver. They are molecular complexes containing a central core (composed of cholesterol, cholesterol ester, and triglycerides) wrapped in bark of cholesterol, phospholipids, and apolipoproteins (ApoA-I being the most abundant one) (Diotel et al., 2018; Meilhac et al., 2020; Scherer et al., 2011). The possibility of molecular interaction between HDLs and E2 in vivo is also suggested by the fact that HDLs isolated from women contain E2, while those from men do not (Gong et al., 2003). As well, male and female HDLs show different physiological properties probably supported by their steroid content. Interestingly, HDLs also show neuroprotective effects, with intravenous injections reducing the cerebral infarct volume in rats (Lapergue et al., 2010; Paternò et al., 2004).

The BBB is conserved across vertebrate organisms. Zebrafish have recently emerged as interesting models to understand the physiological and pathological functions of the BBB. In zebrafish, the BBB shares striking similarities in organization and function with higher vertebrates (Diotel et al., 2018; O'Brown et al., 2018). General tight junction markers are expressed in endothelial vascular cells within adults and during brain development. Two paralogs of *claudin 5* (*cldn5a* and *cldn5b*) and *zonula occludens 1* encoding genes (and *tjp1a* and *tjp1b*) were identified in zebrafish (Abdelilah-Seyfried, 2010; Jeong et al., 2008). Many studies show their expression in brain endothelial cells. Moreover, the BBB in zebrafish gradually matures between the ages of 3 and 10 days in larvae coinciding with *cldn5* and *tjp1* gene expression as early as 24 and 48 hpf (hours post-fertilization) for *clnd5b* and *clnd5a*, respectively, and 72 hpf for *tjp1a* and *tjp1b* (Jeong et al., 2008; Kim et al., 2017; Quiñonez-Silvero et al., 2020; van Leeuwen et al., 2018; Wang et al., 2014; Xie et al., 2010; Zhang et al., 2010).

Currently, in zebrafish, no data are available in the literature regarding the role of E2 on tight junction protein gene expression under physiological conditions. Similarly, no data are provided on the ability of E2 to exert neuroprotective effects in the context of BBB leakage. The aim of the present study was first to determine in zebrafish larvae whether *cldn5* and *tjp1* genes are regulated by E2 signaling. Second, we examined the impact of E2 on *cldn5* and *tjp1* gene expression in the telencephalon of adult zebrafish, both under physiological conditions and after mechanical injury to determine whether E2 can exert protective effects on the BBB. Finally, we investigated the relationship between estrogen-synthesizing neural stem cells and brain endothelial cells, as well as the effects of HDLs, as a potent substrate supplier for estrogen synthesis, on tight junction gene expression.

2 | MATERIAL AND METHODS

2.1 | Chemicals

17β-estradiol (E2) and the estrogen receptor antagonist $ICI_{182,780}$ were purchased from Sigma–Aldrich Chemical Co. Stock solutions of steroids were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Final dilutions were prepared before each experiment.

2.2 | Pharmacological treatment of zebrafish with estradiol and $ICI_{182,780}$

Zebrafish were handled and sacrificed in agreement with the guidelines for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. Adults used for spawning were housed in BIOSIT, Rennes, France (agreement number: B 35-238-6) and CYROI/DéTROI zebrafish facilities (agreement number: 974001) under standard conditions of photoperiod (14 h light and 10 h dark) and temperature (28°C). All experiments were conducted in accordance with the French and European Community Guidelines for the Use of Animals in Research (authorization 2018052821565451_v2-APAFiS #15266).

Eggs obtained from spawning zebrafish were collected immediately after laying and kept for 24 h in glass petri dishes containing water, under standard conditions of temperature and photoperiod. One day postfertilization (dpf), embryos were selected (50 per experimental condition) for exposure experiment with E2 (10^{-6} M), ICI (10^{-7} M), and DMSO vehicle as a control until 3 and 6 dpf. The water supplemented with the respective treatments was changed every day during the experimentation. After exposure, larvae were sacrificed in the MS-222 tricaine anesthetic (300 mg/L) and frozen in liquid nitrogen before being stored at −80°C.

In mature female zebrafish, plasma E2 levels can vary from 3.5×10^{-11} to 1.5×10^{-8} M, depending on the assay used (De Oliveira et al., 2020; Manjunatha & Philip, 2016; Teng et al., 2020). Data are rare in mature males, and there is variability in E2 concentrations, probably due to the measurement method. Nevertheless, the concentrations measured are always much lower than those measured in females (Linderoth et al., 2006; Teng et al., 2020). Based on these data, experiments with adult fish (6-month-old) were carried out on males for estrogenic treatments (to evaluate E2 action) and on females for anti-estrogenic treatments (to inhibit E2 action). First, estrogenic treatment was performed at 10^{-7} and 10^{-6} M for 72 h in males. Then, fish were exposed for a longer period (96 h) to a more physiologically relevant concentration of E2 (10^{-7} M), ICI (10^{-7} M), and DMSO for control fish in glass tanks under standard temperature and photoperiod conditions. The treatments were renewed every day, and zebrafish were sacrificed with MS-222 at the end of the experimentation.

Telencephalons of five zebrafish per experimental condition were extracted and quickly frozen in liquid nitrogen for quantitative polymerase chain reaction (qPCR) analyses.

2.3 | Stab wound injury of the telencephalon, estrogenic treatment, and Evans blue extravasation assay

Stab wound injury of the telencephalon was performed in adult males as previously described (Diotel et al., 2013). Briefly, zebrafish were anesthetized in MS222 anesthetic (300 mg/L) and a sterile needle (BM Microlance 3; $30G^{1/2}$; 0.3 × 13 mm) was inserted within the right telencephalic hemisphere following a dorsoventral axis, with the help of landmarks clearly visible on the zebrafish head. The needle is introduced vertically over 1.5 mm by light pressure. Immediately after injury, zebrafish were placed back in tanks at 28 degrees under standard photoperiod conditions for 1 or 5 days before being sacrificed for telencephalic qPCR analyses. In order to investigate the potential role of estrogens in *cldn5* and *tjp1* gene expression, fish were treated with E2 (10^{-7} M) 8 h after the lesion or with the vehicle (DMSO) before being sacrificed 1 day postinjury (dpi). In parallel, some of these fish were also intraperitoneally injected with 1% Evans blue diluted in phosphate-buffered saline (PBS) (10 μL for 0.1 g of body weight) 30 min before their euthanasia at 1 dpi. They were then fixed overnight at 4°C in PBS containing 4% paraformaldehyde (PFA) and processed for brain vibratome sectioning. In order to determine if the estrogenic treatment has an effect on the function of the BBB, Evans blue fluorescence (red) was measured using ImageJ software in three different sections of injured hemispheres. In total, 13 brains were analyzed (six controls and seven treated with estradiol). The measurements were performed by two different experimenters and the results provided in the study correspond to the average of their quantification. Importantly, the fluorescence measurement was done around the lesioned area, excluding the blood vessels filled with Evans blue.

2.4 | RNA extraction and quantitative real-time polymerase chain reaction

For extraction, frozen brains or larvae were sonicated for 15 s in 250 μL of Trizol Reagent (Invitrogen), and the RNA extractions were then carried out using the Trizol-Chloroform method according to the manufacturer's protocol. Reverse transcription was performed with 0.5 μg of total RNA, with 5 μM of random primer oligonucleotides, 2.5 mM of dNTPs, and 100 U MMLV-RT (Promega) in the appropriate buffer for 10 min at 65°C and 90 min at 37°C. Polymerase chain reaction (PCR) was performed in an iCycler thermocycler coupled to the MyiQ detector (Bio-Rad) using iQ SYBR-Green Supermix (Bio-Rad) according to the manufacturer's protocol. Primers used for the analysis are shown in Table 1. For each experimental condition, the real-time polymerase chain reaction (RT-PCR) quantification was run in triplicate. Melting curve and PCR efficiency analyses were performed to confirm specific amplification. The threshold cycle (Ct) was determined for each gene. The gene *ef1* was used to normalize the expression of other genes. The Delta–Delta CT method was then applied to calculate the relative expression of each gene of interest. The fold induction/inhibition

TABLE 1 Oligonucleotide sequences used in quantitative real-time polymerase chain reaction.

zf-cyp19a1b Fw	5′-TCGGCACGGCGTGCAACTAC-3′
zf-cyp19a1b Rv	5′-CATACCTATGCATTGCAGACC-3′
zf-tjp1a Fw	5′-GAACCCATCAACCGCATC-3′
zf-tjp1a Rv	5′-CGGGGCCCCTACATTTAC-3′
zf-tjp1b Fw	5′-GAGGTCAAAGGGAAAGCTGA-3′
zf-tjp1b Rv	5′-CACGGAGTGGATGTCTGAAA-3′
zf-claudin5a Fw	5′-TCCTGGGTCTGATCCTGTG-3′
zf-claudin5a Rv	5′-CTCGATGAAGGCGGTGAC-3′
zf-claudin5b Fw	5′-CTCAATGCACCAATTGCATC-3′
zf-claudin5b Rv	5′-TTTTTGGCGTAGGGAACTTG-3′
Zf-ef1 Fw	5′-AGCAGCAGCTGAGGAGTGAT-3′
Zf-ef1 Rv	5′-CCGCATTTGTAGATCAGATGG-3′

was determined and expressed as a fold change compared to the normalized control condition (DMSO).

2.5 | RNA sequencing analysis

For RNA sequencing analyses, data were reanalyzed from RNA-Seq/DeTCT database based on zebrafish development (White et al., 2017) (http://www.ebi.ac.uk/gxa/; accession number: E-ERAD-475). As well, the RNA sequencing data from zebrafish telencephalon and whole zebrafish brain were also reanalzyed from a data set generated by Gourain et al. (2021) and Rodriguez Viales et al. (2015) and Wong & Godwin (2015).

2.6 | Aromatase B immunohistochemistry

To study the links between neural stem cells and blood vessels, Aromatase B (AroB; the enzyme converting testosterone to estradiol and encoded by the *Cyp19a1b* gene) immunohistochemistry was performed on Tg(*fli1a*:EGFP)y1 fish that express GFP in endothelial cells (Lawson & Weinstein, 2002). The Tg(*fli1a*:EGFP)y1 fish were euthanized using Tricaine and fixed overnight at 4°C in 4% PFA in PBS (pH 7.4) before being processed as previously described (Diotel et al., 2015; Rodriguez Viales et al., 2015). Briefly, after several washes with 1× PBS followed by 1× PBS containing 0.2% Triton X100 (PBS-T), 50-μm-thick, free-floating, transverse sections were made using a vibratome (VT1000S). Then, sections were blocked for 45 min with PBS-T containing 2% bovine serum albumin (BSA) and incubated overnight at room temperature with the primary antibodies: rabbit anti-Aromatase B (1/500 kindly provided by Dr. François Brion). The next day, sections were washed and incubated for 1.5 h at room temperature with secondary antibodies (goat anti-rabbit Alexa Fluor 594; 1/500; REF: A11012) and then with DAPI (final concentration: 1 μg/mL) to label cell nuclei. Finally, the slides were rinsed and mounted with an anti-fading medium (IMM Ibidi; REF: 50001).

2.7 | HDL injection, brain sampling, qPCR, and ApoA1 immunohistochemistry

To investigate the links between HDLs, endothelial cells, and neural stem cells, reconstituted HDL solution was intraperitoneally injected (80 mg/kg) as previously described (Sulliman et al., 2021) in Tg(*fli1a*:EGFP)y1 zebrafish or Tg(*cyp19a1b*:GFP) zebrafish expressing GFP in aromatase B radial glial cells (RGCs) (Tong et al., 2009). After 1.5 h postinjection, fish were euthanized and fixed for processing to cryostat embedding and sectioning (12 μm thickness). ApoA1 immunohistochemistry was performed on these cryostat sections using a specific antibody (Anti-ApoA1, 1:100; REF: Calbiochem 178422, RRID:AB_564222) as previously described (Sulliman et al., 2021). The specificity of the anti-ApoA1 was previously demonstrated in the zebrafish brain tissue in a previous study (Sulliman et al., 2021). To analyze the role of HDLs in BBB gene expression, we injected males with reconstituted HDL (160 mg/kg) (n = 4/group). Brains of adult zebrafish were collected 48 h postinjection for RNA extraction and qPCR analyses.

2.8 | Nuclear estrogen receptor in situ hybridization

To determine whether cerebral endothelial cells express estrogen receptors, we performed in situ hybridization labeling. Plasmids and RNA probe synthesis of the three nuclear estrogen receptors were obtained from previous studies (Menuet et al., 2002). After linearization, digoxigenin (DIG)-labeled antisense riboprobes were synthesized using T7 or SP6 RNA polymerases as previously described (Diotel et al., 2015). Fluorescent in situ hybridization was performed as previously described (Diotel et al., 2015). Briefly, brains were rehydrated and washed several times in PBS containing 0.1% Tween (PTw), incubated for 30 min with proteinase K (10 μg/mL) at room temperature, and next postfixed in 4% PFA. After washes and prehybridization for 3 h, brains were incubated with the respective DIG-labeled probes overnight at 65°C. After several washes, brains were incubated in a blocking buffer (PTw containing 0.2% BSA, pH 7.4) before embedding in 2% agarose. Brain sections were made with a vibratome and incubated with the anti-digoxigenin-AP, Fab fragments (1/2000; Sigma; Reference: RRID: AB_514497) was performed overnight at 4°C and finally stained with fast red staining solution (SIGMA*FAST*™ Fast Red TR/Naphthol AS-MX Tablets; Sigma; Reference: F4648), followed by DAPI counterstaining. Sections were mounted on slides with Aqua-Poly/Mount (Polysciences).

2.9 | Statistical analysis

Student's *t*-tests were performed to compare two groups assuming a normal distribution. If more than two groups were analyzed, multiple tests were corrected by Benjamini–Hochberg. Error bars correspond to the standard error of the mean, and *n*-values correspond to the number of animals in all experiments. A *p*-value <.05 was considered statistically significant.

2.10 | Microscopy

Brain sections were examined with epifluorescence microscopes (Olympus Provis, AX70-TRF equipped with de DP71 digital camera, or Eclipse 80i Nikon microscope equipped with a Hamamatsu digital camera [Life Sciences]) and laser scanning confocal microscopes (Eclipse confocal [Nikon] and Leica [TCS SP8, DMI 6000]). Images were processed with the respective Olympus and Nikon software. Microphotographs were acquired in TIFF format and adjusted for brightness and contrast using Photoshop.

3 | RESULTS

3.1 | Developmental expression of *claudin 5* and *tight junction protein 1* genes

We first investigated the expression of *cldn5* and *tjp1* during development. To this end, we analyzed a previously published RNA data set from White et al. (2017) providing global transcriptomic profiling from zygote stage (1-cell) to 5 dpf. Due to genomic duplication occurring in Teleost, two orthologs for a wide number of genes exist in zebrafish: *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* (www.ensemble.org). The *cldn5a* gene was not expressed during the early stages and began to be expressed significantly during epiboly, increasing sharply at the segmentation (1–4 somites) and decreasing at the hatching long pec stage to remain stable until day 5 of larval development (Figure 1a). In contrast, *cldn5b* was detected from the zygotic stage until 5 days of development. Its expression increased from the segmentation (1–4 somites) stage, peaking at the pharyngula/hatching long-pec stages (Figure 1b). For *tjp1a* and *tjp1b*, both were detected from zygotic stages to the larval day 5. Expression of *tjp1a* remained fairly stable during these developmental periods with a small decrease in expression around the gastrula/epiboly stages (Figure 1c). In contrast, *tjp1b* expression was highest from the segmentation stage (1–4 somites) to the first pharyngula stage before decreasing and remaining stable until day 5 (Figure 1d). These results demonstrate that these four genes are dynamically expressed during zebrafish development and that *cldn5b*, *tjp1a*, and *tjp1b* displayed an important maternal contribution. Interestingly, the reanalysis of different RNAseq performed in the whole brain or restricted to the telencephalon of adult zebrafish (males + females) (Gourain et al., 2021; Rodriguez Viales et al., 2015; Wong & Godwin, 2015) demonstrated that *clnd5a* was more expressed than *cldn5b* and also that *tjp1a* was more expressed than *tjp1b*. This was observed in the whole brain (Figure 1e; $p < .0001$) as well as in the telencephalon (Figure 1f; $p < .0001$).

3.2 | Effects of estrogen signaling on developmental expression of *claudin 5* and *tight junction protein 1* genes

In mammals, estrogens are known to modulate the expression of genes involved in the development and maintenance of tight junctions, namely, at BBB (Bake & Sohrabji, 2004; Burek et al., 2014; Sandoval & Witt, 2011). In order to investigate such an effect during embryogenesis, fertilized eggs were treated with a high concentration of estradiol (10^{-6} M) from 1 to 3 dpf and from 1 to 6 dpf. In parallel, incubation with the nuclear estrogen antagonist $ICI_{182,780}$ (ICI 10^{-7} M) was performed. These drug treatments are well described to modulate estrogen signaling (Mouriec et al., 2009). In order to verify the efficacy of these estrogenic and anti-estrogenic treatments, the expression of the estrogen-responsive gene, *cyp19a1b*, was investigated. As shown in Figure 2, no change in *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* gene expression was observed under estrogenic stimulation from 1 to 3 dpf as well as from 1 to 6 dpf, while *cyp19a1b* was upregulated following E2 treatment as expected (Figure 2a and Figure 2c, $p < .001$ and $p < .05$). Similarly, the inhibition of estrogen signaling through ICI treatment resulted in no change in *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b*, while the *cyp19a1b* gene was downregulated (Figure 2b and Figure 2d, $p < .01$ and $p = .054$, respectively). Consequently, estrogen signaling did not modulate the expression of *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* during these developmental stages, in the whole eleutheroembryos.

3.3 | Effects of estrogen signaling on *claudin 5* and *tight junction protein 1* gene expression in the brain of adult zebrafish

We next decided to investigate the potential effects of estrogen signaling on *cldn5* and *tjp1* gene expression in the adult brain. Interestingly, treatment of male fish with 10^{-6} M of E2 for 3 days led to the upregulation of *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b*, which was significant for *cldn5a* ($p = .023$), *cldn5b* ($p = .035$), and *tjp1a* ($p = .052$) but not for *tjp1b* ($p = .28$) (Figure 3). However, treatment with E2 (10^{-7} M) for 3 days did not alter the expression of *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* gene expression in the whole brain.

The zebrafish telencephalon is one of the most studied brain structures due to its homologies and similarities with its mammalian counterpart (Diotel et al., 2020; Ghaddar et al., 2021; Jurisch-Yaksi et al., 2020; Than-Trong et al., 2020). We, therefore, decided to focus on this structure using a more physiological concentration of estradiol (10^{-7} M) for a longer period (4 days) in adult male zebrafish, as previously described (Diotel et al., 2013). In parallel, females that

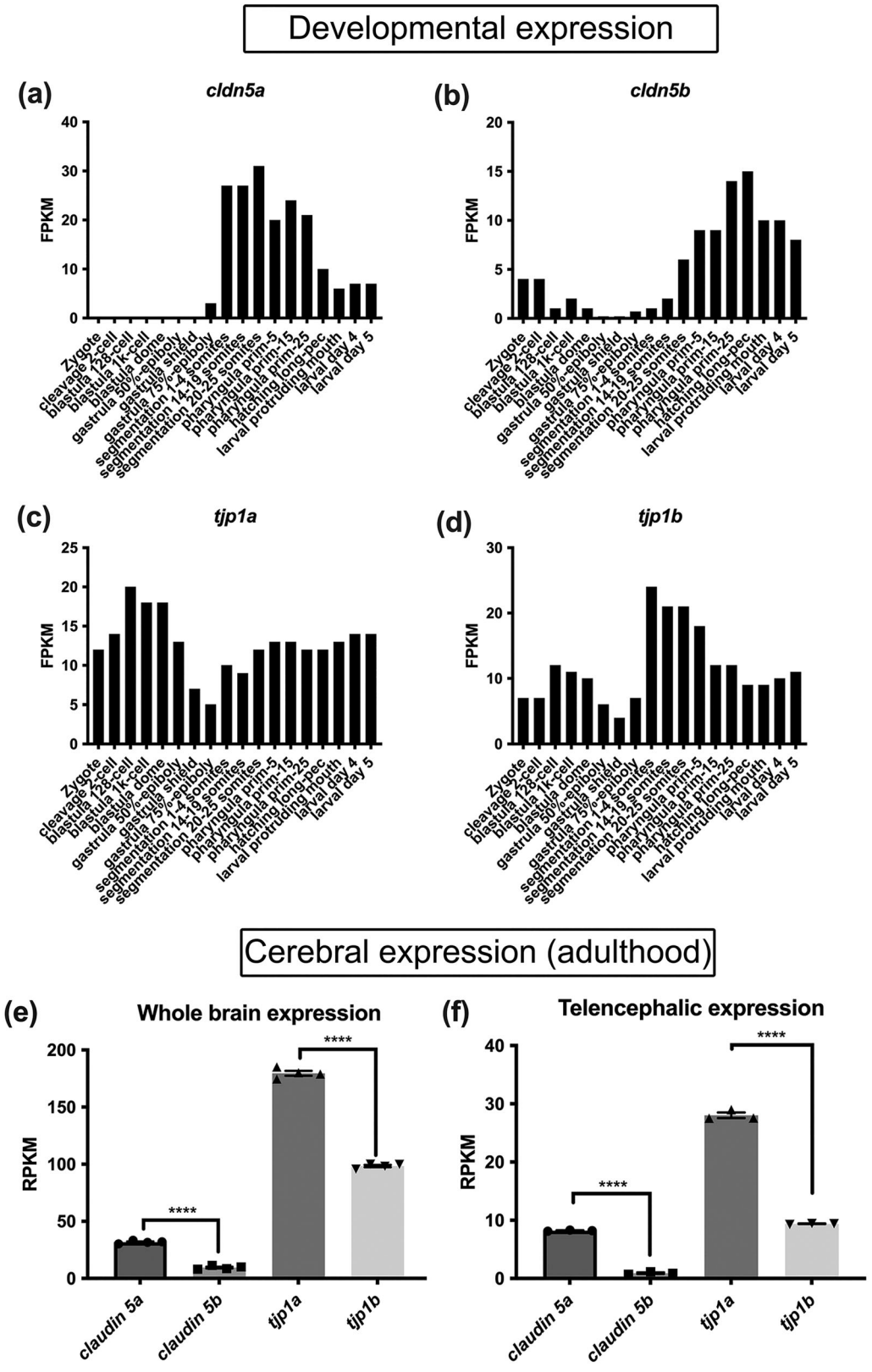

FIGURE 1 *Claudin 5* and *tight junction protein 1* gene expression during zebrafish development and in the brain of adult zebrafish. (a–d) Transcript quantification of *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b1* during zebrafish development between zygote stage (1-cell) to 120 h postfertilization (120 hpf or larval day 5). (e–f) Transcript quantification of *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b1* in the whole adult mixed (male + female) zebrafish brain and telencephalon. Note that these data were obtained from the reanalysis of an RNA seq data set generated by Gourain et al. (2021), Rodriguez Viales et al. (2015), White et al. (2017), and Wong & Godwin (2015). FPKM, fragments per kilobase million; RPKM, reads per kilobase million. $^{****}p < .0001$.

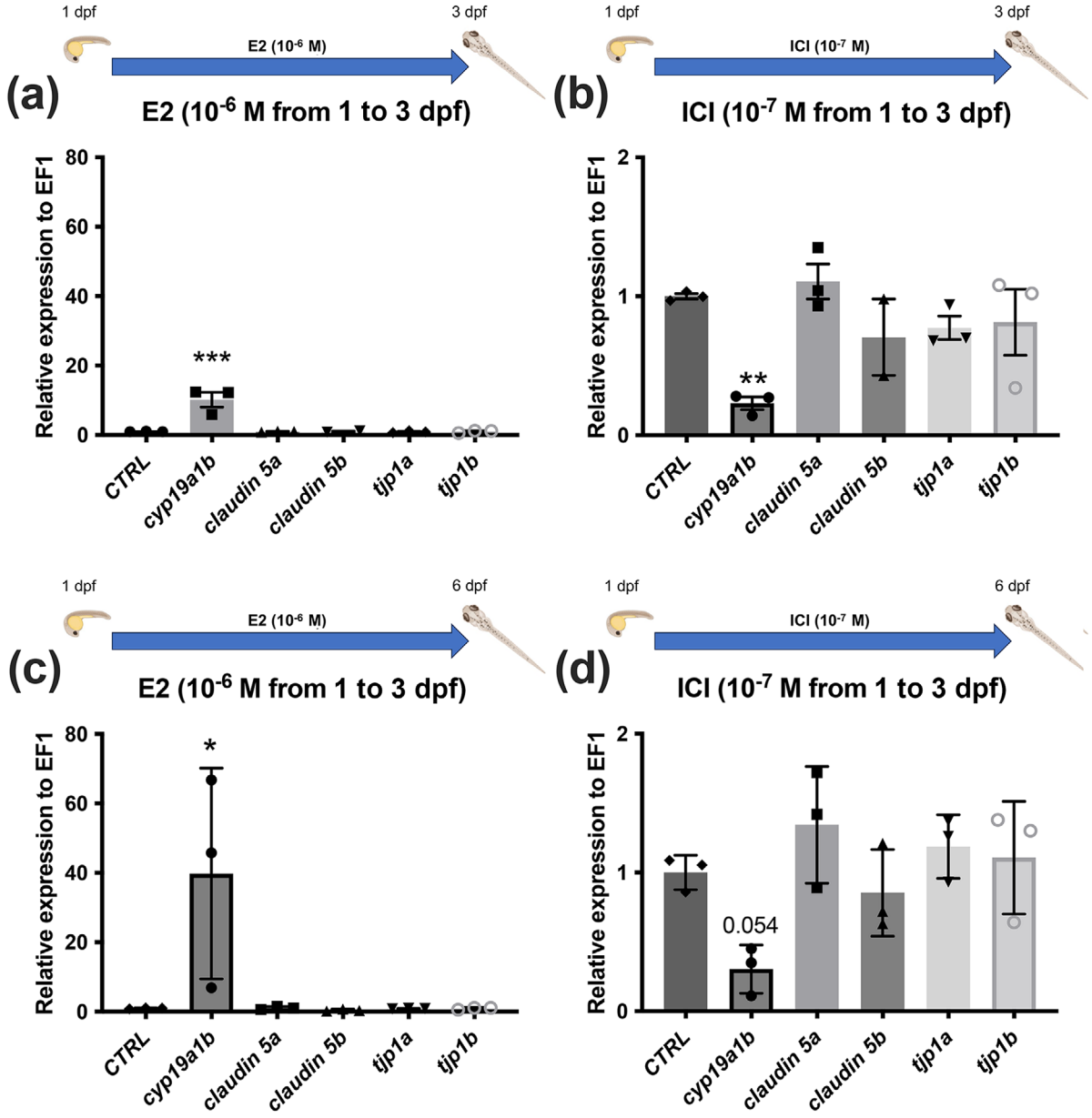

FIGURE 2 Estrogen signaling has no impact on *claudin 5* and *tight junction protein 1* gene expression during zebrafish development. (a, b) *cldn5* and *tjp1* gene expression following treatment with 10^{-6} M of E2 and with 10^{-7} M of ICI from 1 to 3 dpf. (c, d) *cldn5* and *tjp1* gene expression following treatment with 10^{-6} M of E2 and with 10^{-7} M of ICI from 1 to 6 dpf. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

exhibit stronger levels of circulating estrogens than males were treated with ICI (10^{-7} M) to block nuclear estrogen receptors, as previously described (Diotel et al., 2013). These drug concentrations were previously shown to efficiently modulate brain cell proliferation in adult zebrafish (Diotel et al., 2013).

As shown in Figure 4, treatment with E2 (10^{-7} M) in males resulted in a significant increase in the expression of the *tjp1a* ($p = .011$) and *tjp1b* ($p = .005$) telencephalic genes, whereas it did not significantly modulate *cldn5a* and *cldn5b* expression in males. Surprisingly, treatment of females with ICI (10^{-7} M) did not lead to any significant change in the expression of *cldn5a* ($p = .973$), *cldn5b* ($p = .082$), *tjp1a* ($p = .475$), and *tjp1b* ($p = .222$) genes.

3.4 | Claudin 5 and tight junction protein 1 gene expression during brain repair: A role for estradiol?

The expression of the *cldn5* and *tjp1* genes was subsequently studied in a model of telencephalic injury. In this context, a needle was inserted into the right telencephalon, and the fish were allowed to survive for 1 and 5 dpi. Analysis of the *cldn5* and *tjp1* genes revealed a consistent decrease in *cldn5a* and *cldn5b* expression that did not reach a significant level at 1 dpi ($p = .056$ and $p = .292$, respectively) and 5 dpi ($p = .075$ and $p = .241$, respectively) (Figure 5a,b). In the same line of evidence, the expression of *tjp1a* and *tjp1b* genes was significantly reduced at 1 and 5 dpi (Figure 5c and Figure 5d, $p < .001$ at 1 dpi; $p < .05$

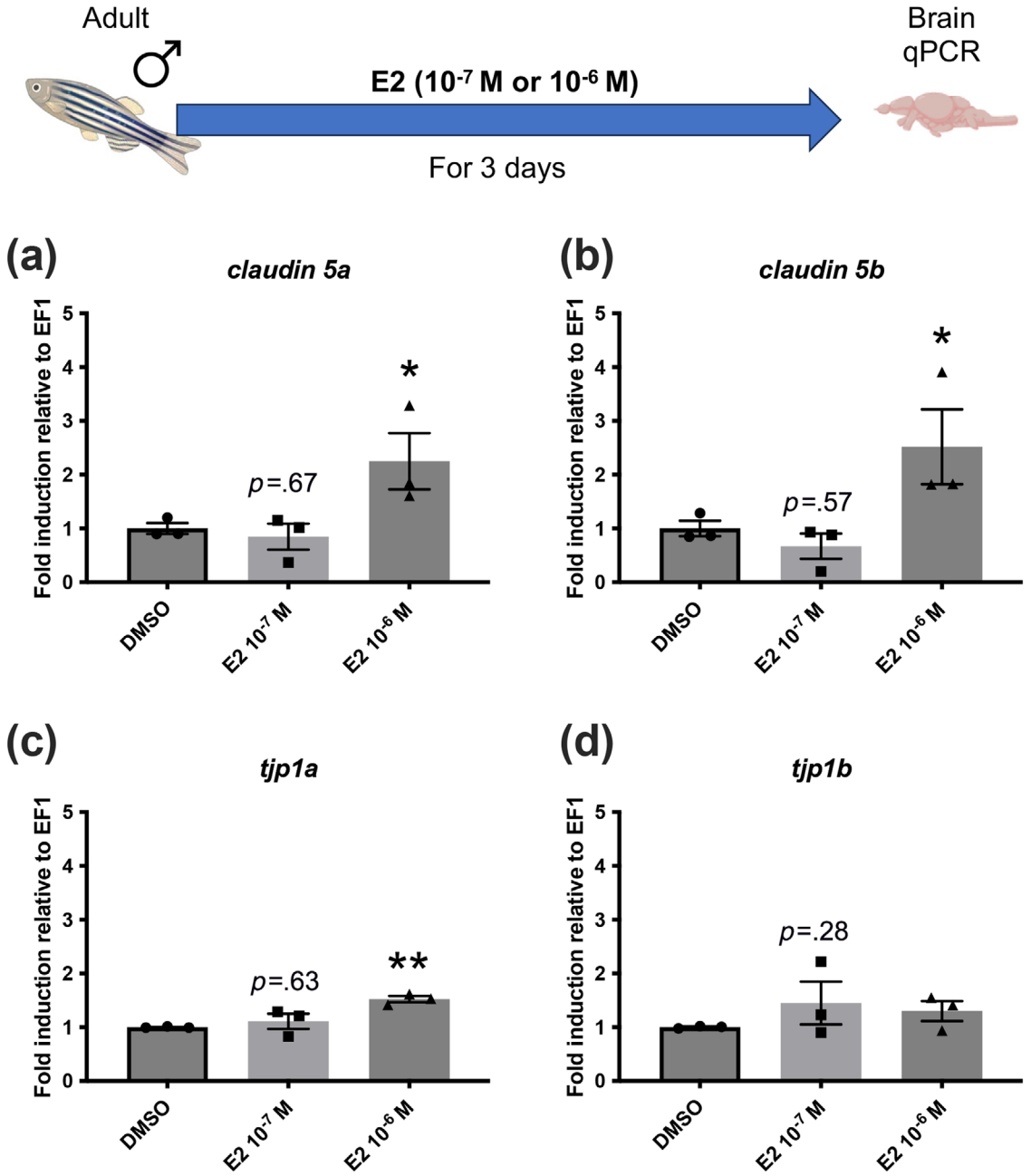

FIGURE 3 High estrogen concentration increases *cldn5* and *tjp1* gene expression in the whole adult male brain. (a–d) *cldn5* and *tjp1* cerebral genes expression following 3 days of treatment with 10^{-7} and 10^{-6} M of E2 compared to controls (dimethyl sulfoxide [DMSO]). $n = 3$ whole brains. $^{*}p < .05$; $^{**}p < .01$.

and $p < .01$ at 5 dpi, respectively). These results demonstrate that stab wounding alters tight junction gene expression and potentially BBB functions. These data also correlate with the fact that the expression of the estrogen-synthesizing enzyme gene (*cyp19a1b*) is decreased from 30 min to 7 dpi (Diotel et al., 2013).

In order to test the potential therapeutic impact of 17β-E2 during brain repair, we decided to create a stab wound in the telencephalon of adult fish and subsequently treat male fish with E2 (10^{-7} M) 8 h after injury for subsequent telencephalic gene analysis at 1 dpi. As revealed by qPCR analysis, injured fish exposed to 17β-E2 exhibited a consistent increasing trend in *cldn5a* ($p = .1$), *cldn5b* ($p = .071$), *tjp1a* ($p = .078$), and *tjp1b* ($p = .158$) gene expression, which did not reach statistical significance (Figure 6a–d).

To reinforce these data and the fact that estradiol could potentially modulate directly the expression of *cldn5* and *tjp1* in endothelial cells, we performed in situ hybridization of nuclear estrogen receptors (*esr1*, *esr2a*, and *esr2b*). As shown in Figure 7, *esr1* and *esr2a* mRNAs were detected in endothelial cells characterized by elongated and flat nuclei bordering blood vessels (see arrows).

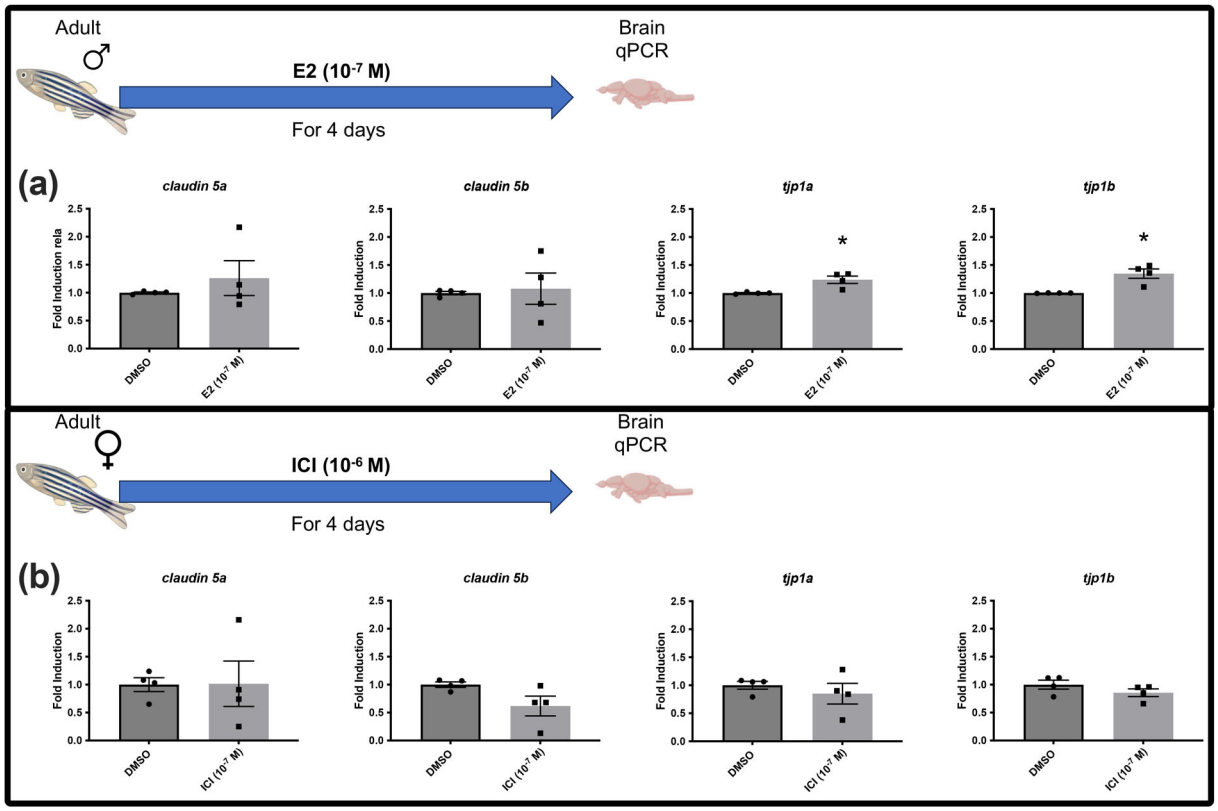

FIGURE 4 Estrogen modulates *tight junction protein 1a* and *1b* gene expression in the telencephalon of adult zebrafish. (a) *cldn5* and *tjp1* telencephalic gene expression in adult males following 4 days of treatment with 10^{-7} M of E2 compared to controls (dimethyl sulfoxide [DMSO]). (b) *cldn5* and *tjp1* telencephalic gene expression in adult females following 4 days of treatment with 10^{-7} M of ICI compared to controls (DMSO). n = 4 pools of five telencephalons. $^{*}p < .05$.

Although a slightly increasing trend in *cldn5* and *tjp1* gene expression was observed under estrogenic treatment after brain injury, the question of its relevance was raised. To determine the impact of estrogenic treatment on the functionality of the BBB after telencephalic lesion, extravasation assay experiments were carried out using Evans blue. Eight hours after the telencephalic lesion, zebrafish were treated with vehicle or 17β-E2 (10^{-7} M). The next day, 30 min before the sacrifice of the fish at 1 dpi, Evans blue was injected and allowed to reach the blood flow for 30 min. Extravasation of this dye into the nervous system reflects leakage from the BBB. The quantification of Evans blue fluorescence in the brain parenchyma (out of the blood vessels) surrounding the lesion site demonstrated a decreased staining in E2-treated fish (Figure 8). This suggests that from a functional point of view, estrogenic treatment limits BBB leakage following injury.

3.5 | Links between the estrogen-synthesizing enzyme Aromatase B and blood vessels

The brain of adult zebrafish is well described to strongly express Aromatase B (AroB), the estrogen-synthesizing enzyme encoded by the *cyp19a1b* gene. Interestingly, the *cyp19a1b* gene and protein expression are restricted to RGCs that behave as neural stem cells during zebrafish development and adulthood (Pellegrini et al., 2007). Given the possible role of estrogens in the regulation of genes involved in BBB functions, we hypothesized that RGCs could be a source of estrogens for endothelial cells. In order to investigate the potential links between AroB-positive RGCs and endothelial cells, we performed AroB immunohistochemistry on Tg(*fli1a*:EGFP)y1 that express the GFP in endothelial cells (nuclear + cytoplasm). AroB-positive RGCs processes enveloped and enrolled blood vessels showing intimate connections with GFP-positive endothelial cells as seen in the ventral nucleus of the ventral telencephalon (Vv) and the dorsomedial region (Dm) of the telencephalon (Figure 9a–c). Considering that AroB-positive neural stem cells are steroidogenic cells and that HDLs could be considered as a source of cholesterol and steroids (namely estrogens) for tissues (Diotel et al., 2018; Höckerstedt et al., 2004; Leszczynski & Schafer, 1991; Sulliman et al., 2021; Tikkanen et al., 2002), the links between endothelial and neural stem cells have been investigated. After intraperitoneal injection of HDLs in Tg(*fli1a*:EGFP)y1 fish, HDLs were detected through ApoA1 immunohistochemistry in endothelial cells (Figure 9d–g). Similarly, the injection of HDLs in Tg(*cyp19a1b*:GFP) fish showed a close association between the endfeet of AroB-positive neural stem cells and blood vessels taking up HDL particles (Figure 9h). These data suggest that HDLs could be a source of cholesterol and estrogen precursors to endothelial cells and neighboring cells including neural stem cells.

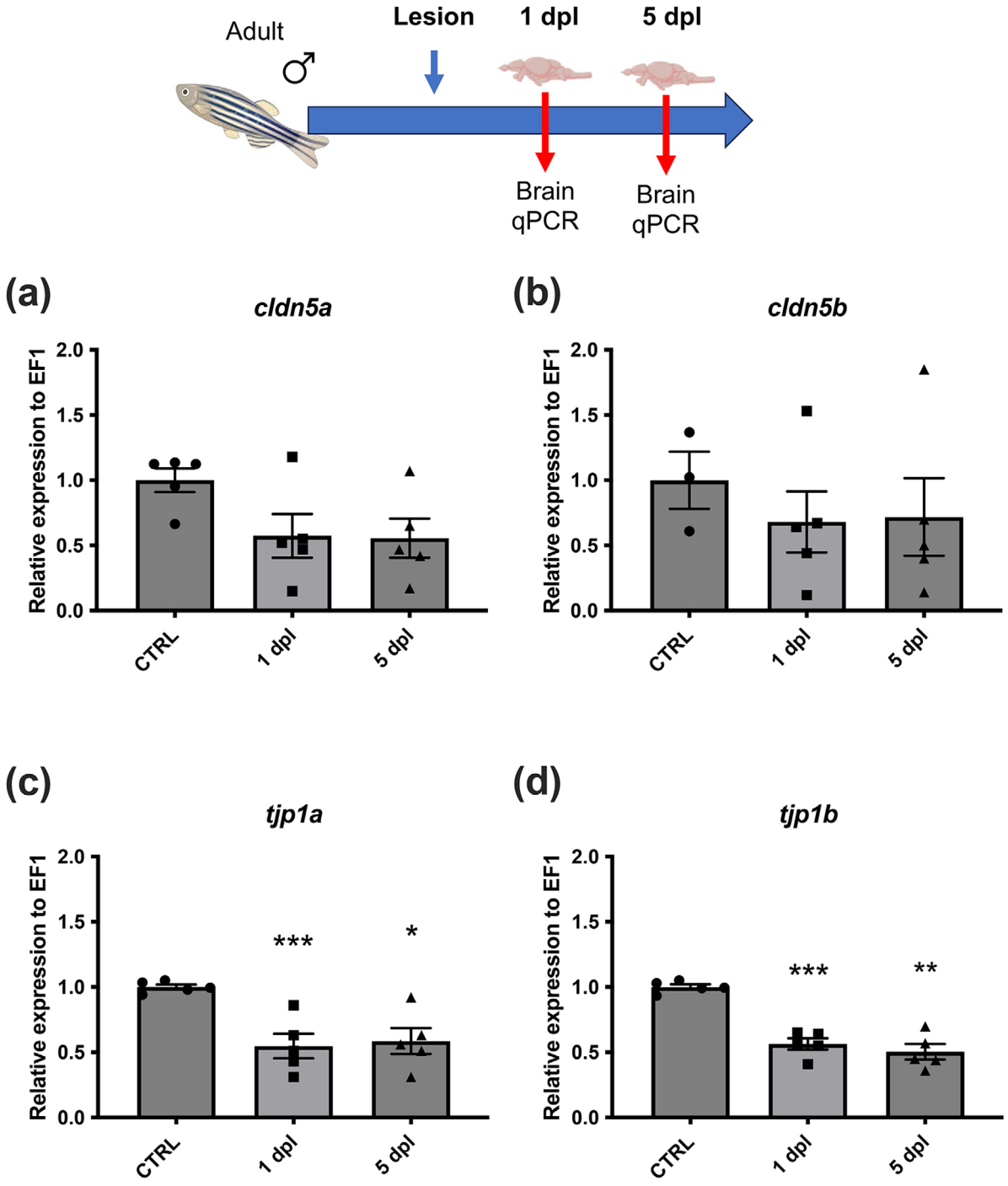

FIGURE 5 Brain injury decreases claudin and tight junction protein gene expression in males. (a, b) *cldn5a* and *cldn5b* gene expressions are not significantly decreased 1 and 5 days after telencephalic brain injury. (c, d) *tjp1a* and *tjp1b* gene expressions are significantly decreased 1 and 5 days after telencephalic brain injury. $N = 5$ pools of three telencephalons. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

3.6 | HDLs did not impact *cldn5* and *tjp1* gene expression in adult male zebrafish brain

It is well known that HDLs can carry different sex steroids depending on gender. To investigate the potential role of male and female HDLs in *cldn5* and *tjp1* gene expression, we decided to inject male zebrafish with HDLs from a human male or female (160 mg/kg). Gene expressions were analyzed 48 h postinjection. As shown in Figure 10, injections with female HDLs tended to increase *cldn5a* and *cldn5b* ($p = .14$ for both genes), *tjp1a* and *tjp1b* ($p = .10$ and $p = 0.051$, respectively), and *cyp19a1b* genes ($p = .056$) without reaching statistical significance. No change was observed when adult males were injected with male HDLs.

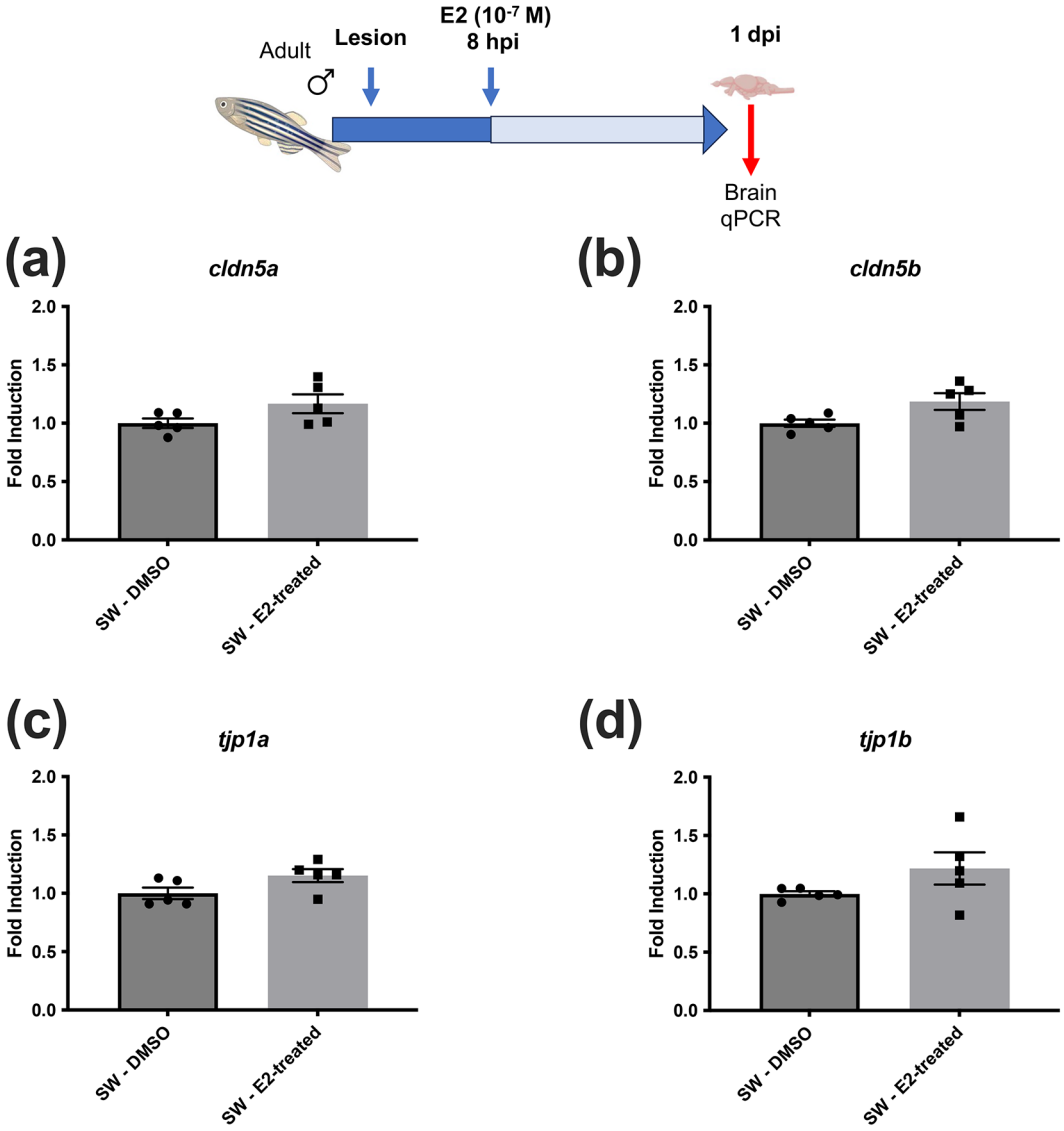

FIGURE 6 Estradiol treatment after brain injury tends to increase claudin and tight junction protein gene expression compared to untreated controls. (a–d) Zebrafish males treated with 10^{-7} M of E2 8 h postinjury (hpi) show an increasing trend in blood–brain barrier (BBB) gene expression 1 day after the lesion (dpi). SW, stab-wounded telencephalon. $n = 5$ pools of three injured telencephalons.

4 | DISCUSSION

Based on transcriptomic data, we analyzed the expression of *cldn5a*, *cldn5b*, *tjp1a* and *tjp1b* during development, and our results showed that modulation of estrogen signaling has no effect on their expression during development. In contrast, in the whole brain and telencephalon of adult zebrafish, estradiol treatment led to the upregulation of these genes in a dose- and time-dependent manner, probably through a direct regulation as endothelial cells express estrogen receptors. Interestingly, after stab wound injury of the telencephalon, *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* were decreased at 1 and 5 days postlesion, reaching statistical significance for *tjp1a* and *tjp1b*. The treatment with 10^{-7} M of estradiol tended to reverse this decreased expression, an effect associated with a significant reduction of BBB permeability. Taken together, these data suggest that estradiol could play a role in the regulation of the BBB physiology by modulating the expression of genes involved in the establishment of tight junctions. They pave the way for therapeutic research to promote the recovery of the BBB after damage.

4.1 | Expression of *cldn5* and *tjp1* transcripts during development

In mammals, BBB development is a gradual process that begins early during embryogenesis, and restrictions of molecule movements are observed before mature postnatal astrocyte ensheathment of vessels appears (Daneman et al., 2010; Mito et al., 1991; Nico et al., 1999; Sohet et al., 2015; Virgintino et al., 2004; Wolburg & Lippoldt, 2002). Previous studies have shown that BBB in zebrafish shares common

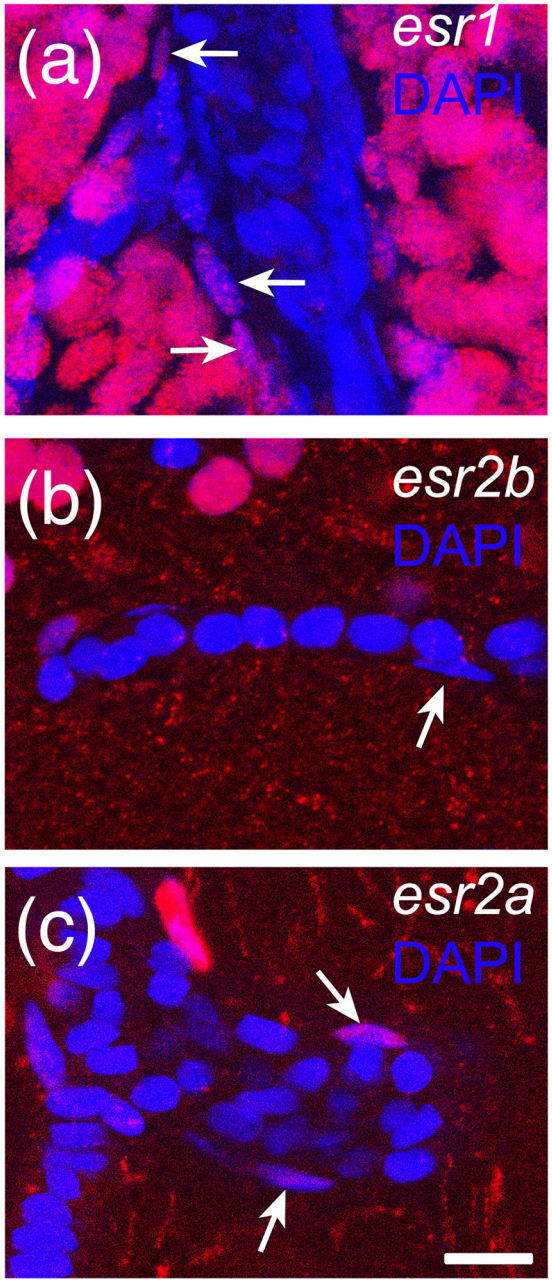

FIGURE 7 Nuclear estrogen receptors *esr1* and *esr2a* are expressed in endothelial cells. Fluorescent in situ hybridization showing *esr1* (a), *esr2b* (b), and *esr2a* (c) in red with DAPI counterstaining. Note that arrows point to endothelial cells characterized by their flat and elongated cell nuclei bordering the blood vessels demonstrating expression of *esr1* and *esr2a* in endothelial cells and no *esr2b* expression. Scale bar: 15 μm.

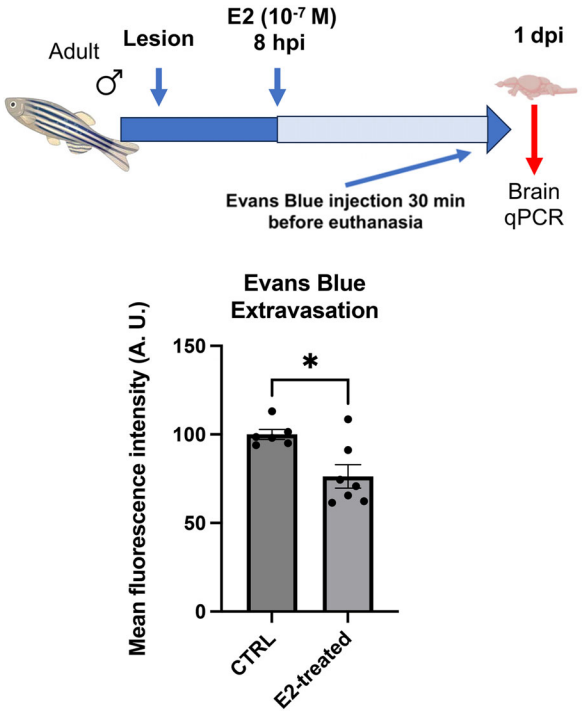

FIGURE 8 Estradiol treatment after brain injury reduces blood–brain barrier (BBB) leakage. Zebrafish males treated with 10^{-7} M of E2 after telencephalic injury show a significant decrease in Evans blue extravasation and consequently a decreased BBB leakage 1 day after the lesion compared to untreated fish. $n = 6$-7 brains (six controls and seven treated with estradiol). $^{*}p < .05$.

structural and functional similarities and gradually matures between the ages of 3 and 10 days in eleutheroembryos coinciding with *cldn5* and *tjp1* expression as early as 24 and 48 hpf for *clnd5b* and *clnd5a*, respectively, and 72 hpf for *tjp1a* and *tjp1b* (Fleming et al., 2013; Jeong et al., 2008; Kim et al., 2017; Quiñonez-Silvero et al., 2020; van Leeuwen et al., 2018; Wang et al., 2014; Xie et al., 2010; Zhang et al., 2010). Our analysis of an RNA data set (generated by White et al., 2017) here demonstrated that these four genes are expressed early during embryonic development. As shown in Figure 1, *cldn5b*, *tjp1a*, and *tjp1b* display different temporal profiles during development, but they are all expressed from zygote stage, suggesting a maternal inherited contribution for these genes, as previously described in mouse, pig, and human (Xu et al., 2012; Zhao et al., 2020). Of note, *cldn5b*, *tjp1a*, and *tjp1b* expression, which is present from the very beginning step of embryonic development, decreases during gastrula-segmentation stages, a decline followed by a rise, probably reflecting a maternal degradation of transcripts that goes along maternal to zygotic transition (Vastenhouw et al., 2019). In contrast, *cldn5a* is not detected before the gastrula-50% epiboly stage, peaks during segmentation and pharyngula stages, and decreases to stable levels in 4- and 5-day-old larvae.

Interestingly, our reanalysis of different RNAseq generated from adult whole brain or telencephalon reveals, for the first time in zebrafish, a stronger expression for *cldn5a* compared to *cldn5b*, and a similar pattern is observed for *tjp1a* and *tjp1b* (Gourain et al., 2021; Rodriguez Viales et al., 2015; White et al., 2017; Wong & Godwin, 2015). So far, it seems that *cldn5a* and *tjp1a* genes have developed or conserved a strong expression in the brain. The significance of these differential expression patterns during development and in adulthood is currently not known, and further experiments will be necessary to provide new information about the functions of *cldn5* and *tjp1* duplicate genes.

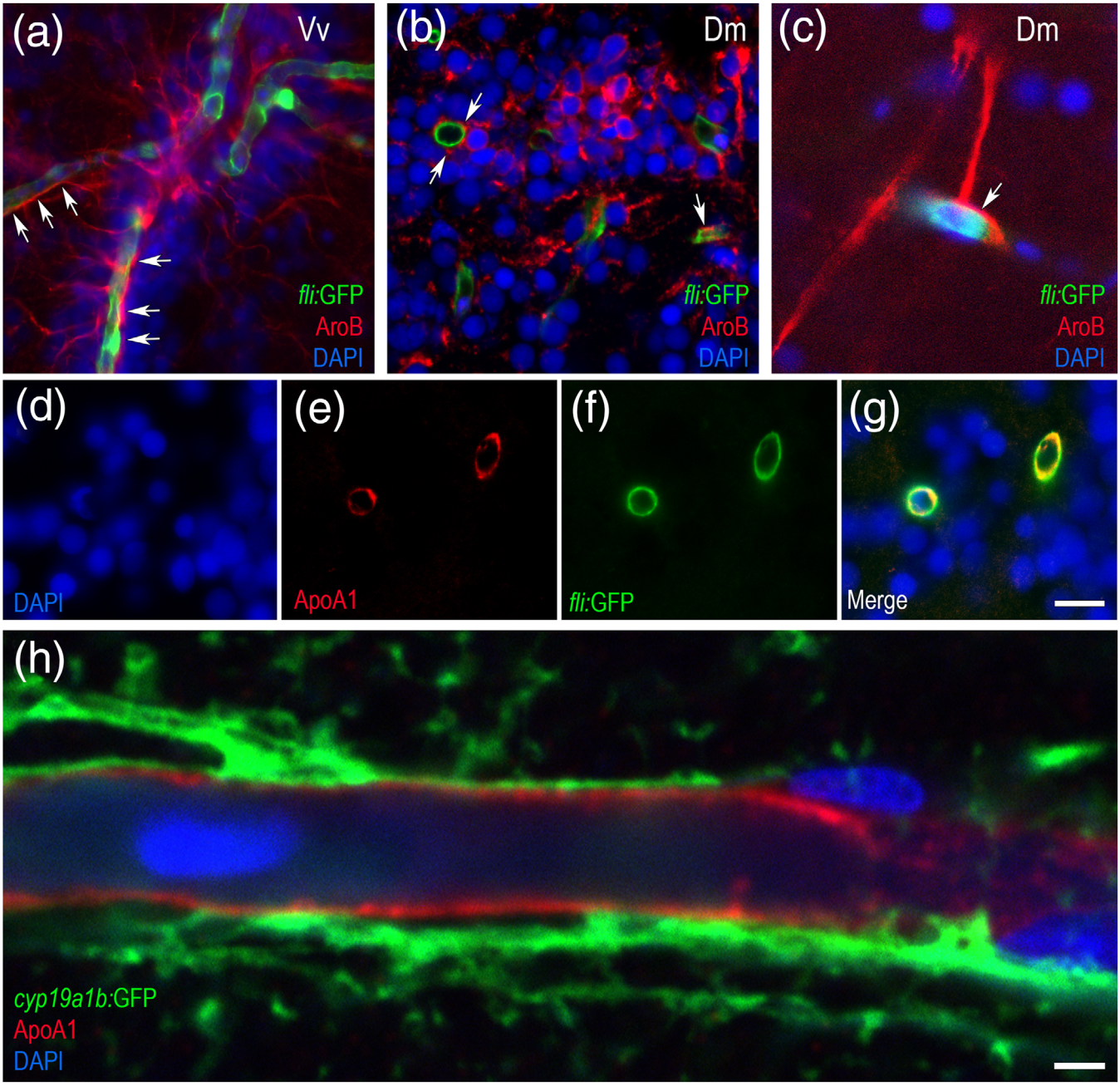

FIGURE 9 Aromatase B-positive radial glial cells strongly interact with endothelial cells being able to take up high-density lipoproteins (HDLs). (a–c) Aromatase B immunohistochemistry (red) in Tg(*fli1a*:EGFP)y1 fish in which endothelial cells express GFP (green) showing strong interactions between neural stem cells and blood vessels in the ventral nucleus of the ventral telencephalon (Vv) and the dorsomedial region (Dm) of the telencephalon. (d–g) Intraperitoneal injection of HDLs in zebrafish allowed HDL uptake by cerebral endothelial cells as revealed by ApoA1 immunohistochemistry (red) in Tg(*fli1a*:EGFP)y1 fish (green) as shown in these transversal sections of two cerebral blood vessels. (h) Intraperitoneal injection of HDLs in *Tg(cyp19a1b*:GFP) showing ApoA1 (red) proximity with AroB-positive neural stem cell endfeets along a sagittal blood vessel section in the telencephalon. *Tg(cyp19a1b*:GFP) zebrafish expressed GFP in radial glial cells (RGCs) (Tong et al., 2009). Scale bar: 21 μm (a–b), 14 μm (c–g), and 3.5 μm (h).

4.2 | Estrogenic regulation of *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* genes expression

The effects of estrogens on cerebral capillary permeability were evidenced in mammals by in vitro *and* in vivo studies, highlighting regulatory actions on BBB integrity (Bake & Sohrabji, 2004; Burek et al., 2014; Cipolla et al., 2009; Saija et al., 1990; Sandoval & Witt, 2011). Our results show that E2 could affect *cldn5* and *tjp1* gene expressions. In males, E2 treatment induced a significant increase in *tjp1a* and *tjp1b* gene expression in the telencephalon, while it did not change *cldn5a* and *cldn5b* ones. In females, the estrogen nuclear receptor antagonist (ICI) did not impact these gene expressions, suggesting sex-specific differences between tight junction protein regulation in mature males and females that should be further investigated. A sex difference in brain

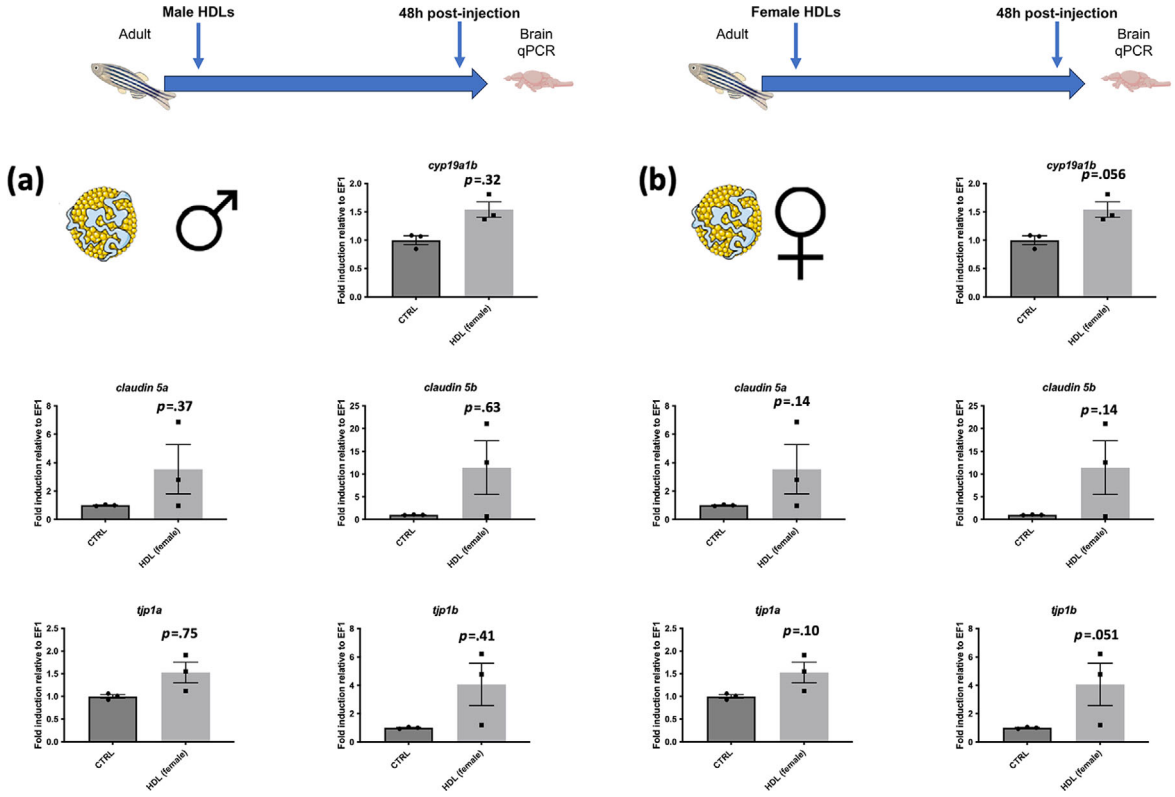

FIGURE 10 Impact of male and female high-density lipoprotein (HDL) injection in claudin and tight junction protein gene expression in adult male brain. (a) Male HDL injection does not change *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* genes expression. (b) Injection of female HDLs tends to increase the expression of the *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* genes, but statistical significance is not reached. Brain analyses were performed on four brains of each sex, 48 h after HDL injection.

expression of estrogen receptors could explain the differential expression and regulation that we observed between males and females. However, even if estrogen receptors are expressed in many different brain areas in adult zebrafish including the telencephalon (Coumailleau et al., 2015; Menuet et al., 2002), no data show differential expression between genders. We also investigated E2 effects at early developmental stages. Currently, there are no data on E2 regulation of TJ protein expression during embryonic development or early postnatal stages in mammals. Treatments of 3 and 6 dpf zebrafish with 10^{-6} M of E2 (a concentration known to upregulate target genes such as *cyp19a1b*) had no effect on the level of *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* transcripts. In the same way, the inhibition of E2 signaling through ICI treatment did not change these levels. Consequently, estrogen signaling does not seem to regulate *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* genes during early development, even if estrogen receptors are described in the brain from the 36th hour of development (Mouriec et al., 2009).

4.3 | Brain mechanical injury effects on *cldn5* and *tjp1* expression and neuroprotective effects of estradiol

Rodent models have revealed that ischemia induced by stroke and traumatic brain injury leads to an opening of BBB through impairment of tight junction protein integrity associated with a significant drop in the expression of claudin 5, occludin, and ZO-1 (Evran et al., 2020; Nag et al., 2007; Wen et al., 2014; Yang et al., 2007). We investigated in the present study the expression levels of *cldn5* and *tjp1* genes in the context of mechanical brain injury. Our results are consistent with data described in mammals and clearly showed that telencephalic stab wound lesion led to a pronounced decrease in the levels of *cldn5a* and *cldn5b* transcripts at 1 and 5 dpi, a decrease that was, however, not statistically significant. It is possible that increased sampling and analysis could erase the heterogeneity of response between animals, until a significant value is reached. Similarly, *tjp1a* and *tjp1b* gene expressions were strongly and significantly reduced at each time investigated. Nevertheless, some data from transcriptomic studies performed on stab-wounded brains at 20 h postinjury showed an increase in *cldn5b* and a decrease in *tjp1a* (Demirci et al., 2020). Strikingly, in 2022, a new analysis revealed that *tjp1a* was not in the list of genes differentially expressed at 24 h postinjury (Demirci et al., 2022). These data are in contradiction with each other and partly with our work. Another study shows that *cldn5a* and *b* are upregulated at 5 dpi (Gourain et al., 2021). Such differences between these studies are complex to understand and seem to occur even within the same group. Maybe it could be due to the heterogeneity between the injuries made and some environmental, gender, or genetic background that may differ between each laboratory as well as the transcriptomic methods. However, in our hands,

we obtained similar results in our two laboratories (UMR DéTROI and UMR_S 1085) using the same set of primers and male fish from AB strain. Further studies are needed to clarify these discrepancies.

In order to evaluate the potential protective properties of E2, we treated zebrafish 8 h after the mechanical injury was inflicted with E2 and analyzed tight junction gene expressions 1 day after the lesion. Our results clearly showed that E2 treatment of stab-wounded zebrafish increased *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* mRNA levels compared to control injured zebrafish, but the weak upward trend we consistently observed was not statistically significant. Once again, it is possible that increased sampling and analysis could lead to a significant value and erase the heterogeneity of response between animals. Our data are in line with experimental data in mammals and suggest that estradiol could exert neuroprotective effects on the BBB integrity, through *cldn5a*, *cldn5b*, *tjp1a*, and *tjp1b* expression regulation. In ischemic situations, E2 has been shown to exert neuroprotective effects by rescuing tight junction protein expression through activation of both nuclear (ERα and ERβ) and membrane receptors (GPER1) (Lu et al., 2016; Na et al., 2015; Shin et al., 2013; Xiao et al., 2018). In our traumatic brain injury model, data pointed out a strong decrease in *tjp1a* and *tjp1b* expression and a trend toward fewer *cldn5a* and *cldn5b* transcript levels. Interestingly, using this telencephalon injury model, we have previously shown that the amount of *cyp19a1b* mRNA rapidly drops 30 min after the lesion and remains low until the seventh day (Diotel et al., 2013), suggesting our idea that the locally synthesized E2 could regulate *cldn5* and *tjp1* genes transcription. In agreement with the literature in rodents where E2 is acknowledged to favor tight junction protein expression after injury, our results also showed that E2 reduces the drop of *cldn5* and *tjp1* mRNA levels induced after the lesions. We further demonstrated that estrogenic treatment limits BBB leakage through functional experiments using dye extravasation.

4.4 | Cerebral estradiol synthesis and links with HDLs

Our experiments showed that AroB RGCs extend processes to cerebral blood vessels in a manner similar to astrocytic terminals in mammals. In the adult zebrafish brain, RGCs persist and do not develop into astrocytes, unlike mammals at the end of embryonic development. The functions normally performed by astrocytes in the establishment of the BBB could therefore be performed by RGCs, as previously reported (Diotel et al., 2020; Lyons & Talbot, 2014; Nagai et al., 2021). Interestingly, we also demonstrated that HDLs can easily reach the cerebral vasculature and be in close contact with the RGC processes covering the blood vessels. So far, these results suggest that HDL particles may be a source of cholesterol and steroids for AroB-positive RGCs. Indeed, as in mammals, the brain of adult teleost fish was shown to be a steroidogenic organ, able to de novo synthesize a wide diversity of steroids due to the expression and activity of the main key steroidogenic enzymes such as *17b-hsd*, *3a*, and *3b-hsd*, *cyp17*, *5a-reductase*, and *cyp19a1b* (Diotel et al., 2011; Mindnich et al., 2005; Pellegrini et al., 2016; Sakamoto et al., 2001). The brain is also well known to be a cholesterol-rich organ. In mammals, the main cholesterol-synthesizing cells are glial cells (astrocytes, oligodendrocytes, and microglia) and to a lesser extent neurons (Do Rego et al., 2009; Zwain & Yen, 1999). However, small HDL particles composed of cholesterol are suggested to cross the BBB and be transported within the brain (Koch et al., 2001; Ladu et al., 2000). These particles are also proposed to transport some steroids (androstenediol, E2, DHEA, DHT, pregnenolone, and progesterone) (Höckerstedt et al., 2004; Leszczynski & Schafer, 1991; Tikkanen et al., 2002). Consequently, although the brain expresses the complete set of steroidogenic enzymes allowing the synthesis of estrogen, steroid hormones are lipophilic and can easily cross the BBB by diffusion (Witt & Sandoval, 2014). Thus, peripheral androgens such as testosterone could be locally aromatized by neural stem cells after crossing the BBB. As well, HDL particles and other lipoproteins could eventually bring cholesterol and steroids to the brain in order to facilitate steroidogenesis. We showed that HDLs can target endothelial cells and could thus serve as a carrier. Thus, HDLs could provide cholesterol, cholesterol esters, and steroids to endothelial cells. However, in our experimental conditions, HDL injection did not show any significant change in *cyp19a1b* gene expression (a target gene of estrogen signaling) and in *cldn5* and *tjp1* gene levels. Nevertheless, women HDLs injected into zebrafish led to an increasing trend of *cldn5* and *tjp1*gene expression without reaching statistical significance levels. One limitation of our experiment is that estrogen quantification should have been performed; it may be that the concentration contained in these HDLs is not sufficient to stimulate *cldn5* and *tjp1*gene expression.

5 | CONCLUSION

In this study, we demonstrated that estrogen signaling could impact the expression of *cldn5* and *tjp1* genes in the adult zebrafish brain in a region-, time-, and dose-dependent manner. Furthermore, our data argue for a potential role for estrogen in preventing the decreased expression of these genes rapidly after telencephalic injury. Taken together, these results reinforce (1) the idea of evolutionary conserved regulation of BBB physiology between mammals and fish and (2) that the modulation of estrogen signaling could be considered as a therapeutic target to limit BBB leakage and promote brain repair. Thus, it may be of interest to artificially reconstitute HDLs with high amounts of estradiol to target endothelial cells and the brain to promote vascular and neuroprotection. Also, it would be of great interest to determine how estrogenic modulation similarly or differently affects tight junction expression and BBB functionality according to the sex of the fish.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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