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RESEARCH REPORT

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Gene expression patterns of the LDL receptor and its inhibitor Pcsk9 in the adult zebrafish brain suggest a possible role in neurogenesis

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Abstract

The low-density lipoprotein receptor (LDLr) is the first member of a closely related transmembrane protein family. It is known for its involvement in various physiological processes, mainly in the regulation of lipid metabolism, especially in the brains of mammals and zebrafish. In zebrafish, two *ldlr* genes (*ldlra* and *b*) have been identified and their distribution in the brain is not well documented. Recently, the roles of ldlr and its inhibitor pcsk9 in regenerative process after telencephalic brain injury have been discussed. In this study, we explored the expression patterns of these genes during zebrafish development. We found that *ldlra* expression was detected at the end of the pharyngula period (48 hpf) and increased during the larval stage. Conversely, *ldlrb* expression was observed from zygotic to larval stages. Using techniques like *in situ* hybridization and taking advantage of transgenic fish, we demonstrated the widespread distribution of *ldlra*, *ldlrb* and *pcsk9* in the brain of adult zebrafish. Specifically, these genes were expressed in neurons and neural stem cells and also at lower levels in endothelial cells. As expected, intraperitoneal injection of fluorescent-labelled LDLs resulted in their uptake by cerebral endothelial cells in a homeostatic context, whereas they diffused within the brain parenchyma after telencephalic injury. However, after intracerebroventricular injections into animals, LDL particles were not taken up by neural stem cells. In conclusion, our results provide additional evidence for LDLr expression in the

Abbreviations: AroB, aromatase B; Apo, apolipoprotein; Blbp, Brain lipid binding protein; DAPI, 4′,6-diamidino-2-phenylindole; DIG, digoxigenin; Dil, 3,3′-dioctadecyl-indocarbocyanine; Dm, dorsomedial part of the telencephalon; Dl, dorsolateral part of the telencephalon; Dp, dorsoposterior part of the telencephalon; Dpf, day post-fertilization; EGFP/GFP, enhanced green fluorescent protein/green fluorescent protein; GFAP, glial fibrillary acidic protein; HDL, high-density lipoprotein; Hpf, hour post-fertilization; Hv, mediobasal hypothalamus; Hc, caudal hypothalamus; ISH, *in situ* hybridization; LDL, low-density lipoprotein; LDLr, LDL receptor; LR, lateral recess of the hypothalamus; PR, posterior recess of the hypothalamus; PBS, phosphate-buffered saline; PCSK9, proprotein convertase subtilisin/kexin type 9; PFA, paraformaldehyde; PPa, anterior part of the preoptic area; PPp, posterior part of the preoptic area; PTw, PBS containing 0.1% Tween-20; VLDL, very-low-density lipoprotein; VLDLr, VLDL receptor; Vv, ventral nucleus of the ventral telencephalon; Vd, dorsal nucleus of the ventral telencephalon; Vc, central nucleus of the ventral telencephalon.

brain of adult zebrafish. These results raise the question of the role of LDLr in the cholesterol/lipid imbalance in cerebral complications.

KEYWORDS
LDL receptor, lipid metabolism, lipoprotein, nervous system, neurogenesis, PCSK9, zebrafish

1 | INTRODUCTION

In the 1970s, Brown and Goldstein's study of the internalization and degradation of low-density lipoprotein (LDL) by human fibroblasts led to the identification of the LDL receptor (LDLr) (Goldstein & Brown, 2009). They showed that normal fibroblasts have high-affinity binding sites on the cell surface for radiolabelled LDL particles, compared to cells from patients with familial hypercholesterolaemia who have LDLr mutation (Goldstein & Brown, 1974). Subsequent studies characterized other members of the LDLr superfamily, including the LDLr-related protein (LRP1), LRP1B, megalin (LRP2), very-low-density lipoprotein receptor (VLDLr), apolipoprotein E receptor 2 (ApoER2/LRP8) and multiple epidermal growth factor-like domain 7 (MEGF7/LRP4), which share different regions of the extracellular domain (Strickland et al., 2002). Other receptors in the same family with less structural homologies, have also been described, including LRP5, LRP6 and lipoprotein receptor LR11 (SorLA) (Herz, 2001).

In mammals, most members of the LDLr family are found in different organs. However, their tissue expression levels vary, reflecting their functional relevance (Hussain et al., 1999). In rodents, LDLr are predominantly expressed in the liver, intestine and adrenal gland and play a critical role in cholesterol metabolism (Fong et al., 1989; Srivastava et al., 1991). However, these receptors have also been detected at low densities in the brain, heart and muscles of rodents (Srivastava et al., 1991) and in the brain of adult monkeys (Pitas et al., 1987). A few studies have also reported the occurrence of the LDLr on endothelial cells of brain capillaries (Méresse et al., 1989) and rat astrocytes (Dehouck et al., 1997).

The primary role of the LDLr is to facilitate cellular endocytosis of cholesterol-rich lipoproteins by binding primarily to two physiological ligands: apolipoprotein B-100 (ApoB100) for LDL particles and apolipoprotein E (ApoE) for VLDL particles (Jeon & Blacklow, 2005). In addition to regulating cholesterol homeostasis, several studies have shown that LDLr are involved in many important physiological processes. These include intercellular signalling during embryonic development, regulation of the expression of some proteases and growth factors, regulation of Ca^{2+} homeostasis and transport of nutrients and vitamins (May et al., 2007; Strickland et al., 2002). Under physiological conditions, LDLr regulation is modulated by the endogenous proprotein convertase subtilisin/kexin type 9 (PCSK9), which binds directly to LDLr and induces its lysosomal degradation (Lagace, 2014; Poirier et al., 2009). Similar to LDLr, PCSK9 is primarily expressed in the liver but also, to a lesser extent, in the intestine, kidneys, lungs and brain of mice (Rousselet et al., 2011; Zhang et al., 2023). However, PCSK9 protein remains predominantly secreted by the liver. Given the importance of the delivery of essential lipids, including cholesterol to the brain, the potential functions of LDLr in the nervous system have been investigated but remain poorly documented. Lack of LDLr expression results in normal brain development in mammals, whereas other LDLr family members such as ApoER2, VLDLr and megalin appear to be more essential for neuronal migration and embryonic neurodevelopment, as their knock-out results in brain and/or cerebellar defects (Hirota et al., 2018; Trommsdorff et al., 1999; Willnow et al., 1996). *In vitro*, the LDLr allowed the transcytosis of LDL particles across a blood–brain barrier model, consisting of brain capillary endothelial cells cocultured with astrocytes (Dehouck et al., 1997).

In the field of lipid research, zebrafish (*Danio rerio*) has gained interest due to the high degree of genomic homology with humans (Howe et al., 2013). Zebrafish shares many well-conserved molecular pathways of lipid metabolism including cholesterol homeostasis (Anderson et al., 2011). Additionally, zebrafish possesses several gastrointestinal organs (liver, intestine, pancreas, gallbladder) as well as different cell types involved in lipid metabolism (enterocytes, adipocytes, hepatocytes and acinar cells) (Wallace et al., 2005). Interestingly, similar to mammals, the lipid profile of zebrafish can be modulated by diet. Several dyslipidemic models, including high-fat diets, have been developed to study pathologies involving lipid abnormalities such as atherosclerosis, obesity and type 2 diabetes (Schlegel, 2016). For instance, a high-cholesterol diet in zebrafish resulted in hypercholesterolaemia, which was characterized by elevated LDL-cholesterol (LDL-c) levels and accumulation of vascular lipid deposits in larvae (Stoletov et al., 2009). Similar to

higher vertebrates, lipid transport in zebrafish is facilitated by lipoproteins, mainly represented by high-density lipoproteins (HDLs) (Hölttä-Vuori et al., 2010).

Numerous studies have reported the expression of relatively conserved genes required for lipid transport in zebrafish including the apolipoprotein (*apoA-I*, *apoB*, *apoE*), cholesterol transporter (*npc1l1*), HDL receptor (*scarb1*, *abca1*, *abcg1*, *cd36*) and LDL receptor (*ldlr*) genes (O'Hare et al., 2014; Quinlivan & Farber, 2017; Sulliman et al., 2021). Recently, the role of cholesterol metabolism, HDLr, LDLr and Pcsk9 during brain regeneration after telencephalic injury in zebrafish has been discussed (Gourain et al., 2021; Rodriguez Viales et al., 2015; Sulliman et al., 2021). Indeed, genes involved in cholesterol metabolism as well as *ldlr*, *vldlr* and *pcsk9* were differentially regulated after brain injury, in agreement with other studies (Gourain et al., 2021). Therefore, zebrafish seem to be an excellent candidate for a better understanding of the molecular pathways involved in cholesterol and lipid metabolism, especially in the brain.

The aim of this study was to further characterize LDLr in the nervous system and explore its potential impact on neurogenesis using zebrafish. To achieve this goal, we first examined the gene expression of *ldlr* and its inhibitor *pcsk9* during zebrafish development and in adult zebrafish brain by RNA sequencing analysis and *in situ* hybridization (ISH), respectively. In addition, we analysed *ldlr* expression and uptake in different cerebral cell types using Tg (*fli1*:*EGFP*) and Tg (*GFAP*::*GFP*) transgenic fish to label blood vessels (endothelial cells) and neural stem cells, respectively. We then monitored the uptake of LDLs by brain cells and further investigated the role of LDLr in neurogenesis by conducting intracerebroventricular injections of labelled LDLs and inducing stab wound injury in the telencephalon.

2 | MATERIAL AND METHODS

2.1 | Zebrafish husbandry and ethics

Adult zebrafish (*D. rerio*) of the wild type (AB) and transgenic lines Tg (*fli1*:*EGFP*) and Tg (*GFAP*::*GFP*) were maintained in the DéTROI laboratory. These lines allow to visualize endothelial cells (Lawson & Weinstein, 2002) and neural stem cells (Lam et al., 2009; März et al., 2010; Sulliman et al., 2021).

Fish were housed under standard conditions of temperature (28.5°C), pH (7.4), conductivity (400 μS) and photoperiod (14 h dark/10 h light). All experiments were carried out on zebrafish in compliance with French and EU directives on the use of animals in research (APAFIS #2018040507397248; APAFIS #20200908140689).

TABLE 1 *ldlra* and *ldlrb* cloning sequences.

zf-ldlra	GCCTGAAAAATGTGGCCCTGGAACATCTAAG CCCACCAAAAATCCCTGCACTTCCATGGAGTT CCATTGCGGAAGTGGGGAATGCATACACGGT AGCTGGAAGTGTGATGGAGGAGCGGATTGTTT GGATCACTCGGATGAGCAAAACTGCTCCTTGC CAACATGCCGTCCAGATGAGTTCCAGTGTGGT GATGGGTCTTGCATCCACGGCAGTCGGCAGTG CAACCACGTTTATGACTGCAAAGACATGAGCG ATGAGCTGGGCTGCGTCAATGCAACTCACTGT GAGCCACCATACAGGTTTAAGTGCCGCAGCG GTGAATGCATAAGCATGGAAAAGGTTTGCAA CAAGCAACGGGACTGCAGGGATTGGTCTGATG AGCCGCTACGAGAATGTGACTCTAATGAGTGT CTTTACAACAACGGTGGCTGCTCTCATATCTG TAATGACCTGAAGATTGGTTATGAGTGCTTAT GTCCTACTGGCTTCCGGTTGGTGGACAAGAG ACGCTGTGA
zf-ldlrb	CACACATTATCAGCGCCGCTGTTTATCAGAGG ATCTGACACAGACAGGGTGAAACACACACACA CACACACACACTCTCCTCATGCTAATGACTCC TCTGCGTCTGCAGGACAGAAGACATGTGTGTC GGGTCAGTTCAGCTGTGGTGACCGGCTGAAT CAGTGTGTGTCCTCCAGGTGGCGCTGTGACG GGAAGTCTGACTGTGAGAACGGCGCAGACGA GCAGAACTGCGCGCAGAAGAACTGCAGCGCT GAGGAGTTCCGCTGCGGCAGCGGGCAGTGTG TGTCGCTCTCCTTCGTGTGTGACGGCGACAG CGACTGCAGCGACGGCAGTGATGAGGCAGCG TGTCCCACACACACACACCTGCGGCCCCA CAGCATTCCAGTGCAGCAGCCCAGCGGTGTG TGTGCCGCAGCTG
zf-pcsk9	AACAGAGTGCCTGAGGAGGACGGGGTCAGAG TTCACAGGCAGGCCAGTCAGTGTGACAGTCAT GGCACACACATTGCAGGGGTGATCAGTGGAC GGGACTCGGGTGTCGCTCGAGGTGCCAGTGT GAACAGCGTCCGAGTGTTGAACTGCCAGGGC AAGGGTACTGTGTCTGGAGCTTTGGCAGGTC TGGAATATATCCAGTCATCTCTGGCCTCTCAG CCTGTCAGTCCTGTTATCCTGCTGCTGCCATT TGTAGGGGGCTTCAGTCGCACCCTAAACACC GCCTGCCGGAAAATTGTTGAGTCTGGTGCAG TGCTTATTGCTGCAGCGGGAAACTATAACGA TGATGCGTGTCTGTATTCACCTGCCTCAGAG CCAGAGGTGATCACAGTAGGTGCTGTTAATT TTGCCGACCAGCCACTGAACCGTGGGACGA CGGGAACTAACGTGGGCCGCTGTGTGGATGT GTTCGCACCAGGTGATGACATCATTAGCGCA TCCAGTGACTGCCCCACCTGCTTCACCACCA AGAGTGGGACATCGCAGGCAGCCGCGCACG TTGCTGGTGTAGCAGCAGTTCTTCTGAACCT GAGGCCAAACTCCAGCTCTGCTGAGGTTCTT CAGCAGCTCCGCTATCATTCAGTCAAACAGG TCATTAACCCAGAGTCTCTACCAGTGATGCA CCGTCTCACTACACCCAACATGGTGGTGGCT CTGCCTGACCCAACATCCACACTCACAGG

2.2 | Zebrafish brain sampling

For ISH and immunohistochemistry, fish were euthanized with 0.02% tricaine. Immediately afterwards, they were fixed overnight at 4°C with PBS containing 4% paraformaldehyde (PBS-PFA). Next, the brains were removed from the skull and dehydrated stepwise in a methanol series before being stored in 100% methanol at −20°C until use.

2.3 | Probe synthesis, chromogenic and fluorescence ISH

Probe synthesis was carried out as previously described (Diotel et al., 2015; Gence et al., 2023). Briefly, DNA fragments of *ldlra*, *ldlrb* and *pcsk9* were cloned into the pGEM-T easy vector (Promega) (Table 1). After linearization, the plasmids were used to synthesize digoxigenin (DIG)-tagged antisense and sense riboprobes using T7 or SP6 RNA polymerases.

For chromogenic hybridization and fluorescence ISH, experiments were carried out as previously described (Diotel, Rodriguez Viales, et al., 2015; Rodriguez Viales et al., 2015). First, brains were rehydrated and washed several times in PBS containing 0.1% Tween-20 (PTw). Next, incubation in PTw containing proteinase K (10 μg/ml) was performed at room temperature for 30 min. Brains were immediately post-fixed in PBS-PFA for 20 min and rinsed with PTw. After a 3-h prehybridization step, brains were incubated in hybridization buffer (pH 6) containing one of the DIG-labelled probes (*ldlra*, *ldlrb* or *pcsk9*) overnight at 67°C. The brains were then washed several times before being embedded in 2% PBS agarose. They were then sectioned using a vibratome (Leica VT1000S) and blocked again for 1 h with blocking buffer (PTw containing 0.2% BSA, pH 7.4) at room temperature.

Incubation with Fab anti-DIG-AP fragments (1/2000, Sigma, Reference: 110932744910) was carried out overnight at 4°C. Next, brain sections were washed with PTw and stained with NBT/BCIP buffer (pH 9.5) or fast red staining solution (SIGMAFAST™ Fast Red TR/Naphthol AS-MX Tablets, Sigma, Reference: F4648) for chromogenic or fluorescence ISH, respectively. Finally, sections were mounted on slides with Aqua-Poly/Mount (Polysciences) or treated for immunohistostaining.

2.4 | Immunohistostaining

Following fluorescence ISH, immunostaining against HuC/D was performed to label neurons and against aromatase B (AroB) or brain lipid binding protein (BLBP) to label neural stem cells. To this end, sections processed for fluorescence ISH were incubated with anti-HuC/D (1/100; Invitrogen, A21271, Clone 16A11), anti-Blbp (1/500; Abcam, Ab32423) or anti-AroB antibodies (aromatase B encoded by the *cyp19a1b* gene; 1/500; kindly provided by F. Brion and previously used in Menuet et al., 2005; Pellegrini et al., 2007). After overnight incubation at 4°C, sections were washed several times in PTw and incubated for 1 h 30 min with the respective secondary antibodies: donkey anti-mouse Alexa Fluor 488 (1/500; A-21202) and donkey anti-rabbit Alexa Fluor 488 (1/500; A-21206), as well as with DAPI (final concentration: 1 μg/ml) to label cell nuclei. After several washes in PTw, sections were mounted with anti-fading medium (IMM Ibidi; REF: 50001).

2.5 | LDL preparation and injection

To investigate the links between LDL, endothelial cells and neural stem cells, human LDLs were isolated from blood samples of healthy individuals before being labelled with fluorescent lipid dye 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ($DiIC_{18}$, referred as DiI in the manuscript, 300 μg, Molecular Probes) as previously described (Bonneville et al., 2021; Ingueneau et al., 2009).

Intraperitoneal injection (80 mg/kg body weight) of LDLs was performed under anaesthesia (0.02% tricaine) in Tg (*fli: EGFP*). In parallel, an intracerebroventricular injection of LDLs was performed in Tg (*GFAP::GFP*) fish. For this, fish were anaesthetized (0.02% tricaine), and a hole was made in the skull above the junction between telencephalon and diencephalon. Approximately 2 nL of DiI-LDLs (10 mg/ml) was injected into the telencephalic ventricle with a glass capillary using a microinjector (FemtoJet, Eppendorf) as previously described (Gence et al., 2023; Rodriguez Viales et al., 2015; Sulliman et al., 2021). Fish were euthanized 90 min post-injection and fixed overnight at 4°C. After dissection, the brains were directly processed for embedding and vibratome sectioning without methanol treatment (avoiding consequently the decreased of DiI fluorescence).

2.6 | LDL biodistribution in brain injury conditions

LDL biodistribution during brain damage was evaluated by intraperitoneal injection (80 mg/kg) of LDLs 30 min before the stab wound injury with fish euthanasia occurring 1 h 30 min after the injection. Concerning the telencephalic lesion, it was performed in adult males as previously described (Diotel et al., 2013; Pellegrini

et al., 2023). Briefly, after anaesthesia in tricaine, the right telencephalic hemisphere was stab wounded with a sterile needle (BM Microlance 3; $30G^{1/2}$; 0.3 × 13 mm) following a dorso-ventral axis. Immediately after injury, zebrafish were placed back in tanks at 28°C under standard photoperiod.

2.7 | RNA sequencing analysis

Data from previously published RNA sequencing analyses were reanalysed from RNA-Seq/DeTCT database based during zebrafish development: ebi.ac.uk/gxa/; accession number: E-ERAD-475 (White et al., 2017). Also, data from (Gourain et al., 2021; Rodriguez Viales et al., 2015) were also used to study the expression of genes of interest in the uninjured hemispheres.

2.8 | Microscopy

Pictures were acquired with a Nikon SMZ18 stereomicroscope, a Zeiss Microscope Apotome 2 and a laser scanning confocal microscopes (Eclipse confocal [Nikon, Tokyo, Japan]). Microphotographs were acquired in TIFF format and adjusted for brightness and contrast using Photoshop before being assembled on plates.

3 | RESULTS

3.1 | Developmental expression of *ldlra*, *ldlrb* and *pcsk9*

A limited amount of data is available regarding the developmental expression of the *ldlra*, *ldlrb* and *pcsk9* genes in zebrafish. To gain a better understanding of their embryonic expression, we initially analysed a previously published RNA dataset by (White et al., 2017), which provides global transcriptomic profiling from the zygotic stage (one cell) to 5 days post-fertilization (dpf). Due to the genomic duplication that occurs in teleosts, two orthologues of the *ldlr* genes exist in zebrafish, namely, *ldlra* and *ldlrb*, whereas only one *pcsk9* gene has been conserved (www.ensembl.org). Interestingly, *ldlra* expression is almost not detected from the zygote stage to the pharyngula prim-25 stage (Figure 1a). Then, at the long-pec hatching stage, *ldlra* expression started to increase to a maximum in the larva (5 days) (Figure 1a). This expression parallels that of *pcsk9*, which began in the larva (protruding mouth stage) and remained elevated at 5 days (Figure 1c). In contrast, *ldlrb* exhibited strong inheritance, and its expression, though variable during development, remained significant throughout the analysed period (Figure 1b).

3.2 | Expression of *ldlra*, *ldlrb* and *pcsk9* in the brain and telencephalon of adult zebrafish

In the adult telencephalon, reanalysis of previous RNA-sequencing data (Gourain et al., 2021; Rodriguez Viales et al., 2015) showed that *ldlra* was expressed at lower levels than *ldlrb*, with *pcsk9* being also significantly expressed in the brain subdivision (Figure 1d). To further delineate the expression patterns of these three genes, we performed ISH on brain samples.

Initially, we incubated the brain without any probe and no staining was observed (data not shown). However, when we incubated the brain with the *id1* probe, which is well characterized for labelling neural stem cells along the ventricles of the brain, we observed the expected positive staining (data not shown) (Diotel, Rodriguez Viales, et al., 2015; Rodriguez Viales et al., 2015). Subsequently, *ldrla*, *ldlrb* and *pcsk9* ISH was performed. All these probes gave a positive signal in all brain subdivisions (tel-, di- and rhombencephalon) (Figure 2). Interestingly, the *ldlra*, *ldlrb* and *pcsk9* probes exhibited strong overlap in all brain areas studied.

Briefly, these genes were expressed in the telencephalon in both the subpallium and pallium, with clear detection in the ventral (Vv), dorsal (Vd) and central (Vc) nuclei of the ventral telencephalon. Expression was also observed in the dorsomedial (Dm), dorsolateral (Dl) and dorsoposterior telencephalon (Dp). In the diencephalon, *ldrla*, *ldlrb* and *pcsk9* were also detected in the anterior (PPa) and posterior (PPp) part of the preoptic area. Intense staining was also observed for each gene in the anterior (Hv), mediobasal (Hv LR) and caudal (Hc) hypothalamus, namely, around the lateral (LR) and posterior (PR) recesses of the hypothalamus. The thalamus was also strongly stained as well as the habenula, the optic tectum and the valvula cerebelli. Together, these data show that *ldrla*, *ldlrb* and *pcsk9* genes were widely and almost ubiquitously expressed in the adult zebrafish brain, both in parenchymal cells and in the cells lining the different cerebral ventricles, where neural stem cells are localized. Furthermore, we observed that the expression of *ldlrb* is stronger than that of *ldlra*, which is consistent with the RNA-sequencing data.

The general distribution of *ldlra*, *ldlrb* and *pcsk9* suggests significant neuronal expression in the brain parenchyma, as well as possible expression in neural progenitors (radial glial cells) located along the cerebral ventricles. To investigate these different hypotheses,

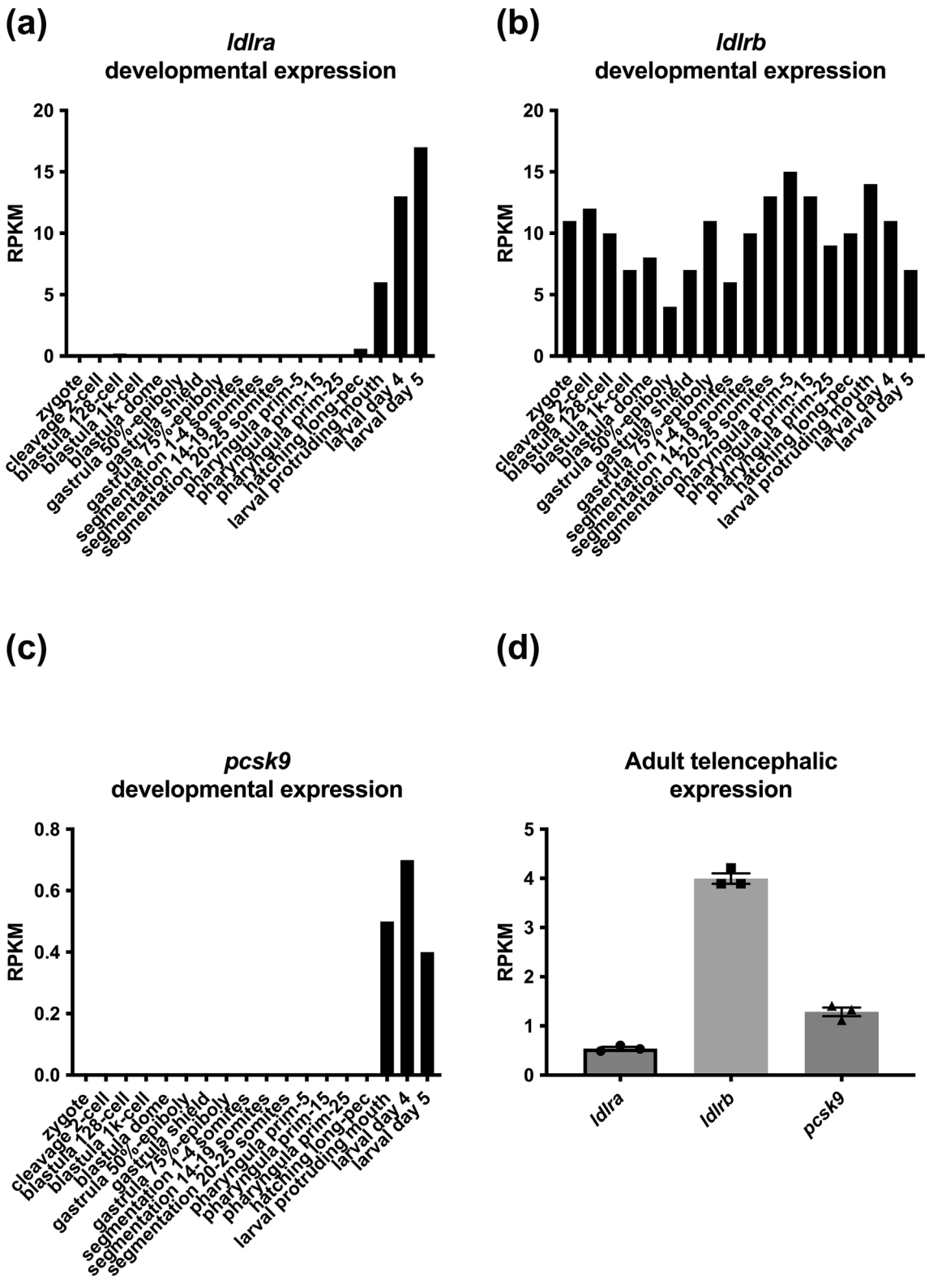

FIGURE 1 Developmental expression of *ldlra*, *ldlrb* and *pcsk9* and cerebral expression during adulthood. (a–c) Transcript quantification of *ldlra*, *ldlrb* and *pcsk9* during zebrafish development between zygote stage (one cell) to 120 h post-fertilization (120 hpf or larval day 5). Note that these data were obtained from the reanalysis of an RNA seq dataset performed by (White et al., 2017). (d) Transcript quantification of *ldlra*, *ldlrb* and *pcsk9* in the telencephalon of adult zebrafish (male + female). Note that these data were obtained from the reanalysis of an RNA seq dataset performed by (Gourain et al., 2021; Rodriguez Viales et al., 2015). RPKM, reads per kilobase million.

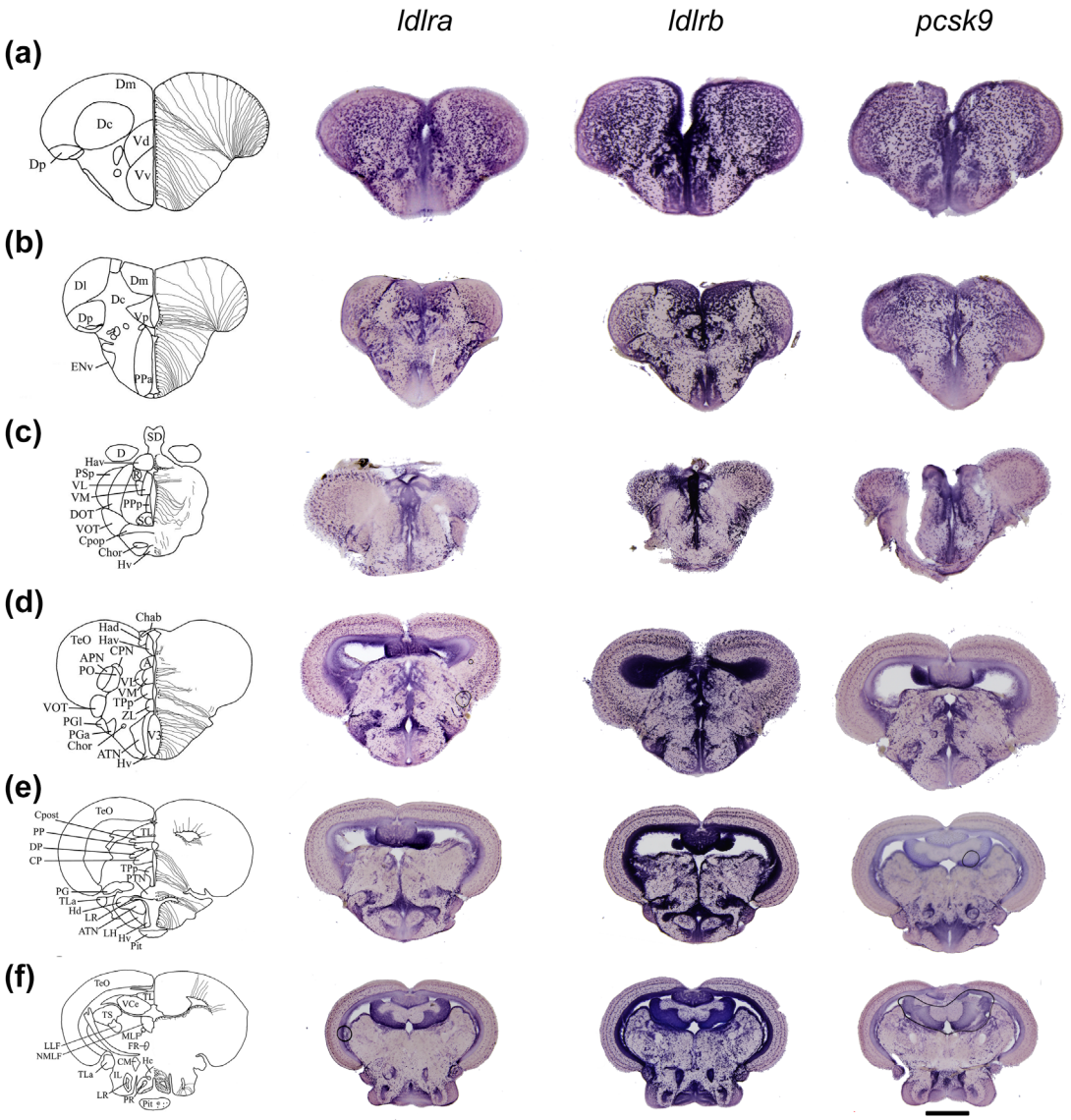

FIGURE 2 *ldlra*, *ldlrb* and *pcsk9 in situ* hybridization in the brain of adult zebrafish. (a–f) The schemes (left columns) provide the localization of the transversal brain sections with the name of the different brain nuclei and/or domains. (a–c) *ldlra*, *ldlrb* and *pcsk9 in situ* hybridization in the telencephalon (a,b), diencephalon with the anterior (b; PPa) and posterior (c; PPp) parts of the preoptic area. (d–f) *ldlra*, *ldlrb* and *pcsk9 in situ* hybridization in transversal brain section through the anterior part of the hypothalamus (D, Hv), the mediobasal hypothalamus (e; Hv LR) and the caudal hypothalamus (f; at the level of LR PR). Note that staining for each gene was detected in the anterior part of the hypothalamus, the periventricular nucleus of the posterior tuberculum (TPp), the central posterior thalamic nucleus (CP), the torus semi-circularis (TS) and midbrain parenchyma. The general overall pattern of expression for *ldlra*, *ldlrb* and *pcsk9* was almost similar between the different genes and was almost ubiquitous within the brain. Bar: 400 μm (c–f), 100 μm (b) and 70 μm (a).

fluorescence ISH was performed for the *ldlra*, *ldlrb* and *pcsk9*, followed by AroB and brain lipid binding protein (Blbp) staining to label radial glial cells (Figures 3 and 4) and by HuC/D immunohistofluorescence to label neurons (Figure 5).

Along the brain ventricles where neural stem cells are localized, numerous AroB-positive cells were detected as expected (Diotel et al., 2016; Menuet et al., 2005; Pellegrini et al., 2007; Pellegrini et al., 2016; Vosges et al., 2010) (Figure 3c,g,k). Double labelling against *ldlra, ldlrb* and *pcsk9* (Figure 3a,e,i) and AroB clearly demonstrated the expression of these genes in AroB-positive neural stem cells from the telencephalon (Figure 3d,h,l; see arrows). As shown in Figure 4, the expression of *ldlra*, *ldlrb* and *pcsk9* genes in radial glial cells was confirmed using Blbp staining, known to label neural stem cells (Diotel et al., 2010; Diotel et al., 2016; März et al., 2010). Similar results were obtained in radial glial cells from the posterior brain, particularly around the posterior ventricular cavity (Figure S1).

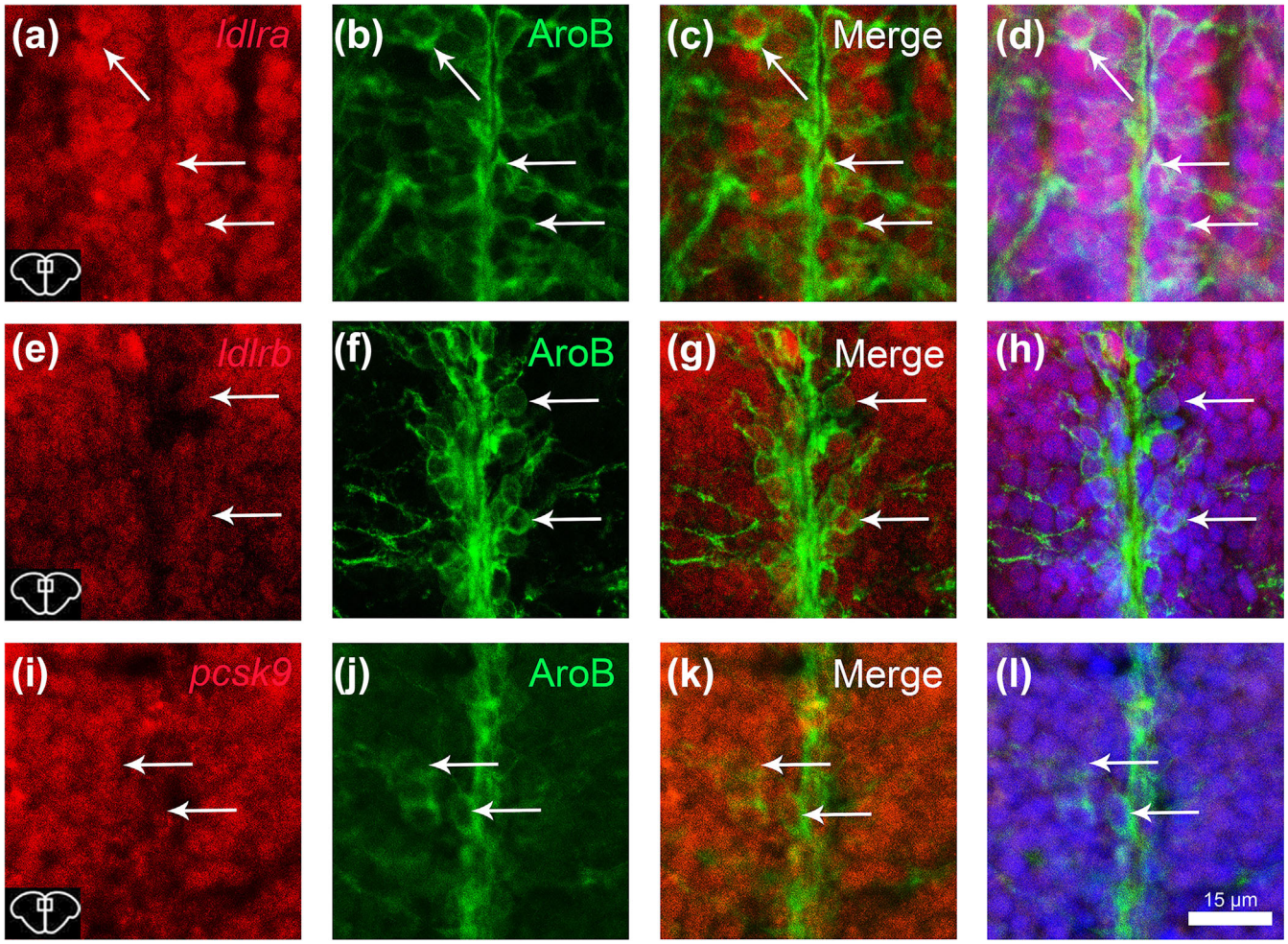

FIGURE 3 *ldlra*, *ldlrb* and *pcsk9* are expressed in AroB-positive neural stem cells in the telencephalon. (a–l) Fluorescence *in situ* hybridization (red) for *ldlra*, *ldlrb* and *pcsk9* (a,e,i), followed by AroB immunodetection (b,f,j) and DAPI counterstaining (blue). The merged pictures demonstrated the general expression of *ldlra*, *ldlrb* and *pcsk9* in AroB-positive neural stem cells without (c,g,k) or with DAPI staining (d,h,l) (see arrows). Bar: 15 µm.

As shown, in the parenchyma of the telencephalon, we observed that most *ldlra*-positive cells were HuC/D-positive (Figure 5a–d). The same pattern was observed for *ldlrb* (Figure 5e–h) and *pcsk9* (Figure 5i–l). However, not all the cells were positive for *ldlra*, *ldlrb* and *pcsk9* staining in the brain parenchyma (data not shown). In order to further identify these cells, we decided to investigate *ldlra*, *ldlrb* and *pcsk9* expression in endothelial cells using cell nuclei morphology and in microglia using the Tg (*mpeg1.1:GFP*) line. Interestingly, *ldlra*, *ldlrb* and *pcsk9* gene expression was studied in endothelial cells (Figure 6). As previously shown (Pellegrini et al., 2023), elongated cell nuclei lining blood vessels correspond to endothelial cells. These cells were generally positive for these different transcripts, but at low levels for *ldlra* and *pcsk9* (Figure 6; see arrows). In contrast, *ldlrb* expression appeared to be slightly stronger (Figure 6h).

In addition, we investigated expression in microglia using the Tg (*mpeg1.1*:GFP) fish. We observed that most microglia did not express the *ldlra*, *ldlrb* and *pcsk9* (data not shown).

Since LDLs are known to be taken up by endothelial cells and given the fact that we demonstrated *ldlr* gene expression in these cells, we decided to investigate whether intraperitoneal injection of human LDLs could be taken up by zebrafish cerebral endothelial cells from zebrafish. To achieve this, Dil-labelled LDLs were injected into the intraperitoneal cavity of Tg(*fli1:EGFP*) fish, in which endothelial cells are GFP-positive. After 1 h 30 min, the fish were euthanized, fixed and dissected to perform vibratome sectioning and confocal microscopy. As shown in Figure 7, fluorescent LDLs were found within the lumen of the blood vessels and also in endothelial cells (see arrows) (Figure 7).

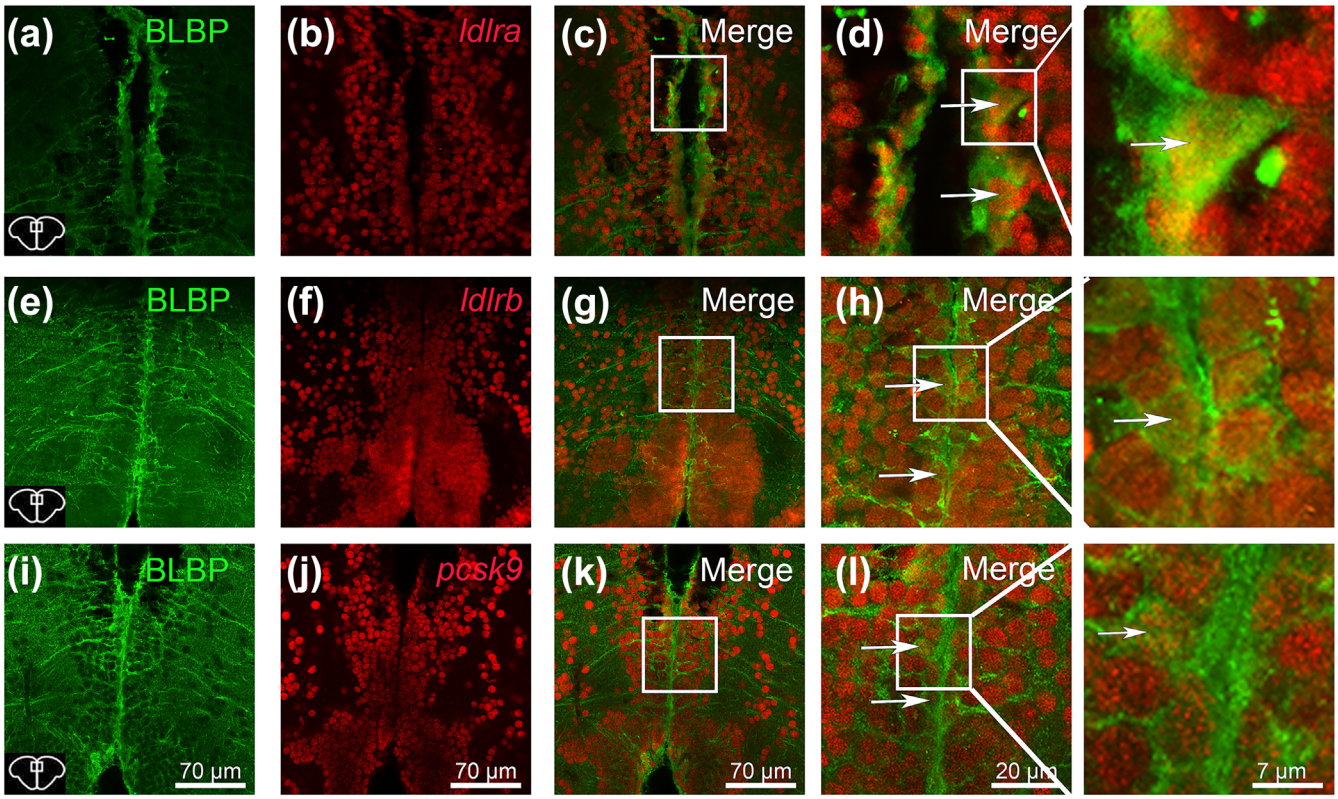

FIGURE 4 *ldlra*, *ldlrb* and *pcsk9* are expressed in BLBP-positive neural stem cells in the telencephalon. (a–l) Fluorescence *in situ* hybridization (red) for *ldlra*, *ldlrb* and *pcsk9* (b,f,j), followed by BLBP immunodetection (green) (a,e,i). The images shown in (c), (g) and (k) are the respective merged images of ab, ef and ij. (d,h,l) High magnifications of the corresponding white squares. The merged images showed the general expression of *ldlra*, *ldlrb* and *pcsk9* in BLBP-positive neural stem cells (see arrows). Bar: 70 µm (a,b,c,e,f,g,i,j,k), 20 µm (d,h,l) and 7 µm for high magnifications (right panels).

Next, given the expression of *ldlra* and *ldlrb* in the neurogenic niches, and in particular in neural stem cells, we investigated the possible uptake of LDLs by these cells. Fluorescent LDLs were injected via intracerebroventricular injection, a method previously employed in zebrafish studies for HDLs (Sulliman et al., 2021). We analysed the transport of LDLs in neural stem cells using *Tg (GFAP::GFP)* fish. As shown in Figure 8, most LDLs injected into the telencephalic ventricle were not taken up by GFAP::GFP-positive neural stem cells, except in a few isolated cases highlighted by arrows (Figure 8a–h). In our previous studies, we demonstrated that after brain injury, circulating fluorescent HDLs (from the bloodstream) diffuse within the brain parenchyma and were also strongly taken up by neural stem cells. However, in this study, intraperitoneal injection of LDLs followed by stab wound injury resulted in diffusion of LDLs within the brain parenchyma without increased uptake by neural stem cells (Figure 8i–p).

Very interestingly, for the extraction of RNA sequencing data from telencephalic stab wound injury of the telencephalon (5 days post lesion, dpl), *ldlra*, *ldlrb* and *pcsk9* were strongly downregulated in the injured hemisphere compared to the intact hemisphere (Gourain et al., 2021; Rodriguez Viales et al., 2015) as shown in Figure 9.

4 | DISCUSSION

In this study, we characterized the temporal expression levels of *ldlra*, *ldlrb* and *pcsk9* during zebrafish development and demonstrated their expression in the adult zebrafish brain. Using ISH experiments, we documented for the first time the distribution of *ldlr* and *pcsk9* in the adult zebrafish brain. Our results show their widespread presence across all brain subdivisions and territories, with overlapping expression profiles. We have shown that *ldlr* and *psck9* are primarily expressed by neurons, with additional expression in neural stem cells. Our use of heterologous LDLs allowed us to show that LDLs can be taken up by cerebral endothelial cells but not by neural stem cells. Interestingly, plasma LDLs can diffuse into the brain parenchyma in the setting of brain injury, suggesting a compromised integrity of the blood–brain barrier.

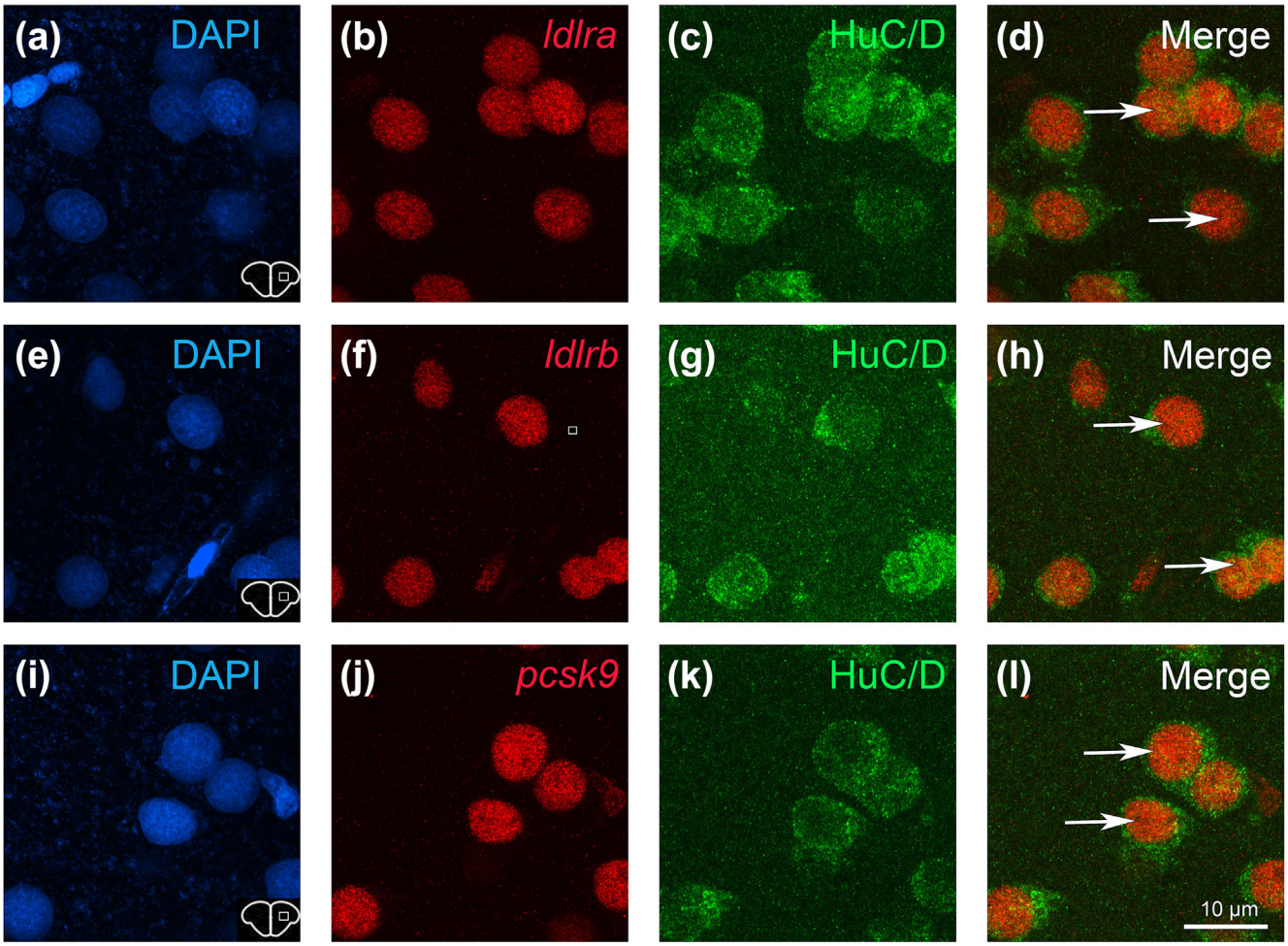

FIGURE 5 *ldlra*, *ldlrb* and *pcsk9* are mainly expressed by neurons in the telencephalon. (a–l) Fluorescence *in situ* hybridization for *ldlra*, *ldlrb* and *pcsk9* (b,f,j), followed by HuC/D immunodetection (c,g,k) with DAPI counterstaining (a,e,i). The merged pictures demonstrated the general expression of *ldlra*, *ldlrb* and *pcsk9* in HuC/D-positive neurons (d,h,l; see arrows). Bar: 10 µm.

4.1 | Specificity and nuclear localization of *ldlr* and *pcsk9*

An interesting aspect of the ISH staining obtained in this work is that *ldlr* and *pcsk9* appear to be widely expressed within the brain. However, not all cells express *ldlra*, *ldlrb* and *pcsk9* (see asterisks in Figure 6), which reinforces the fact that the staining is specific. Furthermore, the use of established probes (*id1* and *her4.1*) produced the anticipated staining, whereas incubation without probes resulted in no staining (data not shown), consistent with our previous findings (Fernezelian et al., 2024; Diotel, Rodriguez Viales, et al., 2015). Moreover, in certain brain regions, the high density of cells expressing these transcripts makes it somewhat challenging to distinguish individual cells. However, this cannot be regarded as background noise, especially since in areas where cells are more sparsely distributed, the staining remains clear.

The cellular localization of both *ldlr* and *pcsk9* appeared to be predominantly nuclear and/or perinuclear although a cytoplasmic detection was observed in posterior part of the brain (Figure S1), suggesting a different cellular localization/trafficking of the mRNA in these brain regions. In contrast with the prevailing view that messenger RNA (mRNA) is plentiful and readily detectable in the cytoplasm, an increasing body of evidence indicates that mRNA may also be retained within the nucleus (Dahlberg et al., 2003). It has been postulated that this nuclear retention may serve as a mechanism to buffer gene expression noise. By compartmentalizing mRNA, cells may be able to mitigate fluctuations in cytoplasmic mRNA levels associated with bursts of transcription, which could ultimately influence protein production (Bahar Halpern et al., 2015; Rambout & Maquat, 2024). In light of these findings, it is pertinent to inquire into the specifics of nuclear detection and the relative translation of *ldlr* and *pcsk9* within the brain. A

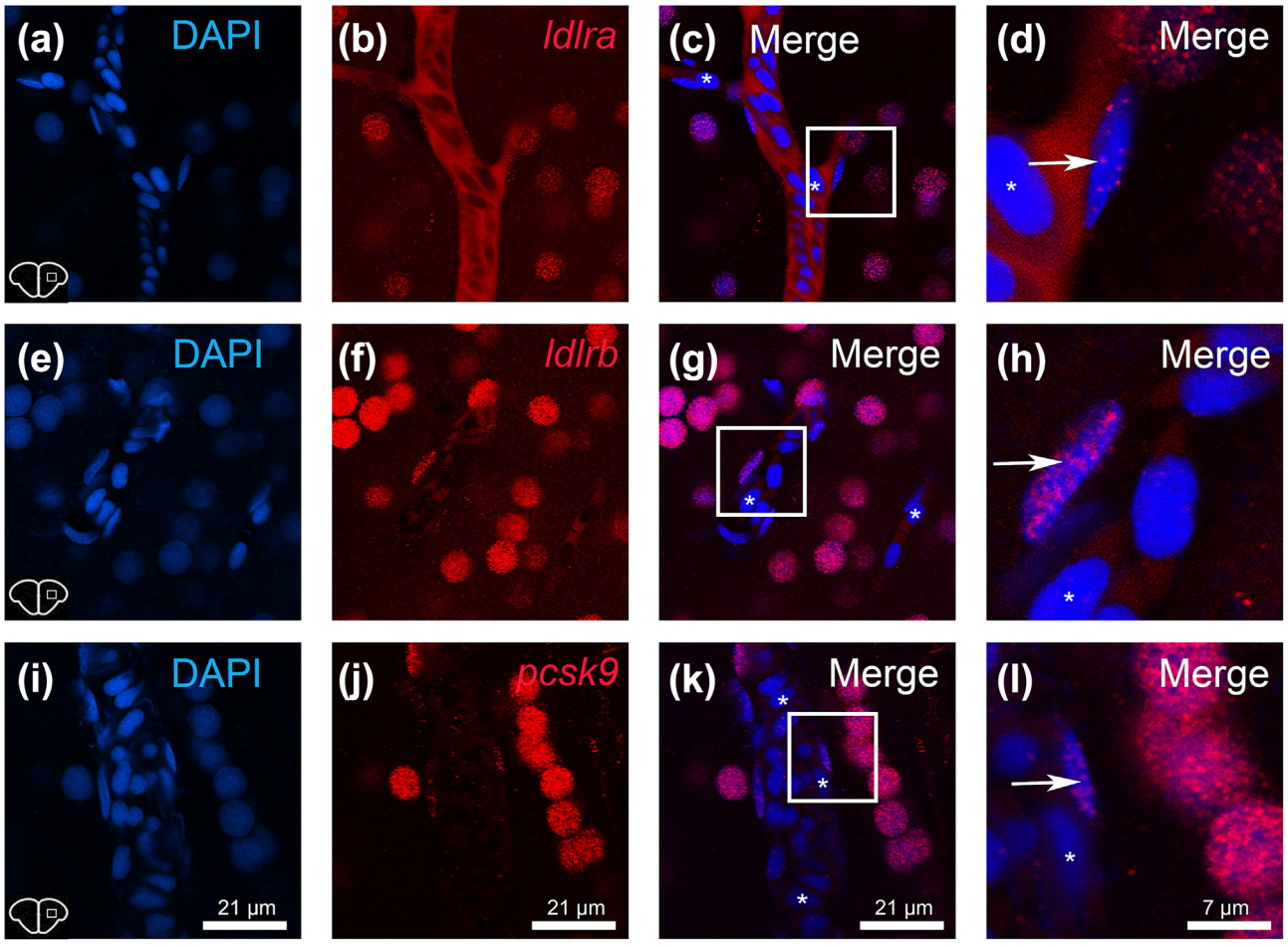

FIGURE 6 *ldlra*, *ldlrb* and *pcsk9* gene expression in endothelial cells. (a–l) Fluorescence *in situ* hybridization (red) for *ldlra*, *ldlrb* and *pcsk9* (B, F and J) in the telencephalon of adult zebrafish with DAPI counterstaining (blue, a,e,i). (c,g,k,d,h,l) Merged images with high magnifications of the corresponding white squares. The arrows indicate the detection of transcripts in some cells exhibiting an elongated and flat nucleus localized along the blood vessels, corresponding to endothelial cells. Note that a weak expression is shown in endothelial cells for *ldlra* and *pcsk9*, whereas *ldlrb* appears qualitatively more expressed in these cells. Asterisks show DAPI nuclei without ISH staining. Bar: 21 µm (a,b,c,e,f,g,i,j,k) and 7 µm (d,h,l).

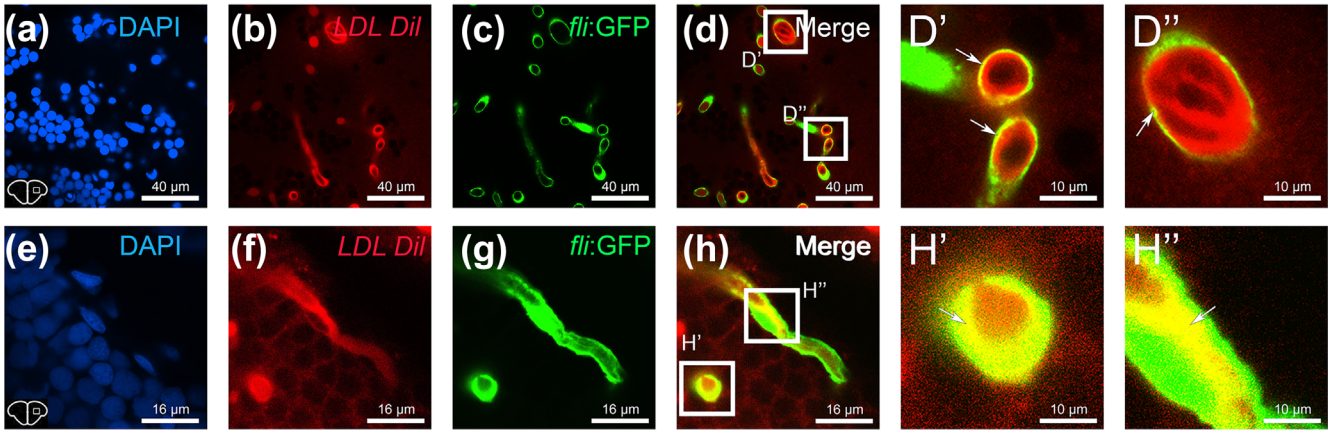

FIGURE 7 LDL are taken up by cerebral endothelial cells. (a–h″) intraperitoneal injection of DiI-labelled LDL in Tg(*fli1:EGFP*) fish (green) allowing to show the vascular distribution of LDLs (in red) in the telencephalon. White squares in d and h identify the high magnifications provided in D′, D″, H′ and H″. The arrows indicate the colocalization of GFP (green) and stained LDL (red), demonstrating that a subset of plasma LDL are taken up by endothelial cells (yellow colour). Bar: 40 µm (a–d), 16 µm (e–h). 10 µm (D′, D″, H′ and H″).

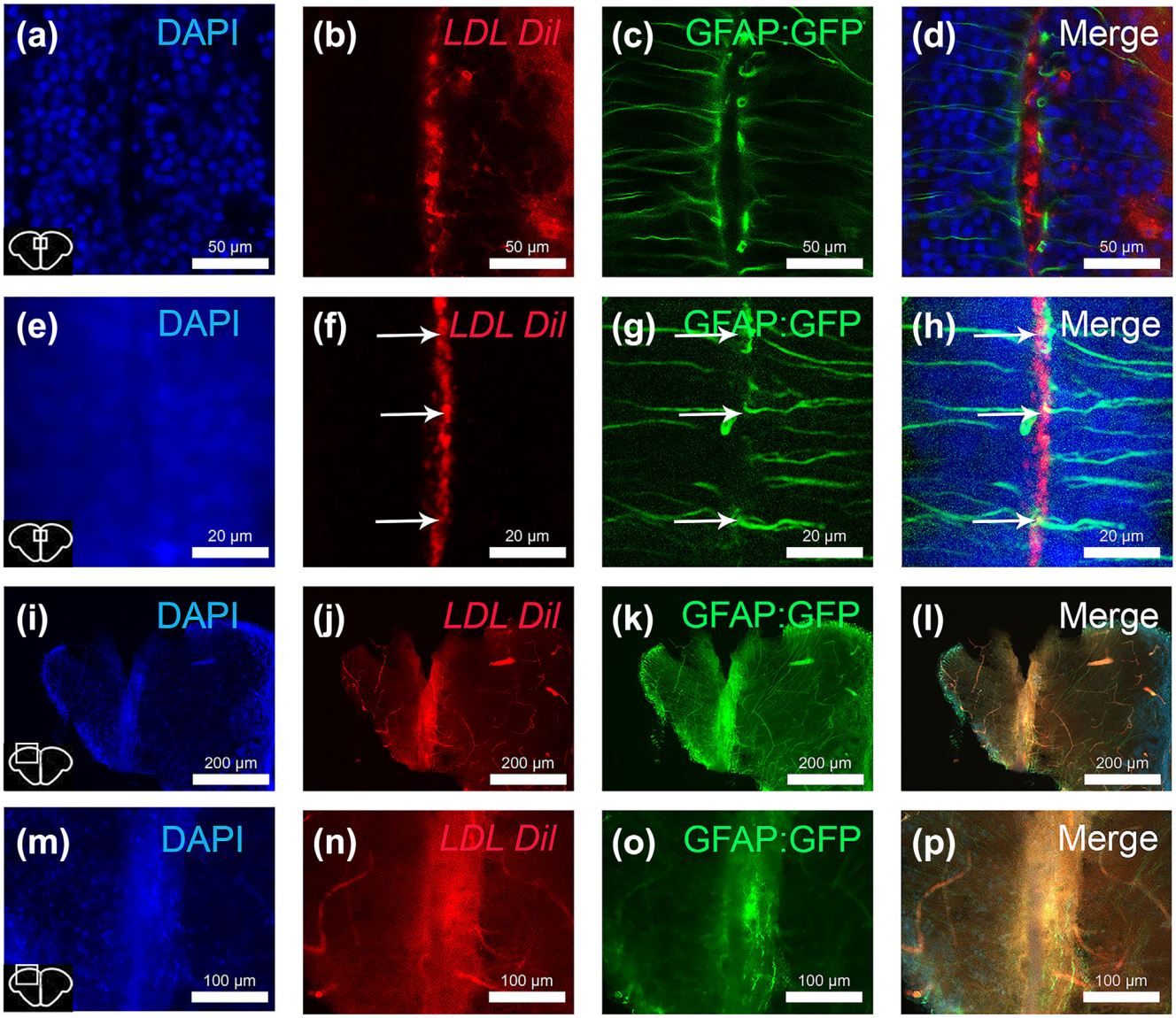

FIGURE 8 LDL are not taken up by neural stem cells in homeostatic and regenerative conditions. (a–h) Intracerebroventricular injection of Dil-labelled LDLs in *Tg (GFAP::GFP)* fish allowing to show that LDLs (in red) are not widely taken up by neural stem cells in homeostatic conditions. Arrows show little LDL detection in GFP positive neural stem cells. (i–p) Intraventricular injection of fluorescent LDLs followed by stab wound injury allows the diffusion of LDL particles within the brain parenchyma but not their increased uptake by neural stem cells. In this context, LDLs were intraperitoneally injected 30 min prior the stab wound and euthanized 1 h30 after this injury. (m–p) Magnification of above pictures (i–k). Bar: 200 µm (i–l), 100 µm (m–p), 50 µm (a–d) and 20 µm (e–h).

nuclear staining has previously been observed in zebrafish, particularly in a screen of over 1000 transcription factors in the telencephalon (Diotel, Rodriguez Viales, et al., 2015) and more recently for *vegf* and their receptors (Fernezelian et al., 2024). In this latter work, it was demonstrated that Vegfr3 and Vegfr4 immunostainings exhibited strong overlap with *vegfr3* and *vegfr4* ISH, despite nuclear detection, suggesting an efficient translation.

Taken together, the non-ubiquitous expression of the investigated transcripts, the *in situ* controls performed (i.e. *id1* and no probe incubation) and the literature on nuclear detection of mRNA reinforce the specificity of our staining.

4.2 | Ldl receptor and *pcsk9* expression in the brain

Cholesterol synthesis is essential for brain development and function (Diotel et al., 2018; Orth & Bellosta, 2012; Zhang & Liu, 2015). In the brain, cholesterol plays a

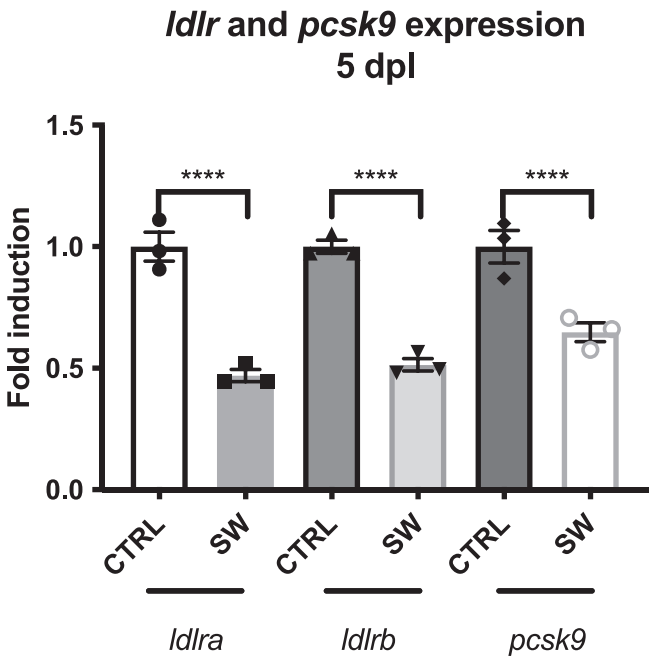

FIGURE 9 Downregulation of *ldlr* and *pcsk9* gene expression after telencephalic injury. Quantification of *ldlra*, *ldlrb* and *pcsk9* transcripts in the contralateral hemisphere (CTRL) and the stab wounded hemisphere (SW) at 5 days post lesion (dpl). Note that these data were obtained from a re-analysis of an RNA seq dataset performed by Gourain et al. (2021) and Rodriguez Viales et al. (2015). FPKM, fragments per kilobase million; RPKM, reads per kilobase million.

critical role in the formation of membranes, including dendrites, axons and synapses (Goritz et al., 2005; Pfenninger, 2009). It has been well documented that both neurons and glia are involved in cholesterol synthesis (Li et al., 2022). In addition, the LDLr is involved in the regulation of blood cholesterol levels by removing cholesterol from the bloodstream to peripheral tissues, including the brain. Consequently, the LDLr is a key player in cholesterol metabolism in the central nervous system, facilitating the uptake of cholesterol and cholesteryl esters from LDLs. In fact, a deficiency of the LDLr has adverse effects on brain function, particularly in neurogenic regions such as the hippocampus. For example, under a high-cholesterol diet, the hippocampus of LDLR−/− mice experiences neuroinflammation, impaired blood–brain barrier transport and disrupted of neural progenitor/stem cell activity (Engel et al., 2019; Mulder et al., 2007; Rutkowsky et al., 2018; Thirumangalakudi et al., 2008).

In zebrafish, due to a genomic duplication that occurred in teleosts (Steinke et al., 2006), two *ldlr* genes have been described: *ldlra* and *ldlrb*. Under our experimental conditions, these genes showed wide distribution and expression throughout the brain, including the telencephalon, diencephalon and midbrain, with both receptors showing overlapping patterns. In humans and mice, *ldlr* gene expression also appears to be widespread, with expression in the telencephalon (i.e. cortex and hippocampal formation), diencephalon (i.e. hypothalamus and thalamus), midbrain and hindbrain (i.e. cerebellum) (https://www.proteinatlas.org). Fluorescence ISH of *ldlra* and *ldlrb* combined with immunohistochemistry showed that both genes were expressed in most neurons and also in numerous neural stem cells. In the mammalian central nervous system, LDLr is also mainly expressed by neurons but is also detected in astrocytes and oligodendrocytes (Saher & Stumpf, 2015; Sun et al., 2020) (https://www.proteinatlas.org). We also showed that microglia do not express these genes, or only weakly for some of them, whereas endothelial cells express *ldlr* and *pcsk9* at significant levels. In mice and humans, brain RNA sequencing also shows a lower expression of LDLr in endothelial cells and microglia compared to neurons (Bennett et al., 2016; Clarke et al., 2018) (https://brainrnaseq.org). Together, our data are in line with the general mammalian literature and demonstrate that LDLr expression in the brain is evolutionarily conserved.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is known to bind to the LDLr and promote its degradation through endosomes/lysosomes (Blanchard et al., 2019; Sobati et al., 2020). In zebrafish, a single *pcks9* gene has been reported in the genome (www.ensembl.org). We demonstrated a broad distribution of cells expressing the *pcsk9* gene in the brain, overlapping with that of *ldlra* and *ldlrb*. Moreover, *pcsk9* gene expression was detected mainly in neurons but also in neural stem cells and endothelial cells. This expression seems to be consistent with data obtained in humans and mice (Bennett et al., 2016; Clarke et al., 2018) (https://brainrnaseq.org). Indeed, PCSK9 expression is observed in the different subdivisions of the mouse brain, mainly in the cortex, hippocampus, amygdala and basal ganglia, whereas in humans it is mainly detected at the level of the cerebellum, pons and medulla oblongata. In zebrafish, however, the expression of Pcsk9 protein is unknown and has not been documented.

In our analysis, re-evaluation of RNA-sequencing data from (Gourain et al., 2021; Rodriguez Viales et al., 2015) revealed that *ldlrb* is more highly expressed than *ldlra* in the brain. Similarly, brain expression of *pcsk9* appears to be lower than that of the predominantly expressed *ldlrb* gene. In mammals, data also showed lower expression of the *PCKS9* gene in human and mouse brains compared to the *LDLR* gene (Bennett et al., 2016; Clarke et al., 2018) (https://brainrnaseq.org; https://www.proteinatlas.org).

4.3 | Role of cholesterol and Pcsk9 in neurogenesis

The expression of *ldlr* and *pcsk9* in radial glial cells, which correspond to *bona fide* neural stem cells, raises the question of the role of LDL/cholesterol in zebrafish neurogenesis. Interestingly, in the case of familial hypercholesterolaemia, clinical and preclinical studies have shown an association between hypercholesterolaemia, mood and memory disorders (Engel et al., 2019; Zambon et al., 2010). Recently, it has also been shown that the metabolic state of neural stem cells influences their neurogenic activity (Knobloch et al., 2017; Sakayori et al., 2013). Interestingly, the work of Mulder et al. (2007) found that LDLr-deficient mice had weaker proliferating cells and synaptogenesis than control mice in the dentate gyrus of the hippocampus at 14 months of age. In young animals (3–4 months old), the number of proliferating cells and the total quantity of newly generated cells (neurons) in the dentate gyrus were lower than in older animals and controls (Engel et al., 2019). Furthermore, *in vitro* studies have shown that downregulation of LDLr leads to decreased proliferation of adult hippocampal precursor cells *in vitro*. In addition, treatment of these neuronal precursors with 27-hydroxycholesterol (an oxidized metabolite of cholesterol that is produced in excess in hypercholesterolaemic disease) induces a decreased cell survival (Engel et al., 2019). Similarly, disruption of cholesterol biosynthesis during embryonic development results in early forebrain neurogenesis in mice, which is partially rescued by treatment with statins and dietary cholesterol in pregnant dams (Driver et al., 2016). Taken together, these data from rodent models suggest a role of LDLR and cholesterol for brain development and neurogenesis.

During embryogenesis, zebrafish *pcsk9* expression is observed at the onset of neurogenesis (three-somite stage, 10.33 hpf), whereas in mice, it is detected at the telencephalic and cerebellar neurogenesis (E12.5 and E17-P15, respectively) (O'Connell & Lohoff, 2020). Interestingly, initial experiment using specific knockdown of *pcks9* mRNA in zebrafish resulted in generalized brain/cerebellar malformation, leading to embryonic death (at 96 hpf) (Poirier et al., 2006). In contrast, recent CRISPR/CAS9 studies targeting zebrafish *pcsk9* did not affect brain morphology and viability (Jacome Sanz et al., 2021). Such differences may be due to the toxicity of morpholinos initially used. Nevertheless, numerous studies have demonstrated a link between PCSK9 and neurogenesis in mammals (Poirier et al., 2006; Rousselet et al., 2011; Seidah et al., 2003). For example, overexpression of PCSK9 in mouse neural progenitor cells during embryogenesis leads to an increase in the number of neurons (Seidah et al., 2003). As reviewed by O'Connell and Lohoff (2020), the involvement of PCSK9 in neurogenesis and neuronal differentiation does not appear to be related to its role in LDLR/cholesterol metabolism. It would therefore be interesting to determine the role of PCSK9 in adult zebrafish neurogenesis.

Interestingly, 5 days after telencephalic stab wound injury of the telencephalon, *ldlra*, *ldlrb* and *pcsk9* are strongly downregulated in the ipsilateral compared to the contralateral hemisphere (Gourain et al., 2021; Rodriguez Viales et al., 2015). Consistent with these observations, the expression of the master regulator of cholesterol synthesizing enzymes *srebf2* is decreased after brain lesion in zebrafish, as well as its target genes encoding cholesterol-synthesizing enzymes (Gourain et al., 2021). It appears that brain injury in zebrafish leads to a reprogramming of cholesterol metabolism from synthesis to translocation of cholesterol (Gourain et al., 2021). However, it would be interesting to study the expression of these genes at later time points (5–30 days after injury). Indeed, we could hypothesize that the regeneration processes could be different with time, with newborn neurons generated after stab wound completing their differentiation (axogenesis, synaptogenesis) to functionally integrate the parenchymal neuronal network.

4.4 | Heterologous LDLs are taken up by endothelial cells but not by neural stem cells

Interestingly, injection of fluorescently labelled human LDLs resulted in their uptake by cerebral endothelial cells in zebrafish. This is not surprising given that brain endothelial cells are well known to express LDLr and bind LDLs in mammals (Dehouck et al., 1997; Kakava et al., 2022). Similar data have been obtained with HDLs, demonstrating their uptake by cerebral endothelial cells in zebrafish (Sulliman et al., 2021). In mammals, the SR-BI HDL receptor is also able to regulate LDL transcytosis in adult brain endothelial cells (Armstrong et al., 2015).

Surprisingly, injection of Dil-labelled LDLs into the cerebroventricular cavity did not result in LDL uptake by neural stem cells, which we have shown to express *ldlra* and *ldlrb* genes. This is surprising, given that it has been established in the literature that LDLs bind to neural progenitors (Engel et al., 2019). The same experiments with HDLs resulted in internalization or binding of HDLs to neural stem cells expressing a subset of HDL receptors, including SR-BI. These results may support the fact that neural stem cells preferentially take up HDLs, whose levels are known to be higher in zebrafish (Hölttä-Vuori

et al., 2010). Several hypotheses can be put forward: (1) Zebrafish neural stem cells do not express Ldlr proteins; (2) only a duplicate, *ldlra* or *ldlrb*, could mediate human LDL uptake, suggesting that neural stem cells would express the isoform unable to bind human LDL; and (3) human LDLs are unable to bind zebrafish Ldlr (*ldlra* and *ldlrb*). The latter would imply that fluorescent LDL detection in zebrafish brain endothelial cells would be mediated by receptors other than LDLr, possibly including SR-BI, as previously demonstrated in mammals, and other LDLr family receptors that are preferentially expressed in the brain (ApoER2, megalin and VLDLr). The use of specific knock-out fish lines will allow us to sort out these different possibilities.

5 | CONCLUSION

Therefore, for the first time to our knowledge, we report the distribution of *ldlr* and *pcsk9* gene expression in the adult zebrafish brain and demonstrate their expression specifically in neurons and neural stem cells. This raises the question of the impact of LDLs and cholesterol metabolism as well as of PCSK9 in constitutive and regenerative neurogenesis. It also prompts inquiries to the role of LDL dysregulation in metabolic disorders such as diabetes and/or obesity on the brain and neural stem cell homeostasis. Therefore, zebrafish may be an interesting model to better understanding the role of LDL cholesterol and metabolism in neurogenesis and brain repair, especially concerning metabolic diseases.

AUTHOR CONTRIBUTIONS

Laura Gence and Nicolas Diotel designed the experiments. Laura Gence and Nicolas Diotel performed all the experiments. Elena Morello performed additional ISH experiments, and Marie Laurine Apalama provided fluorescent LDLs. All authors contributed to the analysis of the experiments and/or in the writing and reviewing of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16586.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding authors (Nicolas Diotel and Laura Gence).

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