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Insulin signaling promotes neurogenesis in the brain of adult zebrafish

Laura Gence[1] | Danielle Fernezelian[1] | Olivier Meilhac[1,2] | Sepand Rastegar[3] | Jean-Loup Bascands[1] | Nicolas Diotel[1]

[1]Université de La Réunion, INSERM, UMR 1188, Diabète athérothrombose Thérapies Réunion Océan Indien (DéTROI), Saint-Denis, La Réunion, France

[2]CHU de La Réunion, Saint-Denis, La Réunion, France

[3]Institute of Biological and Chemical Systems—Biological Information Processing (IBCS-BIP), Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

Correspondence
Nicolas Diotel, Université de La Réunion, INSERM, UMR 1188, Diabète athérothrombose Thérapies Réunion Océan Indien (DéTROI), Saint-Denis, La Réunion, France.
Email: nicolas.diotel@univ-reunion.fr

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Abstract

Insulin is a peptide hormone that plays a central role in the regulation of circulating blood glucose in vertebrates, including zebrafish. Increasing evidence has demonstrated the important role of insulin in many brain functions. In zebrafish, two insulin receptor genes (*insra* and *insrb*) have been identified. However, their biodistribution in the adult brain as well as their cell-specific expression pattern has not been well described. Using gene expression analysis, in situ hybridization and transgenic fish, we confirmed the expression of *insra*, *insrb*, and *irs1* (*insulin receptor substrate 1*, the downstream effector of insulin receptor) in the brain of adult zebrafish and characterized their specific expression in neurons and neural stem cells (radial glia). After demonstrating that intracerebroventricular (ICV) injection resulted in the diffusion of the injected solution within the ventricular system, we analyzed the effect of insulin ICV injection on neurogenesis. We showed that insulin promotes ventricular cell proliferation 24 h postinjection. This neurogenic effect appeared to be independent of neuroinflammatory processes. Also, after a mechanical telencephalic stab-wound injury, we highlighted the overexpression of *irs1* gene 5 days postlesion notably in the ventricular zone where radial glial cells (RGCs) are localized, suggesting key roles of insulin signaling in regenerative processes. Finally, our results reinforced the expression of insulin-related proteins in the brain of adult zebrafish, highlighting the potential role of insulin signaling on neurogenesis.

KEYWORDS
brain injury, insulin, insulin receptor, insulin receptor substrate, neurogenesis, zebrafish, RRID: AB_2160651, RRID: AB_221448, RRID: AB_2732856, RRID: AB_2734146

1 | INTRODUCTION

Insulin is a peripheral hormone primarily synthesized by the pancreatic β-cells. It belongs to a superfamily of structurally related proteins including Insulin-like Growth Factors (IGF) and Relaxin-like proteins (Shabanpoor et al., 2009). Insulin plays an important role in the regulation of circulating blood glucose (Aronoff et al., 2004), acting in particular on the liver, muscle and adipose tissue by regulating glucose absorption, production, and release (Girard, 2006; Magkos et al., 2010). It was long assumed that insulin did not reach the brain. However, in 1967, Margolis and Altszuler provided evidence for the presence of insulin in the cerebrospinal fluids of dogs, at a concentration of

approximately 30% of the serum concentration (Margolis & Altszuler, 1967), suggesting that insulin was able to cross the blood-brain barrier (BBB). This process was subsequently confirmed by many other studies performed in different vertebrates, including humans (Daniel & Henderson, 1967; Greco et al., 1970) and was shown to be mainly mediated by a saturable transporter mechanism (Banks et al., 1997; Baura et al., 1993). Controversially, the possibility of insulin production directly by brain cells was not excluded. Numerous studies have shown the presence of C-peptide (the proinsulin connecting peptide) in human neurons as well as *insulin* mRNA in rat brain (reviewed by Ghasemi et al., 2013).

Interestingly, the expression of insulin receptors (IR) as well as other insulin-related proteins such as Insulin Receptor Substrates (IRS) were largely investigated in the mammalian brain. In rodents, these proteins are widely distributed in the central nervous system and are detected in neurons and glial cells (Unger et al., 1989). Of interest, in some neurogenic regions including the olfactory bulbs, hippocampus (dentate gyrus) and hypothalamus, IR appear to be strongly expressed (Baskin et al., 1993; Kamei et al., 2016; Marks et al., 1990; Unger et al., 1989).

The discovery of IR in the brain has clearly highlighted the possible roles of insulin on neurogenic processes and its effects on neural stem cell proliferation (Erickson et al., 2008). Therefore, many studies have shown the action of insulin on various physiological processes including BBB integrity, neuronal survival and brain plasticity (Banks et al., 2012). Indeed, insulin contributes to the maintenance of BBB homeostasis by regulating the integrity of the tight junction (Ito et al., 2017). It also exerts a neuroprotective action on neurons and glial cells by reducing the activation of protein-mediated apoptosis such as caspases and cathepsins (Mielke et al., 2006; Valenciano et al., 2006). On the other hand, it has been proposed that brain alterations in insulin-related processes lead to neuronal degeneration which is subsequently implicated in cognitive decline as well as in the pathogenesis of neurodegenerative diseases such as Alzheimer's (Banks et al., 2018; Bilotta et al., 2017; Bosco et al., 2011). Insulin deficiency has been associated with deregulation of IRS1 and a decrease in the number of synapses resulting in altered neuronal network and then to cognitive disorders (Chiu et al., 2008; Talbot et al., 2012).

Although adult neurogenesis was considered impossible for over a century, it is now well accepted that neurogenesis persists throughout life in the brain of vertebrates, including mammals and humans (Gage, 2019). Similarly, after brain damage, injury-induced neurogenesis occurs to repair the brain (Dos Santos et al., 2021; Gemma & Bachstetter, 2013). Neural progenitor cells are able to proliferate, differentiate, and migrate to replace damaged and/or dead cells (Parent, 2003). However, the mechanisms underlying these processes remain unclear and it is hypothesized that one possible pathway to activate homeostatic and regenerative neurogenesis involves Tyrosine Kinase receptors such as IR (Anderson et al., 2002; Shioda et al., 2009).

In the field of neurosciences, adult zebrafish has emerged as an important model to investigate neurogenesis and brain plasticity because of the significant brain homology with mammals, as well as the relatively evolutionary conserved neurogenic and regenerative mechanisms (Diotel et al., 2020; Howe et al., 2013; Schmidt et al., 2014, 2013). In zebrafish, two isoforms of IR have been characterized encoded by two distinct genes (*insra* and *insrb*), and their function were found to be highly conserved compared to humans, activating the same signaling cascades, notably via Irs1 (Toyoshima et al., 2008). However, little is known about their expression and roles in the brain of adult zebrafish.

The aim of this study was therefore (1) to investigate the expression and distribution of *insr* genes in the brain of adult zebrafish, (2) to determine the impact of insulin in neural stem cell proliferation, and (3) to provide insight on the possible role of insulin signaling in neurogenesis after telencephalic injury. To this end, we first performed RNA sequencing reanalysis and in situ hybridization to determine the distribution and expression of *insra*, *insrb* and *irs1* in different cell types of the adult zebrafish brain. In parallel, we examined the effect of insulin on constitutive neurogenesis by microinjecting insulin into the brain ventricle. Finally, after telencephalic stab-wound injury, we analyzed the expression of *insulin*, *insulin receptors*, and *irs* at 5 days postlesion, a key time for brain regeneration.

2 | MATERIAL AND METHODS

2.1 | Animals and ethics

Zebrafish (*Danio rerio*, AB strain) were housed in the zebrafish facility of the CYROI/DéTROI, La Réunion (A974001). Adult wild-type (WT), transgenic tg(*GFAP::GFP*) and tg(*mpeg1.1:GFP*) zebrafish (3-6 months old) were maintained under standard conditions of temperature (28.5°C), photoperiod (14 h dark/10 h light), pH (7.4) and conductivity (400 μS). Fish were fed 3 times a day (Gemma Micro 300, Skretting, France). All the animal experiments were conducted in accordance with the French and European Community Guidelines for the Use of Animals in Research (86/609/EEC and 2010/63/EU) and approved by the local Ethics Committee for animal experimentation of CYROI and the French Government (APAFIS# 2022111511426148 v4).

2.2 | Traumatic brain injury on adult zebrafish telencephalon

The traumatic brain injury was performed by inserting a needle (30G ½″ 0.3 × 13 mm, BD Microlance™ 3) into the medial region of the right telencephalic hemisphere, as previously described (Diotel et al., 2013; Schmidt et al., 2014) under anesthesia with 0.02% tricaine (MS-222). After telencephalic injury, fish were placed back in water for 5 days before sacrifice and histological analyses.

2.3 | Intracerebroventricular (ICV) injection of dye and insulin

To study the effects of insulin on neurogenesis, we performed ICV injections of insulin in the ventricle. However, we first ascertained the diffusion of injected solution within the ventricular system using dyes (Evans blue and high-density lipoproteins (HDLs) labeled with DiIC18) as previously described (Sulliman et al., 2021). Then, to assess the

TABLE 1 Primary and secondary antibodies.

Primary antibodies				
Antibody	**Dilution**	**Host**	**Reference**	**RRID**
PCNA	1/500	Mouse	Dako, M0879	RRID:AB_2160651
HuC/D	1/300	Mouse	Invitrogen, A21271, Clone 16A11	RRID:AB_221448
Secondary antibodies				
Antibody		**Dilution**	**Reference**	
Alexa Fluor 488 donkey anti-mouse		1/500	Abcam, ab150105	RRID: AB_2732856
Alexa Fluor 594 donkey anti-mouse		1/500	Abcam, ab150108	RRID: AB_2734146

PCNA: proliferating cell nuclear antigen.

impact of insulin on neural stem cell proliferation and neuroinflammation, fish were anesthetized (0.02% tricaine) and a hole was made using a needle into the skull, at the junction between the telencephalon and diencephalon (Sulliman et al., 2021). Around 2 nL of, vehicle (1× PBS) and insulin (Umuline®, 100 UI/mL, injection of 10 UI/mL after 1/10 dilution in 1× PBS) were injected into the ventricle with a glass capillary using a microinjector (Femtojet, Eppendorf). After injection, fish were placed in water before being sacrificed 24 h postinjection. Then, fish were fixed in 4% paraformaldehyde (PFA), overnight at 4°C, prior to brain dissections. Next day, brains were carefully removed and stored in 100% methanol until brain cell proliferation analyses. This experiment was done on a total of 4–6 fish/condition. Note that fish injected with the dyes were euthanized in similar conditions.

2.4 | Immunohistochemistry

Brain cell proliferation analysis was done according to previous protocols (Dorsemans, Lefebvre d'Hellencourt et al., 2017; Gence et al., 2022). Briefly, adult zebrafish brains were rehydrated, permeabilized with PTw (1× PBS containing 0.1% Tween 20, pH 7.4) and embedded in 2% agarose before performing 50-μm-thick sections using a vibratome (VT1000S, Leica). After 1 h of blocking with PTw containing 0.2% BSA and 1% DMSO, sections were incubated with primary antibodies overnight at 4°C (Table 1). Next day, the sections were washed 3 times with PTw and incubated with Alexa Fluor® secondary antibodies and DAPI counterstaining (1 ng/mL, ThermoFisher, Reference: D1306) for 2 h at room temperature. Finally, the sections were washed with PTw and mounted on slides with Aqua-Poly/Mount (Polysciences).

2.5 | Probe synthesis and in situ hybridization

Probe synthesis was done as previously described (Sulliman et al., 2021). Briefly, specific primers (Table 2) were designed to amplify the required DNA fragments and clone them into the pGEM-T easy vector (Promega). Plasmids were then linearized for the synthesis of digoxigenin (DIG)-labeled antisense and sense riboprobes using T7 or SP6 RNA polymerases.

For ISH, brains were rehydrated through methanol/PBS gradient series and washed several times in PTw. They were next incubated for 30 min in PTw containing proteinase K (10 μg/mL) at room temperature. After postfixation in 4% PFA for 30 min and washes in PTw, brains were prehybridized for 3 h and incubated in hybridization buffer (pH 6) containing the DIG-labeled probes, overnight at 65°C. The second day, brains were washed several times and incubated briefly in blocking buffer (PTw containing 0.2% BSA, pH 7.4) before embedding in 2% agarose. They were then sectioned using a vibratome and blocked again for 1 h at room temperature. Incubation with the anti-digoxigenin-AP, Fab fragments (1/2000, Sigma, Reference: 110932744910) was performed 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 (SIGMA*FAST*™ Fast Red TR/Naphthol AS-MX Tablets, Sigma, Reference: F4648) for chromogenic or fluorescent ISH, respectively. Finally, the sections were mounted on slides with Aqua-Poly/Mount (Polysciences) or processed for additional immunohistochemistry before mounting.

2.6 | Microscopy and cell counting

Pictures were acquired using an Axio Observer microscope equipped with the ApoTome 2 (Carl Zeiss), a Nikon SMZ18 stereomicroscope equipped with a Nikon DS-Fi3 Camera and a confocal microscope (Confocal Nikon C2si). The ZEN lite software and NIS elements software were used for Zeiss and Nikon equipment, respectively.

For brain cell proliferation, the PCNA-positive cells were counted on 1–2 vibratome sections/region/fish for each condition. The cell counting provided graphs corresponding to the PCNA-positive cells normalized to the vehicle condition in %.

For quantification of fluorescence, pictures were analyzed using the ImageJ software.

2.7 | Statistical analysis

Data were presented as means ± *SEM* (standard error of the mean). Statistical analyses were performed using GraphPad Prism 8 software. Comparisons between groups were done using Student t-test considering values as significantly different when p value < .05.

TABLE 2 Cloning primers.

ZF gene	Amplicon
insra	TCTTCAACTATGCGCTGGTGCTCTTTGAGATGCTGCAGCTGAAGGAGATCGGCCTTCACAGCCTGATGAC ATCACTCGCGGAGCCGTGCGTGTGGAAAAGAACCCGGACCTCTGCTACCTCTCCACCCTCGACTGGTCCA TGATCCTGGACTCTGTGGAGGACAACTACATTGTGGCCAATAAGAATGATCGGGAGTGTGGAGACATCTG TCCAGGCACGGTTCTGGGCAAAACAACATGCCCCCTCACCACTATCAACGGCGACTTCAGCGAGCGCTGC TGGAACCAGAAGTACTGCCAGAGAATGTGTCCATCCTCATGCAAGCACCGCGCCTGCACTAAAGACAGCC AGTGCTGCCACGACCAGTGTCTGGGCGGCTGTTTGGAGCCCCACTCGTCTAACAAATGCGTGGCCTGCCG CAACTTCATGCACAACAACATGTGTGTCGAAAAGTGCCCACATGGTTTCTACACCTTCAAGGGCTGGCGC TGCGTCAGCTTCAGCTTTTGCCAGGAGCTCCACAACAAGTGCAAGCAAGGCAAAGGTGACTGCCACGAA TACGTCATTCACAACGGCGCCTGCATCCCTGAGTGCCCTTCAGGATACACCACTGTCAACTCCACTACGTT GAACTGCACCCC
insrb	CCGCAGAGACCTAGTGAAGGTAAAGAGGTGGAGCCGGGGTACCTGATCATGCCTCTCAAGCCCTGGACT CAATACGCCGTCATGGTGAAAGCCCAGCTCGCAGCCTCAGAAGACCATCAGGTCAGAGGAGCCAAGACC GAGATCATCTACGTCAGGACCAACGCCACACGGCCTTCAGTGCCGCTGGATCCTCACTCCTGGTCAAACT CGTCCTCTCAGATCATCCTCAAGTGGAAACCGCCGTCAGACCCCAATGGAAACATCACACACTACATCAT CTACTGCCAGAAGCAGAGCGAGGACATCACACACTACAAGTTTGACTACTGCCAGCAAGGTATGAAAGT GCCGTCCCACACGCCGACGCACCACGACGTCGAGGAGGACCAGAAGCTTAACCAGACGGAGGTTCCAG ACCAGAAGGGGCACTGCTGCGCCTGTCCCAAAACCGAAATGCAGCTCAAGAAAGAGGCAGAGGAAACT GAGTTTCGCAAGACCTTCGAGAACTACCTGCACAATGAAGTGTTCGAGCCCAGGCCCCACCGTCGGCGTC GCTCAGTTGGCGTAGCCAATGCAACGTTGCCAAATTTTGGAACCACACCAACTATAATACCCAACTTTCC AACCACTTCAATCCCAGAGGAAGAAAAGAGCCGGAAGATGGTGTTCAAAGCCAGCTCCAAAG
irs1	ACTTCCGGACGAAACCAGGGCGCTAAAGTAATACGTGCTGACCCACAGGGTCGACGGCGGCACAGTTCA GAGACTTTTGCATCCACAGCGACCAGAGGGTCAGTCGACACATCGAGCACCTGTCAATCAGGGGATGTA AAGCGCCACAGCTCCGCCTCCTTCGAGAATGTGTGGTTGAGACCTGATCAGGCCTCTGCTGCTTCTACCG CTCCATCTGCATCGACAGTAACCAACGGACGAAGGGAAACCGCTTCAGGCCAAATTCCCACATTAACAA ACCATGACCAGAACGGCCTGAACTACATTGATCTGGATCTAATGCAGAATGGAGAGCAACATGTTCCTG ATTGGAATGCTTTC

3 | RESULTS

3.1 | Expression of *insulin receptors* (*insr*) in the adult zebrafish brain

Although the expression of insulin and its receptors has been widely reported in the rodent brain, virtually no data exist for the adult zebrafish brain. By reanalyzing RNAseq data published in the studies of Gourain et al. (2021), Rodriguez Viales et al. (2015), and Wong and Godwin (2015), we investigated the cerebral expression of *insr* in zebrafish (Figure 1). In the whole brain, *insrb* gene expression was significantly lower than that of *insra*. In contrast, the two isoforms (*insra* and *insrb*) appear to be similarly expressed in the telencephalon of adult zebrafish.

To describe more precisely the distribution of *insr* in the brain of adult zebrafish, we performed in situ hybridization (ISH). Firstly, the specificity of the probes generated for *insra* and *insrb* was tested. The sense probes resulted in no or very low staining as shown in Figure 2. In parallel, we also performed an ISH for *id1* (Figure 2c) and observed the expected expression in the ventricular zone where neural stem cells are localized (Diotel, Rodriguez Viales et al., 2015; Rodriguez Viales et al., 2015).

Interestingly, using *insra* and *insrb* antisense probes, the distribution between their respective transcripts appeared to overlap widely in the major subdivisions of the brain including the tel-, di-, and mesencephalon (Figure 3). Specifically, in the telencephalic region, we observed that *insra* and *insrb* expression was detected in the ventral (Vv), central (Vc), and dorsal (Vd) nuclei of the ventral telencephalon as well as in the dorsomedian (Dm), dorsolateral (Dl), and dorsoposterior (Dp) domains of the pallium. In the diencephalon, *insr* transcripts were also detected in the preoptic, thalamic, and hypothalamic areas. Indeed, expression of both genes was observed in the anterior and posterior parts of the preoptic region (PPa and PPp), and in the ventral zone (Hv), dorsal zone (Hd), and caudal zone (Hc) of the periventricular hypothalamus. Expression was also observed in the ventrolateral (VL) and ventromedial (VM) thalamic nuclei, in the periventricular pretectal nucleus (PPv). In more posterior regions, *insra* and *insrb* were also expressed in the torus lateralis (TLa), preglomerular nucleus (PG), optic tectum (TeO), the valvula of the cerebellum (VCe), and the cerebellum (CCe).

Interestingly, in some brain regions including the preoptic area, periventricular pretectal nucleus (PPv), Hv, TeO, TLa, PG, and CCe, *insrb* seemed less expressed than *insra*. This is consistent with the RNAseq analysis and reinforces the fact that *insrb* gene expression was shown to be less expressed than *insra* in the whole brain (Figure 1).

3.2 | Insulin receptors are expressed in neurons and neural stem cells

Given the general distribution of *insra* and *insrb* in the whole brain, in the parenchyma and along the ventricles where neurons and neural stem cells (NSC) are located, respectively, we suggested that *insra* and *insrb* might be expressed by these cells. Therefore, we first

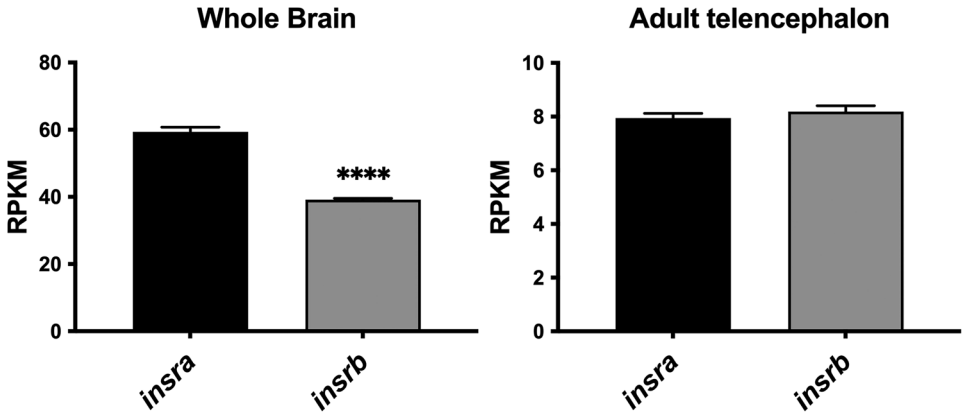

FIGURE 1 Gene expression of *insra* and *insrb* in the whole brain and the telencephalon of adult zebrafish. RNA sequencing data showing the relative gene expression of *insulin receptor a* (*insra*) and *b* (*insrb*) in the whole brain ($n = 4$) and the telencephalon ($n = 3$) of adult zebrafish (data from Gourain et al., 2021; Rodriguez Viales et al., 2015; Wong & Godwin, 2015). RPKM, reads per kilobase per million. Results are expressed as means ± *SEM*. ****$p < .0001$ (Student's *t*-test).

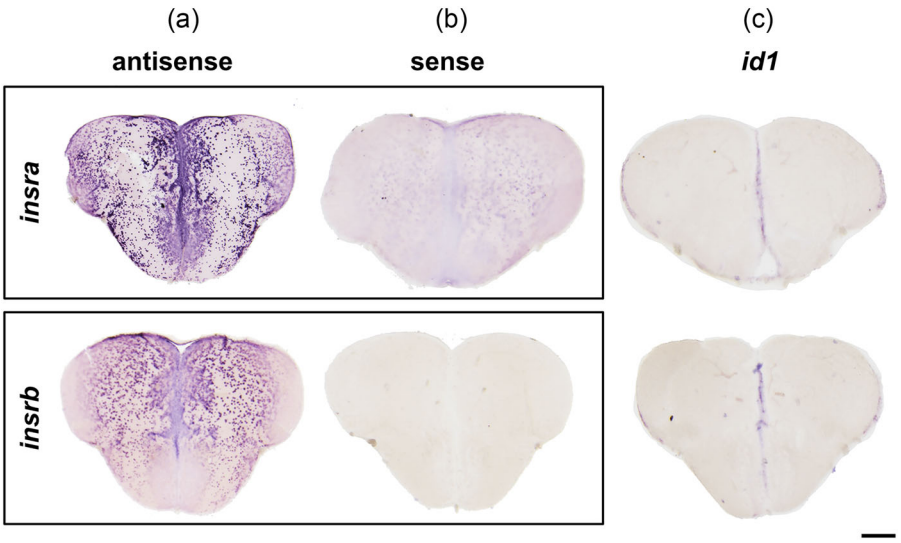

FIGURE 2 Specificity of in situ hybridization staining for *insra* and *insrb*. Transverse sections of zebrafish brain hybridized with *insra* and *insrb* antisense (a) and sense probes (b), with the positive control *id1* probe (c). Note that specific signal was detected for the antisense probes and *id1* probe while no/little signal was observed for ISH with sense probe. Scale bar = 180 μm.

investigated the specific expression of the two genes using fluorescent ISH coupled with HuC/D immunohistochemistry to label neurons. As shown in Figure 4, most *insra*- and *insrb*-positive cells localized in the telencephalon correspond to HuC/D-positive neurons (Figure 4, see arrows). In some cases, few *insr*-positive cells did not colocalize with neurons and could be microglial or oligodendrocyte cells (Figure 4, see arrowheads).

We next performed fluorescent ISH on transgenic Tg(*GFAP::GFP*) allowing us to identify neural stem cells (radial glia) as previously described (Sulliman et al., 2021; Diotel et al., 2015; Lam et al., 2009; Rodriguez Viales et al., 2015). Our in situ hybridization obviously demonstrated the expression of *insra* and *insrb* in GFAP-positive neural stem cells (Figure 5). Together, our data established that *insra* and *insrb* are mainly expressed in neurons in the brain parenchyma and are also expressed by radial glial cells (neural stem cells) along the ventricular layers.

3.3 | Expression of *insulin receptor substrate 1* (IRS1) in the brain of adult zebrafish

We next investigated the expression of the *irs1* gene coding for Irs1, a protein whose phosphorylation is the initial step in the activation of insulin signaling. As expected, we showed that *irs1* was expressed in the same brain regions as *insr* genes (Figure 6). Indeed, a similar expression pattern was observed in the telencephalon, diencephalon, and

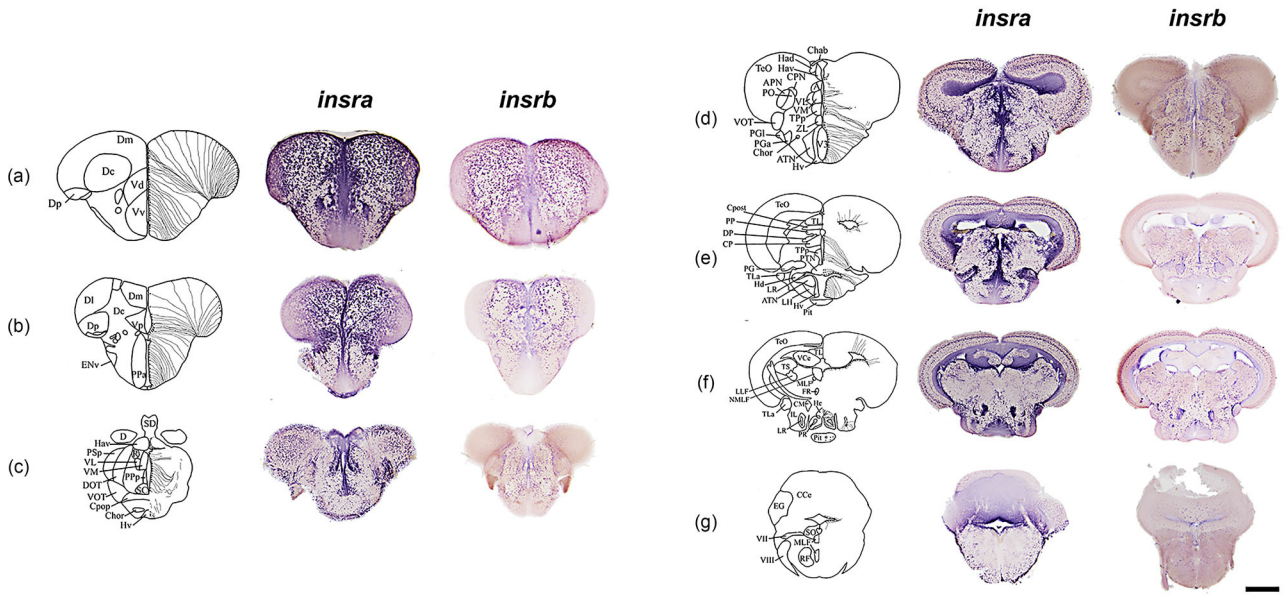

FIGURE 3 *Insulin receptors* (*insra* and *insrb*) are widely expressed in the brain of adult zebrafish within the parenchyma and along the ventricular layers. In situ hybridization for *insra* and *insrb* at the level of the telencephalon, diencephalon as well as the medulla oblongata demonstrating *insr* expression in the brain parenchyma and along the ventricles. The schemes (adapted from the Zebrafish brain atlas; Wulliman et al., 1996) provide the localization of the transversal section performed. Scale bar = 250 μm (a, b), 320 μm (c), 390 μm (d) and 470 μm (e, f, g).

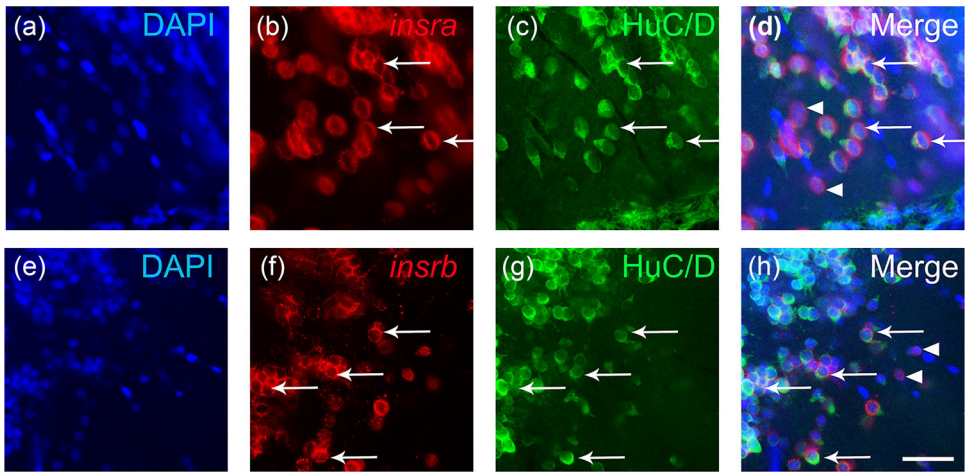

FIGURE 4 *Insulin receptors* (*insra* and *insrb*) are expressed in neurons. Fluorescent in situ hybridization for *insra* (b) and *insrb* (f) genes on zebrafish brain sections followed by HuC/D immunohistochemistry (green, c and g) to label neurons and DAPI counterstaining (blue, a and e). (d and h) Merged pictures showed the colocalization of *insr* (red) with HuC/D immunostaining (green) (see arrows). Note that, other cells in the brain parenchyma (HuC/D-negative) also expressed these *insr* genes (arrowheads). Scale bar = 50 μm.

mesencephalon, in the same brain nuclei and/or domains as *insulin receptors*. For instance, the ISH of *irs1* showed staining in the subpallium (Vv, Vd, and Vc) and pallium (Dm, Dl, Dp, and Vp). Transcripts were also detected in parenchyma and periventricular regions of the preoptic regions (PPa, PPp), in the different thalamic and hypothalamic nuclei (VL, VM, Hv, Hc, and Hd), in the preglomerular nucleus (PG), optic tectum (TeO) and also in the valvula of the cerebellum (VCe).

Finally, analysis of the expression and distribution of insulin-related genes in zebrafish revealed that the *insra* and *insrb* as well as *irs1* genes were widely expressed in the adult fish brain. In addition, we also detected *insra* and *insrb* mRNAs in neurons and neural stem cells. As a next step, we proposed to investigate the role of insulin in neurogenesis and brain injury.

3.4 | Role of insulin in neural stem cell proliferation

Given the general distribution of *insra*, *insrb*, and *irs1* in the brain of adult zebrafish and their expression in neurogenic niches, we hypothesized that insulin could act on constitutive neurogenesis. First, we decided to monitor the diffusion of the injected solution through the

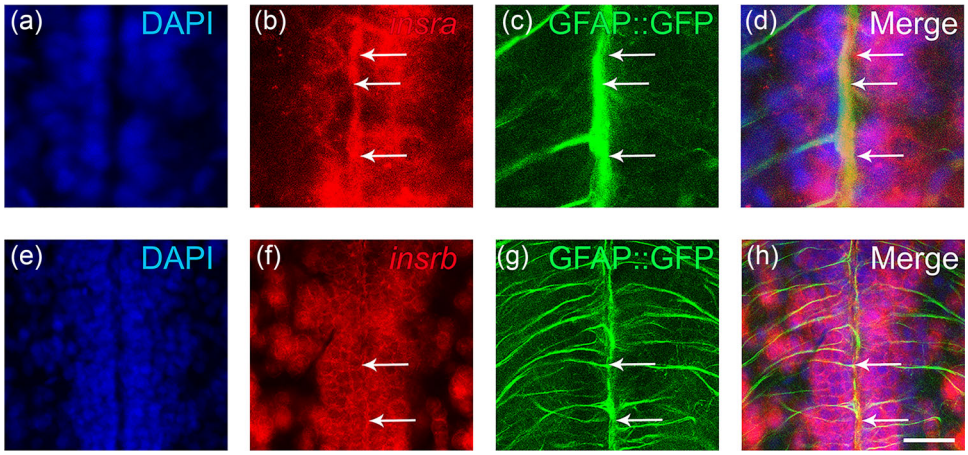

FIGURE 5 *Insulin receptors* (*insra* and *insrb*) are expressed in neural stem cells. Fluorescent in situ hybridization for *insra* (b) and *insrb* (f) genes on brain sections of transgenic tg(*GFAP::GFP*) zebrafish to label NSCs (green, c and g), using DAPI counterstaining (blue, a and e). (d and h) Merged pictures showed the colocalization of *insr* (red) with GFAP::GFP staining (green) (see arrows). Scale bar = 30 μm (a–d) and 70 μm (e–h).

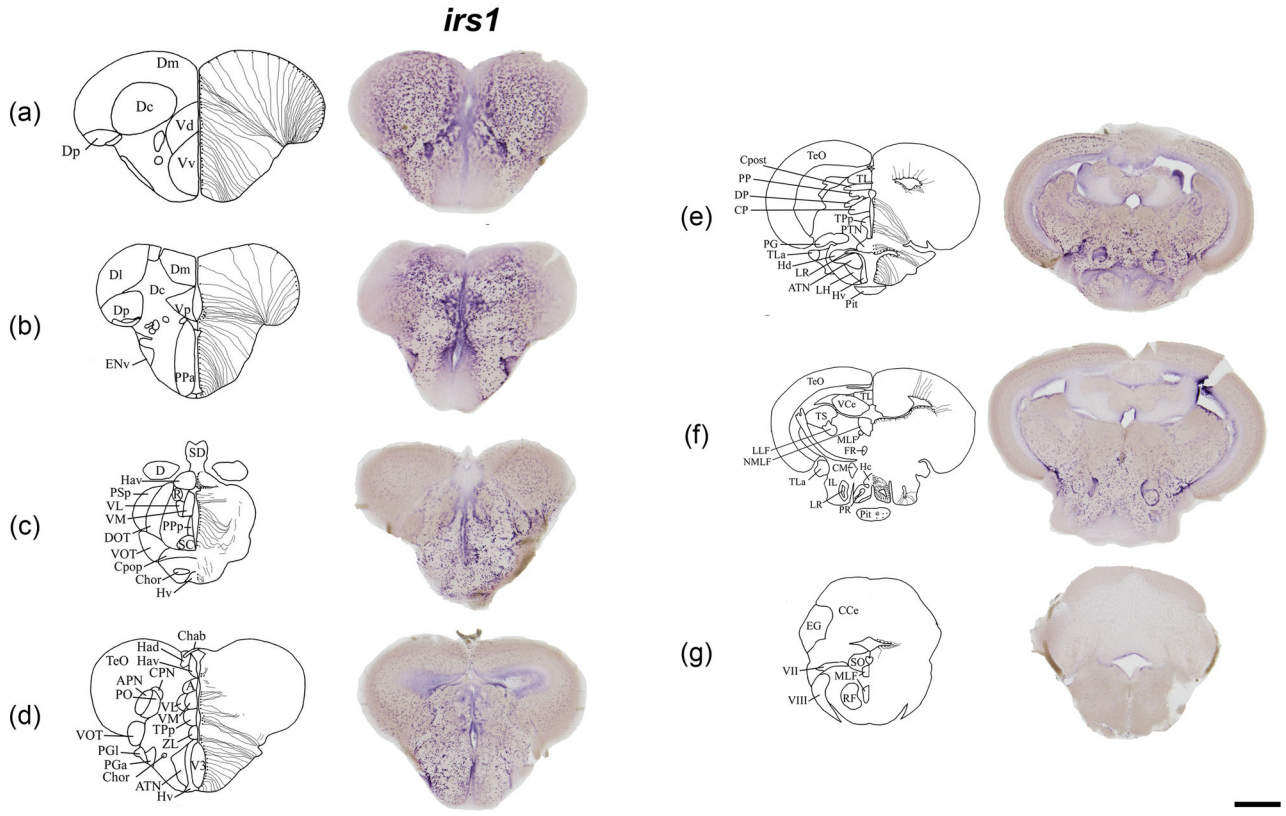

FIGURE 6 *Insulin receptor substrate 1* (*irs1*) is expressed in the brain parenchyma of adult zebrafish. In situ hybridization for *irs1* at the level of the telencephalon, diencephalon as well as the medulla oblongata of adult brain demonstrating *irs1* expression in the brain parenchyma and periventricular regions. The schemes (adapted from the Zebrafish brain atlas; Wulliman et al., 1996) provide the localization of the transversal section performed. Scale bar = 240 μm (a, b), 280 μm (c), 370 μm (d), and 370 μm (e, f, g).

use of dye such as Evans blue and HDL labeled with DilC18, as previously described (Sulliman et al., 2021). As evidenced in Figure 7, Evans blue dye was detected in the brain ventricles from the telencephalon to the 4th ventricle (Figure 7). We noticed that Evans blue diffused within the brain parenchyma probably due to its small size and affinity with tissue. Similarly, HDLs diffused in the brain ventricles as shown in the telencephalon and remained largely within the ventricle. These results clearly demonstrated that the microinjection at the junction between

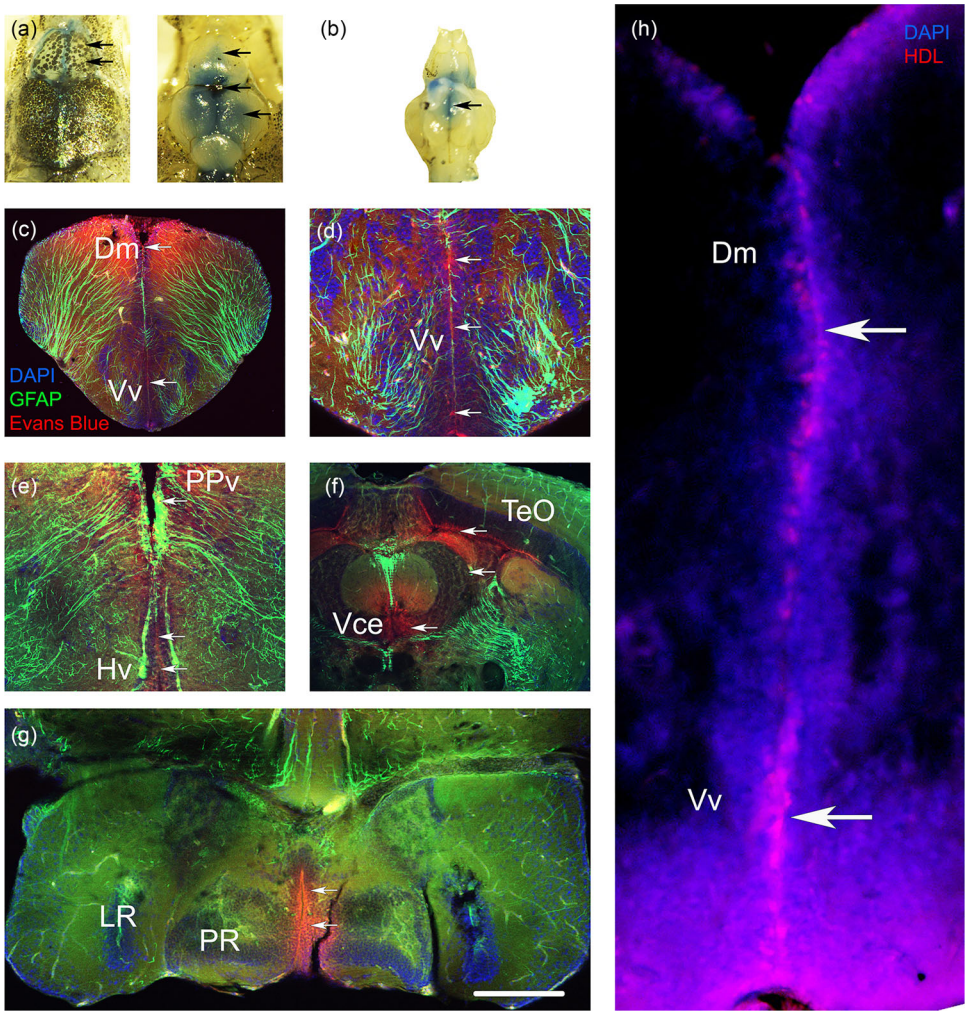

FIGURE 7 Intracerebroventricular injection leads to diffusion of the injected solution through the ventricular system. (a) Dorsal views before and after dissection of the skull allowing to see the Evans blue ICV injection. Arrows show the diffusion of Evans blue in the telencephalic and ventricle at the level of the optic tectum (TeO). (b) Ventral view showing the diffusion of the Evans blue within the hypothalamic ventricle (Arrow). (c–g) Fluorescence imaging performed in Tg(*GFAP::GFP*) zebrafish showing Evans blue (red) staining in the ventricle and periventricular layer of the dorsomedian telencephalon (Dm), ventral telencephalon (Vv), periventricular pretectal nucleus (PPv), anterior hypothalamus (Hv), TeO, valvula of the cerebellum (Vce) and caudal hypothalamus around the lateral and posterior recess (LR PR). (h) HDLs labeled with DiIC18 (red) demonstrating that the injection at the junction of the telencephalon/rhombencephalon well results in the diffusion of HDLs in the anterior part. Note that cell nuclei are counterstained with DAPI.

the posterior telencephalon and rhombencephalon allowed the diffusion of the injected solution throughout the complete ventricular system.

Consequently, we decided to perform intracerebroventricular (ICV) injection of insulin or vehicle (PBS) and allowed the fish to survive for 24 h. Then, brain cell proliferation was studied along the main neurogenic niches of the brain by PCNA immunohistochemistry. In general, in insulin-injected fish, the number of PCNA-positive cells was higher than in vehicle-injected ones (Figure 8). Indeed, a significant increase in PCNA-positive cells was observed 24 h postinjection, in the ventral and dorsal nuclei of the ventral telencephalic area (Vv Vd), the medial nucleus of the dorsal telencephalic area (Dm) and periventricular pretectal (PPv), hypothalamic (LR PR) regions and the valvula of the cerebellum (Figure 8a). Representative staining pictures are provided for the telencephalon and caudal hypothalamus (LR PR) (Figure 8b). As neuroinflammation is known to promote neurogenesis (Diotel et al., 2020; Kizil et al., 2012; Kyritsis et al., 2012), we also investigated the recruitment of microglia along the brain ventricles in neurogenic regions where neural stem cell proliferation was modulated. As shown in Figure 9, no significant change in the total number of microglia, ramified microglia and neuroinflammatory microglia (ameboid form) was detected between sham, PBS-injected and insulin-injected zebrafish. Consequently, ventricular proliferation is induced by insulin injection without impacting inflammatory processes.

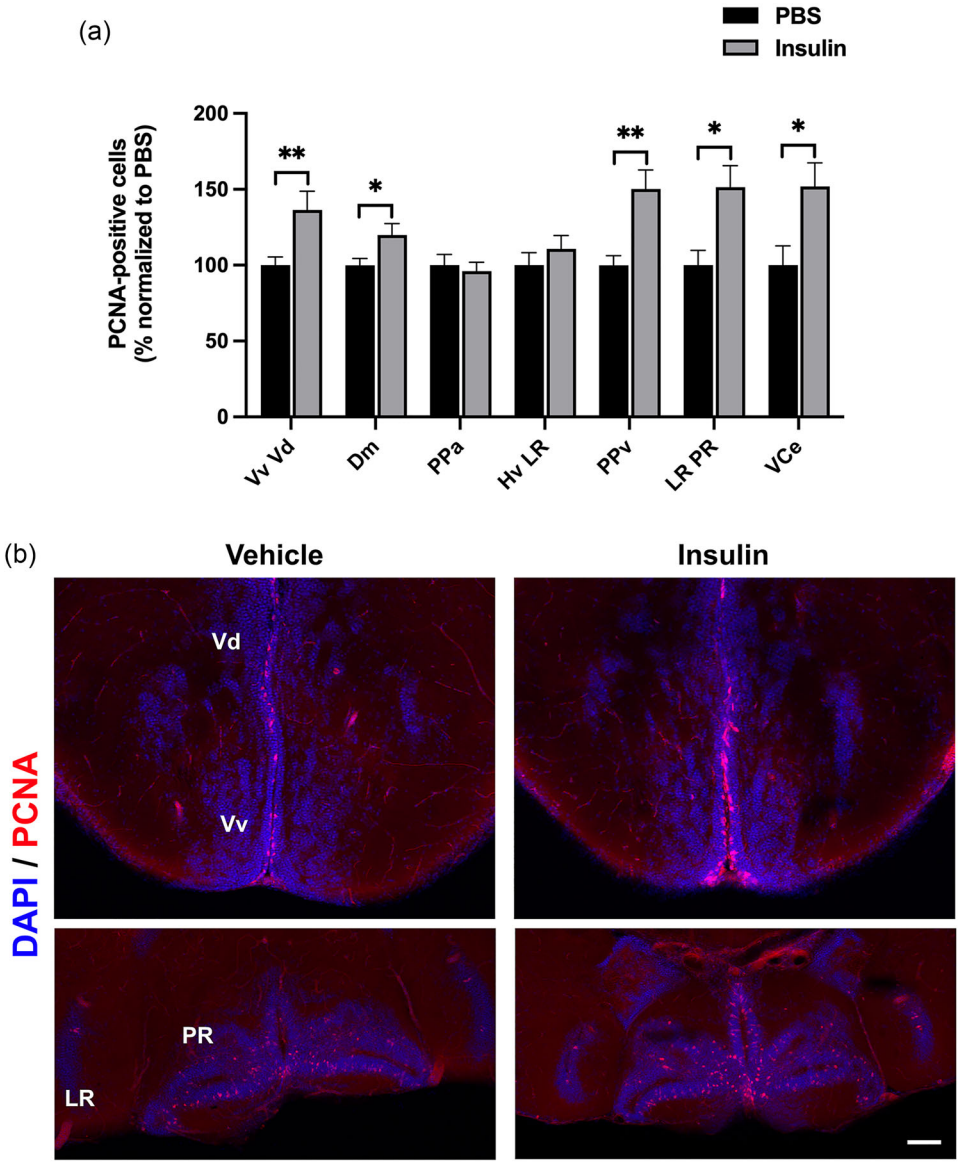

FIGURE 8 Intracerebroventricular injection of insulin induces constitutive neurogenesis in adult zebrafish. (a) Number of PCNA-positive cells in different neurogenic regions in PBS-injected (Vehicle) and insulin-injected fish. (b) Representative pictures of PCNA immunostaining (red) on zebrafish brain sections at the level of the telencephalon (Vv Vd) and caudal hypothalamus (LR PR) with DAPI counterstaining (blue). Dm, medial zone of dorsal telencephalic area. PPa, parvocellular preoptic nucleus, anterior part. Hv, ventral zone of periventricular hypothalamus. LR, lateral recess of diencephalic ventricle. PPv, periventricular pretectal nucleus, ventral part. PR, posterior recess of diencephalic ventricle. VCe, valvula cerebelli. Results are expressed as means ± *SEM* ($n = 4–6$ animals/group). $^{*}p < .05$, $^{**}p < .01$ (Student's *t*-test). Scale bar = 30 μm.

3.5 | Insulin signaling is activated after telencephalic brain injury

In the brain, the protective role of insulin signaling is well documented, particularly in animal models of diabetes and cerebral ischemia, demonstrating the effect of insulin in improving the survival and neurologic scores (Auer, 1998; Zhao et al., 2022). In this context, we examined the expression of genes related to insulin signaling pathway for better understanding the molecular mechanisms that could take place after brain damage. Based on studies by Gourain et al. (2021) and Rodriguez Viales et al. (2015), reanalysis of RNA sequencing data showed similar expression of *ins*, *insra*, *insrb*, *irs2a*, and *irs2b* genes between the contralateral (uninjured) and the stab-wounded (SW) hemispheres at 5 days post lesion (dpl), a key kinetic time point related to high neural stem cell proliferation rate (Figure 10). Interestingly, *irs1* gene expression was significantly increased in the injured hemisphere compared to the control hemisphere.

To verify this result, we performed a stab-wound injury of the zebrafish telencephalon and studied *irs1 expression* at 5 dpl using fluorescent ISH. Quantification of fluorescence at the wound site showed a higher intensity for the stabbed hemisphere compared to the contralateral hemisphere in 4 different fish analyzed (Figure 11). Similarly,

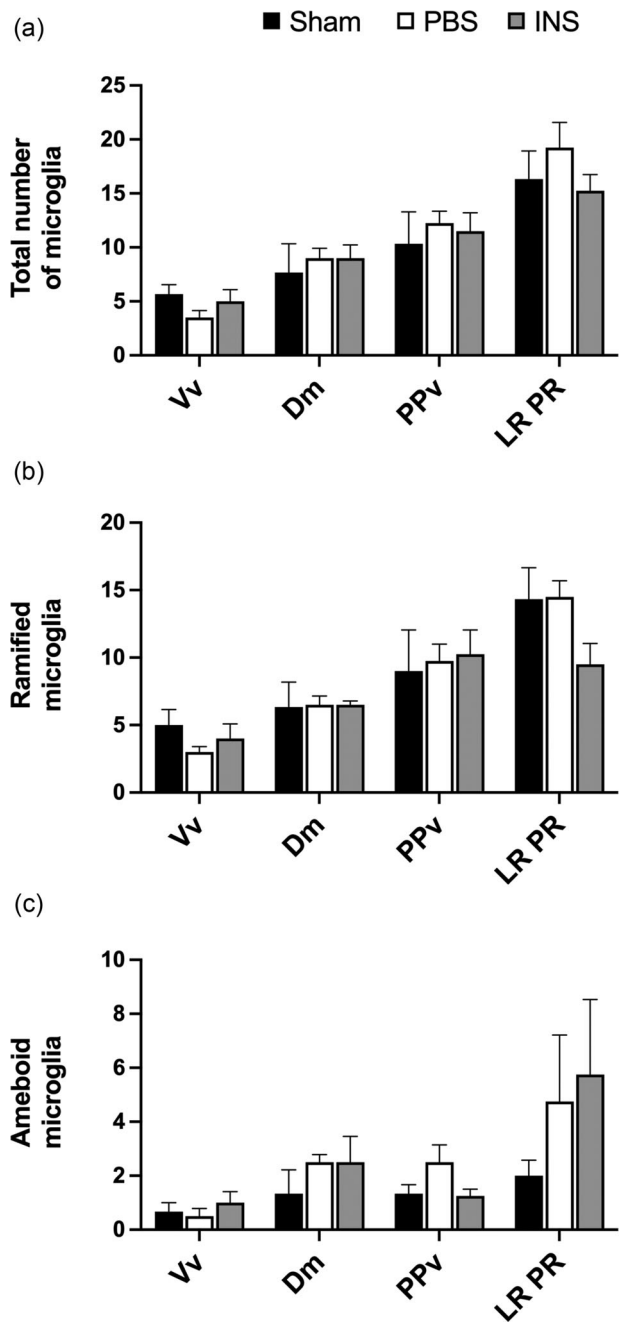

FIGURE 9 Intracerebroventricular injection did not modulate the recruitment and morphology of microglia. (a) Total number of microglia as well as the number of (b) ramified and (c) ameboid microglia in different neurogenic regions in sham, PBS-injected (PBS) and insulin-injected (INS) fish. Dm, medial zone of dorsal telencephalic area. LR PR, region surrounding the lateral and posterior recess of hypothalamic ventricle. PPv, periventricular pretectal nucleus, ventral part. Vv, ventral nucleus of the ventral telencephalon. Results are expressed as means ± *SEM* ($n = 3$–4 animals/group).

fluorescent staining appeared more intense along the ventricular zone in the SW hemisphere (Figure 11). Given that NSC are located along the ventricles and are indeed activated after brain injury, our results suggest that insulin signaling may be activated in these cells and play a role in regenerative/neuroprotective mechanisms that occur in brain injury.

4 | DISCUSSION

In this study, we described for the first time the expression of *insulin receptor* and *insulin receptor substrate 1* genes (*insra*, *insrb*, and *irs1*) in the adult zebrafish brain. We showed that these insulin-related genes were widely expressed in the brain parenchyma and were strongly detected along the ventricles corresponding to neurogenic niches. Using in situ hybridization, immunohistochemistry and transgenic tools, we observed that specific cell populations namely neurons and neural stem cells expressed both *insra* and *insrb*. We also demonstrated that insulin injection in the telencephalic brain ventricle increased NSC proliferation testifying an activation of insulin signaling in neurogenesis. Interestingly, after telencephalic injury, we observed the increase in *irs1* expression at 5 dpl in the brain parenchyma and in the neurogenic niches localized in front of the lesion.

4.1 | Insulin receptors are expressed by neurons and neural stem cells

In zebrafish, the presence of insulin and downstream effectors has already been reported. Previous studies have identified two isoforms of insulin receptors (*insra* and *insrb*) as well as two insulin genes (*insa* and *insb*) during embryogenesis (Papasani et al., 2006; Toyoshima et al., 2008). In adult fish, *insra* and *insrb* were also detected in hepatic and ovarian tissues with high homologies with human genes (65%–68%) arguing for well-conserved functions (Das et al., 2016; Shao et al., 2022). More recently, *irs1* gene has also been reported in zebrafish liver cell line (ZFL cell line) (Shao et al., 2022).

To the best of our knowledge, there are only few data on the expression of IR expression in the adult zebrafish brain (Dos Santos et al., 2020; Obermann et al., 2019). Our results constitute evidence of *insra*, *insrb* and *irs1* expression and distribution in the brain of adult zebrafish. We showed that the expression of these genes was widespread in the adult brain with high densities along the ventricles. Interestingly, *insra* appears more expressed than *insrb* in the whole brain and more specifically, in some specific brain regions including the preotic areas (Ppa), periventricular pretectal (Ppv) and periventricular hypothalamic (Hv) nuclei, as shown by RNA sequencing and ISH analyses. In mammals, *Insr* gene (and respective proteins) is also ubiquitously distributed in the nervous system (Schulingkamp et al., 2000) and abundant in some specific regions comprising the neural stem cell niches. In the brain of rat for example, highest concentrations of insulin receptors were found in the olfactory bulb, dentate gyrus, hippocampus, hypothalamus, cerebellum, and choroid plexus (Marks et al., 1990; Werther et al., 1987; Zhao et al., 1999). As well, up to four IRS have been identified and located at the level of the hippocampus and olfactory bulbs (Baskin et al., 1994; Numan & Russell, 1999). Avian species including chicken also exhibited IR in nervous system, especially in the

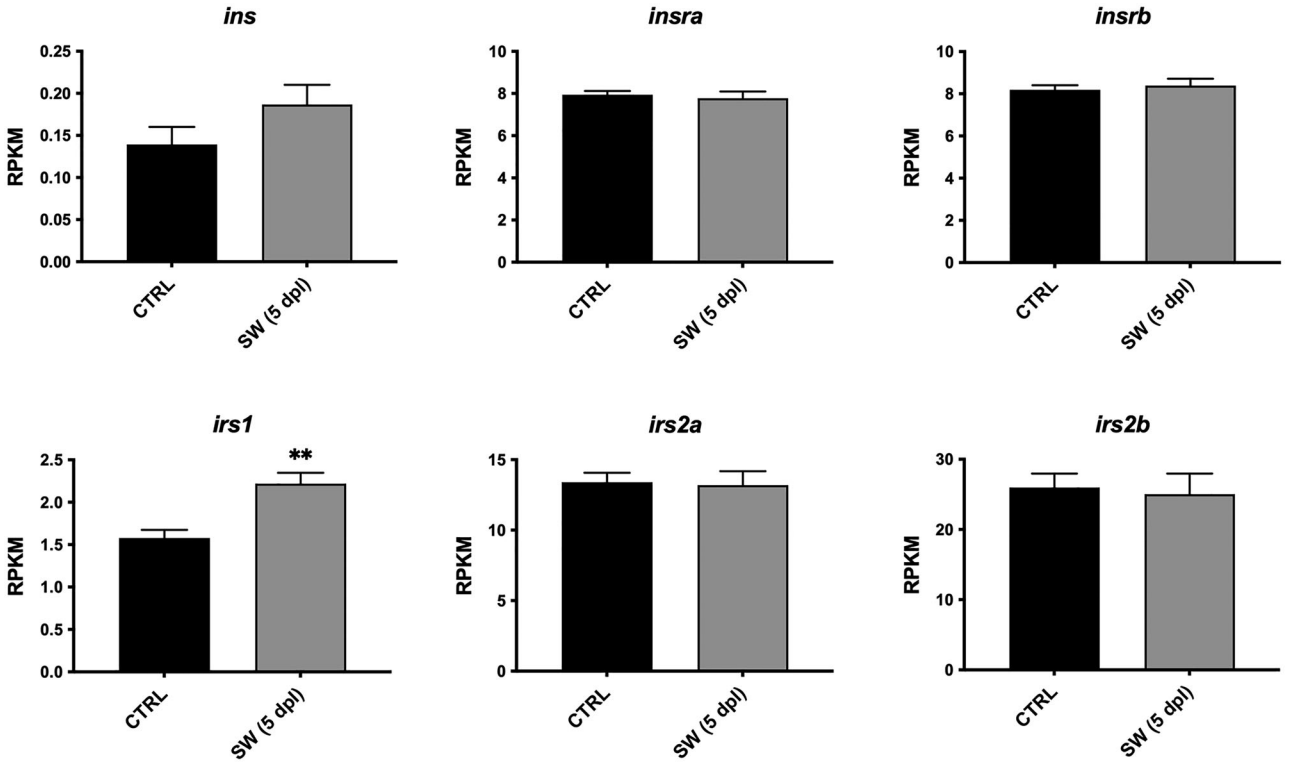

FIGURE 10 Expression of insulin signaling-related genes in zebrafish brain, 5 days after stab-wound injury. RNA sequencing data showing the relative expression of insulin (*ins*), insulin receptor a (*insra*) and b (*insrb*), insulin receptor substrate 1 (*irs1*) and 2 (*irs2a*, *irs2b*) in the uninjured (CTRL) and injured (SW) hemispheres of adult zebrafish ($n = 3$), 5 days postlesion (5 dpl) (data from Gourain et al., 2021; Rodriguez Viales et al., 2015). RPKM, reads per kilobase per million. Results are expressed as means ± *SEM*. $^{**}p < .01$ (Student's *t*-test).

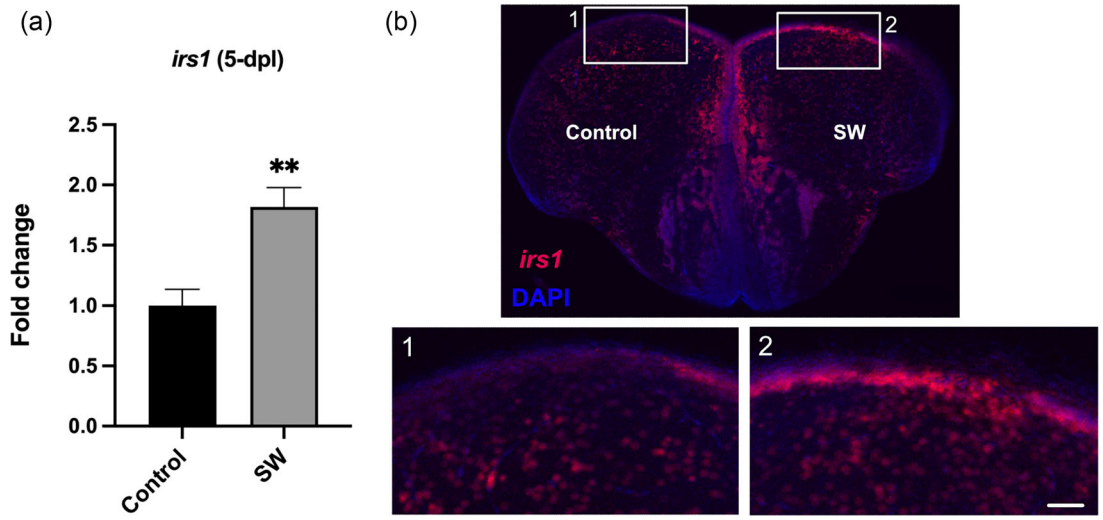

FIGURE 11 *Insulin receptor substrate 1* (*irs1*) expression is increased 5 days post lesion. Fluorescent in situ hybridization for *irs1* (red) of stab-wounded zebrafish with DAPI counterstaining (blue). (a) Quantification of the fluorescent *irs1*-positive area in the contralateral (Control) hemisphere compared to stab-wounded (SW) hemisphere, 5 days post lesion (5 dpl) ($n = 4$). (b) Representative picture of a brain section with ISH staining for *irs1*. Regions of interest (white rectangles) are further magnified in (**1** and **2**). Results are expressed as mean ± *SEM*. $^{**}p < .01$ (Student's *t*-test). Scale bar = 110 μm (b) and 55 μm (b, high magnification).

paraventricular nucleus, ventromedial hypothalamus, lateral hypothalamus, and infundibular nucleus (equivalent of the mammalian arcuate nucleus) (Shiraishi et al., 2011). Together with our results, the expression of IR in these specific neurogenic regions reflects the evolutionary conserved importance of insulin signaling in brain homeostasis and neurogenesis.

We also report here a different expression of *insra* and *insrb* in zebrafish brain (Figure 3). In contrast to zebrafish, mammalian genome contains a single *Insr* gene that is differentially spliced to give two different transcript variants (*Insra* and *Insrb*) (Payankaulam et al., 2019). However, a different regional expression was also described in mice for the two transcripts showing that *Insra* was highly expressed in the subventricular zone whereas *Insrb* was undetectable (Ziegler et al., 2012). So far, the specific role of each *insr* genes in the brain of adult zebrafish as well as the different transcripts in the mammalian brain remains largely unknown. Interestingly, morpholino-mediated selective knockdown in zebrafish embryos demonstrated the key role of *insra* in brain development, while *insrb* was essential for heart development (Toyoshima et al., 2008).

In our work, we observed the expression of both *insulin receptor* genes in neurons (HuC/D-positive cells) and NSCs (GFAP-positive cells) but also by other cells in the brain parenchyma which can probably be microglia, oligodendrocytes, or endothelial cells. This is consistent with many previous studies showing the expression of *Insra* and *Insrb* by different cell populations including neurons, neural stem cells, astrocytes, and endothelial cells in mammals and/or zebrafish (Hersom et al., 2018; Obermann et al., 2019; Zhu et al., 1990; Ziegler et al., 2012). As well, Ziegler and colleagues demonstrated that in mice brain, *Insra* transcript was 5.8 times more abundant than *Insrb* transcript in neurospheres (in vitro-cultured neural stem cells from the subventricular zone), whereas astrocytes and oligodendrocytes exhibited almost equal expression of the two differentially spliced *Insr* (Ziegler et al., 2012). Unexpectedly, the authors also showed the absence of *Insrb* expression by neurons contrary to our results. Finally, other investigations are required to ascertain the expression of these insulin receptor genes (*insra* and *insrb*) by specific cells present in the brain of adult zebrafish.

4.2 | Insulin: a role in constitutive and regenerative neurogenesis

One of the most important metabolic actions of insulin is the stimulation of glucose uptake by cells (Chang et al., 2004). However, over the last few years, it has become clear that insulin displays an important role on the CNS regulating many other key processes including energy homeostasis, reproduction and more importantly neuroprotection (Plum et al., 2005). Here, we focused on the role of insulin signaling on neurogenesis in physiological and pathophysiological conditions. Performing ICV injection of insulin in adult fish, we found that cell proliferation was increased in the main neurogenic niches, independently of the activation of neuroinflammatory processes. As well, 5 days after stab-wound injury, we showed increased expression of the downstream effector of insulin signaling, *irs1*, in the lesioned hemisphere. However, the gene expression of *ins* and *insr* genes was unchanged after brain injury. Then, analysis of protein expression of insulin and its receptors in the brain after injury may provide additional insights into molecular mechanisms involved knowing that a large part of insulin is probably produced in the periphery.

To date, the potential role of insulin and insulin signaling on adult neurogenesis is poorly documented in vivo. Few studies from invertebrates such as insects have shown the beneficial effects of insulin in the brain of adult animals (Malaterre et al., 2003; Siegrist et al., 2010). In cricket, adult neural progenitor cells from mushroom bodies (the main sensorial integrative structures of the brain) increased their proliferative activity in response to insulin (Malaterre et al., 2003). In *Drosophila*, during normal development, decreased insulin/PI3 kinase signaling was correlated with the slowing proliferation and elimination of neural stem cells (neuroblasts) (Siegrist et al., 2010).

Although there is little evidence for a direct action of insulin on neurogenesis, many studies showing the improvement in some cognitive aspects after delivery of insulin to the brain indirectly suggest the influence of insulin on adult brain plasticity (Plum et al., 2005). Indeed, intranasal administration of insulin to healthy human subjects improved memory and mood (Benedict et al., 2004). In the brain of Alzheimer's disease patients, insulin concentration and IR densities were drastically reduced compared with healthy controls (Craft et al., 1998) and the intravenous administration of insulin was shown to improve memory (Craft et al., 1999). Interestingly, a recent study performed in a mouse model of insulin resistance showed cognitive impairment (learning and memory functions) that was significantly restored after intranasal insulin treatment as well as improvement of neurogenesis in the hippocampus (Lv et al., 2020). As well, administration of insulin directly into the brain of mice with chronic neuroinflammation decreased the expression of neuroinflammatory markers in the hippocampus and improved their performance (Adzovic et al., 2015). However, it is important to consider here that the highly related insulin-like growth factor (IGF) axis may also influence brain processes including neurogenesis in adult, through common signaling cascades (Åberg, 2010; Lichtenwalner et al., 2001). Many studies reported the expression of IGF and IGF receptors (IGFR) in the brain of vertebrates including zebrafish and mammals (Dyer et al., 2016; Li et al., 2014; Obermann et al., 2019) and showed their strong structural homology with insulin and IR, respectively, ranging from 45% to 85% for specific binding and substrate recruitment domains (Belfiore et al., 2009; LeRoith et al., 2021). Insulin is thus able to interact directly with the IGFR (essentially IGF1R) whose activation induces the recruitment of adaptor molecules including IRS similarly to the signal transduction through IR (Laviola et al., 2007). In a similar way, IGF can interact with IR, activating the same signaling and evidence also demonstrated the neuroprotective potential of the IGF system after brain injury in rodents (Kazanis et al., 2003; Madathil et al., 2013).

Finally, the beneficial effect of insulin on neurogenesis observed in our work was probably the result of the activation of insulin signaling as well as IGF signaling inducing the overexpression of *irs1*. Importantly, the action of insulin activating either IR or IGFR could be interesting to investigate for a better understanding of insulin action in neurogenesis processes.

4.3 | Impaired insulin signaling: pathophysiological implications for brain disorders?

Given that we showed here the possible impact of insulin on neurogenesis in zebrafish, it can be supposed conversely that insulin disturbances may contribute to the development and progression of brain disorders (Ghasemi et al., 2013). Metabolic disorders including diabetes and obesity are characterized by insulin desensitization (Wondmkun, 2020) and have been associated with impaired neurogenesis as described in zebrafish and mammals (Bayram et al., 2022; Dorsemans et al., 2017; Dos Santos et al., 2020; Gence et al., 2022; Ghaddar et al., 2020; Ghaddar et al., 2021; Kaiyala et al., 2000). Some studies reported that insulin resistance (with and without metabolic alterations) was closely related with the development of neurodegenerative and neuropsychiatric diseases such as Alzheimer's and Parkinson's diseases (Hölscher, 2020). However, how insulin dysregulation affects the development of these pathologies is less discussed. A recent study highlighted that dysregulated insulin/IGF signaling in the human brain (postmortem frontal and temporal lobe regions) mediated brain dysfunctions with altered expression of ligands and receptors (Liou et al., 2019). The alteration in the PI3K/AKT/GSK3 (Phosphatidylinositol 3-kinase/Protein kinase B/Glycogen synthase kinase 3) pathway was also reported in the pathogenesis of brain diseases knowing that these molecules play a crucial role in neuroplasticity by regulating cell survival, neurogenesis, neuronal proliferation and differentiation, and synaptic plasticity (Long et al., 2021; Matsuda et al., 2019). It will be consequently interesting to investigate the specific role of peripheral and central insulin on brain functions and plasticity using specific obese/overweight and/or hyperglycemic zebrafish models (Dorsemans et al., 2017; Gence et al., 2022; Ghaddar et al., 2021).

5 | CONCLUSION

In this work, we provided results on the expression of insulin receptor and insulin receptor substrate 1 genes (*insra*, *insrb*, and *irs1*) in the brain of adult zebrafish showing their specific distribution along the ventricles. After intracerebroventricular injection, insulin was shown to significantly promote cell proliferation in neurogenic niches, supporting its beneficial role on adult neurogenesis. Finally, understanding the complex action of insulin on cerebral processes notably on neurogenesis remains a major challenge and may provide new therapeutical strategies for brain injury.

AUTHOR CONTRIBUTIONS

LG and ND designed the experiments. LG, ND, and DF performed the experiments. All authors contributed to the analysis of the experiments and/or in the writing and reviewing of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions

ORCID

Sepand Rastegar https://orcid.org/0000-0003-4411-5646
Nicolas Diotel https://orcid.org/0000-0003-2032-518X

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