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Impaired Constitutive and Regenerative Neurogenesis in Adult Hyperglycemic Zebrafish

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ABSTRACT

A growing body of evidence supports hyperglycemia as a putative contributor to several brain dysfunctions observed in diabetes patients, such as impaired memory capacity, neural plasticity, and neurogenic processes. Thanks to the persistence of radial glial cells acting as neural stem cells, the brain of the adult zebrafish constitutes a relevant model to investigate constitutive and injury-induced neurogenesis in adult vertebrates. However, there is limited understanding of the impact of hyperglycemia on brain dysfunction in the zebrafish model. This work aimed at exploring the impact of acute and chronic hyperglycemia on brain homeostasis and neurogenesis. Acute hyperglycemia was shown to promote gene expression of proinflammatory cytokines ($il1\beta$, $il6$, $il8$, and $tnf\alpha$) in the brain and chronic hyperglycemia to impair expression of genes involved in the establishment of the blood–brain barrier (*claudin 5a*, *zona occludens 1a* and *b*). Chronic hyperglycemia also decreased brain cell proliferation in most neurogenic niches throughout the forebrain and the midbrain. By using a stab wound telencephalic injury model, the impact of hyperglycemia on brain repair mechanisms was investigated. Whereas the initial step of parenchymal cell proliferation was not affected by acute hyperglycemia, later proliferation of neural progenitors was significantly decreased by chronic hyperglycemia in the injured brain of fish. Taken together, these data offer new evidence highlighting the evolutionary conserved adverse effects of hyperglycemia on neurogenesis and brain healing in zebrafish. In addition, our study reinforces the utility of zebrafish as a robust model for studying the effects of metabolic disorders on the central nervous system. J. Comp. Neurol. 000:000–000, 2016.

Hyperglycemia corresponds to abnormally high blood glucose levels, which may reflect a response to situations of acute stress or to a chronic disorder of insulin secretion and/or resistance (diabetes). The prevalence of type 2 diabetes (characterized by insulin resistance) has increased rapidly in recent decades in association with drastic changes in lifestyle (Lee et al., 2012; World Health Organisation, 2015a,b). Today, 382 million people suffer from type 2 diabetes, and 14,000 people die daily from this disease or its related complications. Thus, type 2 diabetes is a major health problem and is projected to affect 592 million people by 2035 (IDF, 2015).

Hyperglycemia is a complex and multifactorial state caused by both genetic and nongenetic factors. It is associated with an increased risk of mortality related to cerebrovascular and renal dysfunctions, cancers, sepsis, and also neuropathies (Koh et al., 2012; Storey and

Von Ah, 2012; Hafez et al., 2014; Lee et al., 2015). Furthermore, hyperglycemia is an independent risk factor for ischemic stroke and is a predictor of adverse neurological and survival outcomes (Kagansky et al., 2001; Gilmore and Stead, 2006; Desilles et al., 2013). Hyperglycemia has been shown to sensitize the brain against endogenous aggressions (Sander and Kearney, 2009). Both acute and chronic hyperglycemia increase oxidative stress and stimulate the production of proinflammatory cytokines that display deleterious effects on different organs (e.g., eyes, kidney, heart, brain) and that alter various physiological processes (Kodl and Seaquist, 2008; Kayama et al., 2015; Tangvarasittichai, 2015). In the central nervous system, chronic and acute hyperglycemia disrupt energy homeostasis, neural activity, and neural functions, which may alter cognition and promote dementia and depression (Dejgaard et al., 1991; Cukierman et al., 2005; Brismar et al., 2007; Gaudieri et al., 2008; Lang et al., 2009; Lu et al., 2009; Ho et al., 2013; Ojo and Brooke, 2015). In addition, diabetes may impact adult neurogenesis (Ho et al., 2013), a physiological process leading to the generation of functional neurons from neural stem/progenitor cell proliferation.

In mammals, adult neurogenesis occurs throughout life in two main neurogenic regions: the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricle (Lindsey and Tropepe, 2006; Grandel and Brand, 2013). Additional neurogenic niches have also been described such as in the hypothalamus (Migaud et al., 2010; Batailler et al., 2014). Adult neurogenesis is regulated by intrinsic and extrinsic factors (Hsieh, 2012) and is markedly blunted under pathological conditions such as epilepsy and stroke (particularly in cases involving increased blood–brain barrier [BBB] permeability), in the contexts of increased oxidative stress and of inflammation (Zhao et al., 2008; Lin et al., 2015). Unlike mammals, adult teleost fish exhibit intense and widespread neurogenic activity throughout life and maintain a greater capacity for central nervous system repair after injury (Kizil et al., 2012; Schmidt et al., 2013). In teleosts, such capacities are notably due to the persistence of radial glial cells acting as neural progenitors, as well as to further committed progenitors corresponding to neuroblasts (Chapouton et al., 2007; März et al., 2010; Lindsey et al., 2012). In the zebrafish telencephalon, the ventricular zone is described as comprising quiescent radial glial cells (type 1 progenitors), actively divided radial glial cells (type 2 progenitors), and neuroblasts (type 3 progenitors), mainly clustered in the subpallium (März et al.,

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Abbreviations
Anatomical terms used in this study are according to the Wullimann atlas (Wullimann et al., 1996)

A	anterior thalamic nucleus	NIII	oculomotor nucleus
APN	accessory pretectal nucleus	NMLF	nucleus of medial longitudinal fascicle
ATN	anterior tuberal nucleus	NLV	nucleus lateralis valvulae
BBB	blood-brain barrier	PG	preglomerular nucleus
CCe	corpus cerebelli	PGa	anterior preglomerular nucleus
Chab	habenular commissure	PGl	lateral preglomerular nucleus
Chor	horizontal commissure	p.i.	post-injection
CM	corpus mamillare	Pit	pituitary
CP	central posterior thalamic nucleus	PO	posterior pretectal nucleus
CPN	central pretectal nucleus	PPa	parvocellular preoptic nucleus, anterior part
Cpop	postoptic commissure	PPp	parvocellular preoptic nucleus, posterior part
Cpost	posterior commissure	PR	posterior recess of diencephalic ventricle
D	dorsal telencephalic area	PSp	parvocellular superficial pretectal nucleus
Dc	central zone of dorsal telencephalic area	PTN	posterior tuberal nucleus
Dd	dorsal zone of dorsal telencephalic area	PPv	periventricular pretectal nucleus
Dl	lateral zone of dorsal telencephalic area	R	rostrolateral nucleus
Dm	medial zone of dorsal telencephalic area	RF	reticular formation
DOT	dorsomedial optic tract	RV	rhombencephalic ventricle
Dp	posterior zone of dorsal telencephalic area	SC	suprachiasmatic nucleus
DP	dorsal posterior thalamic nucleus	SO	secondary octaval population
DTN	dorsal tegmental nucleus	TeO	tectum opticum
ECL	external cellular layer of olfactory bulb	TL	torus longitudinalis
EG	eminentia granularis	TLa	torus lateralis
ENv	entopendoncular nucleus, ventral part	TPp	periventricular nucleus of posterior tuberculum
FR	fasciculus retroflexus	TS	torus semicircularis
GL	glomerular layer of olfactory bulb	V	ventral telencephalic area
Had	dorsal habenular nucleus	V3	third ventricle
Hav	ventral habenular nucleus	VII	sensory root of the facial nerve
Hc	caudal zone of periventricular hypothalamus	VIII	octaval nerve
Hd	dorsal zone of periventricular hypothalamus	VCe	valvula cerebelli
HG	hyperglycemia	Vd	dorsal nucleus of ventral telencephalic area
Hv	ventral zone of periventricular hypothalamus	VL	ventrolateral thalamic nucleus
ICL	internal cellular layer of olfactory bulb	VM	ventromedial thalamic nucleus
IL	inferior lobe	VOT	ventrolateral optic tract
i.p.	intraperitoneal injection	Vp	post-commissural nucleus of ventral telencephalic area
LH	lateral hypothalamic nucleus	Vv	ventral nucleus of dorsal telencephalic area
LLF	lateral longitudinal fascicle	ZL	zona limitans
LR	lateral recess of diencephalic nucleus		
MLF	medial longitudinal fascicle		

2010). The zebrafish has recently emerged as a relevant and useful model for studying hyperglycemia and type 2 diabetes (Sarras and Intine, 2013; Capiotti et al., 2014a; Connaughton et al., 2016), which may be useful for analyzing the impact of both acute and chronic hyperglycemia on brain plasticity and neurogenic processes.

In the current study, we set up an acute model of hyperglycemia and investigated the effects of both acute and chronic hyperglycemia on brain cell proliferation under homeostatic and regenerative conditions in zebrafish. We show that hyperglycemia upregulates the expression of proinflammatory cytokines in the brain, disturbs the expression of genes involved in the establishment of the BBB, and impairs both constitutive and regenerative neurogenesis.

MATERIALS AND METHODS
Animals and ethics

Adult male and female wildtype zebrafish (*Danio rerio*) of 3–6 months in age were purchased from commercial suppliers and maintained under standard conditions of photoperiod (14/10 hours light/dark) and temperature (28 ± 1°C). The zebrafish were fed daily with commercially available dry food (TetraMin, Germany). Animals were euthanized by rapid chilling on ice to induce a lethal shock before spinal cord sectioning. For all "surgery" procedures and/or injections, fish were deeply anesthetized with 0.02% tricaine (MS-222; REF: A5040, Sigma-Aldrich, Germany). All 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 (APAFIS#2645-2015111308416542; APAFIS#3625-2016011516473942; APAFIS#2646-2015111315373106).

Hyperglycemia models

To assess the impact of hyperglycemia in zebrafish, animals were submitted to acute or chronic exposure to D-glucose (Sigma-Aldrich). Acute hyperglycemia (AHG) was induced by an intraperitoneal (i.p.) injection of D-glucose (2.5 g/kg of body weight) dissolved in a 50 µL saline phosphate buffer (PBS; pH 7.4). The respective control fish were injected with 50 µL of PBS. Chronic hyperglycemia (CHG) was induced by fish water supplementation with 111 mM D-glucose over a period of 14 days, as previously described by Capiotti et al. (2014a). Fish water was changed every 2 days.

For acute HG and chronic HG models, control fish were rigorously maintained in the same conditions (light, temperature, and environment) as treated fish.

Measurement of blood glucose levels

Determination of blood glucose levels was performed after a 12-hour fasting period. Fish were euthanized at the same time of day and gently washed and dried prior to blood glucose level measurement. One eye was removed, and a glucometer test strip (One-Touch Ultra, LifeScan, France) was directly inserted in the ocular cavity. Fish were considered hyperglycemic when glycemia was found to be higher than 100 mg/dl. Fish following hyperglycemic treatment and displaying blood glucose levels <100 mg/dl were excluded from the study.

Stab wound of adult zebrafish telencephalon

Prior to stab wounding, zebrafish were deeply anesthetized with 0.02% tricaine. Mechanical injury to the telencephalon was performed by inserting a sterile needle (BD Microlance 3; 30 G ¹/₂″; 0.3 × 13 mm) in the right telencephalic hemisphere, following a dorsoventral axis and guided by landmarks on the head, as previously described (Diotel et al., 2013). After the procedure fish were put back into their respective tanks and maintained until recovery.

Tissue preparation

For paraffin immunohistochemistry, fish were euthanized before being decapitated. Zebrafish heads were immersed overnight at 4°C in 4% paraformaldehyde (PFA) dissolved in PBS. The next day, brains were extracted from the skull and postfixed in 4% PFA-PBS overnight at 4°C before paraffin embedding. For free-floating vibratome section immunohistochemistry, fixed brains were stepwise dehydrated in a methanol/PBS concentration series and stored at –20°C, as previously described (Adolf et al., 2006).

Immunohistochemistry

For paraffin immunohistochemistry, zebrafish brains were cut into 7-µm-thick coronal sections using a microtome (Microm HM 355 S, Thermo Scientific, Pittsburgh, PA), and double glial fibrillary acidic protein (GFAP)/proliferating cell nuclear antigen (PCNA) immunostainings were performed as follows. The sections were dewaxed in xylene, progressively rehydrated through graded ethanol (100 to 50%), and then rinsed in PBS containing 0.2% Triton X-100 (PBS-T). Antigen retrieval was performed in a sodium citrate buffer (pH 6.0) in a microwave oven (750W, 2 minutes). After washing in PBS-T, the sections were saturated in PBS-T

Primary antibodies			
Antibodies	Reference	RRID	Antigen
Polyclonal rabbit anti glial fibrillary acidic protein	Z033429 (Dako)	AB_2314535	GFAP isolated from cow spinal cord
Proliferating cell nuclear antigen clone PC10	M0879 (Dako)	AB_2160651	Rat PCNA-protein A fusion protein obtained from vector pC2T (Waseem and Lane, 1990)

Secondary antibodies		
Antibodies	Reference	RRID
Donkey anti-mouse Alexa Fluor 488	A21202 (Molecular Probes, Life Technologies)	AB_141607
Donkey anti-rabbit Alexa Fluor 488	A21206 (Molecular Probes, Life Technologies)	AB_10049650
Goat anti-mouse Alexa Fluor 594	A11005 (Molecular Probes, Life Technologies)	AB_141372

containing 0.5% milk powder to avoid nonspecific binding. The sections were then incubated overnight at room temperature with the following primary antibodies: polyclonal rabbit anti-GFAP (1:500; REF: Z033429, Dako, Carpinteria, CA; RRID: AB_2314535) and monoclonal mouse anti-PCNA (1:100; clone PC10, Dako; RRID: AB_2160651). The next day the sections were washed three times in PBS-T and incubated for 90 minutes at room temperature with 4′,6′-diamidino-2-phenylindole (DAPI) for cell nuclei counterstaining and with the respective secondary antibodies: donkey anti-rabbit Alexa Fluor 488 (1:200; REF: A21206; Life Technologies, Bethesda, MD; RRID: AB_10049650) and goat anti-mouse Alexa Fluor 594 (1:200; REF: A11005; Life Technologies; RRID: AB_141372). Finally, the sections were washed three times in PBS-T and the slides were mounted with the antifading medium Vectashield (H-1000, Vector Laboratories, Burlingame, CA).

For free-floating section immunohistochemistry using a vibratome, PCNA immunostaining was performed as follows. Zebrafish brains were progressively rehydrated through graded methanol (75 to 25%) and rinsed in PBS. After washing in PBS containing 0.1% Tween-20 (PTW), the brains were embed in 2% agarose in PBS. Next, the brains were cut into 50-μm-thick free-floating sections on a vibratome (Vibratome Line VT1000S, Leica, Germany) in a blocking buffer (BB; PBS containing 0.1% Tween-10, 0.2% bovine serum albumin, 1% dimethyl sulfoxide). The sections were then incubated overnight at 4°C with the monoclonal mouse anti-PCNA (1:500; clone PC10, Dako) in BB. The next day, the sections were washed three times in PTW and incubated for 2 hours at room temperature with DAPI for cell nuclei counterstaining and with a donkey anti-mouse

Alexa Fluor 488 antibody (1:1000; REF: A21202; Life Technologies; RRID: AB_141607). Finally, the sections were washed three times in PTW and the slides were then mounted with the antifading medium Aqua Polymount (REF: 18606, Polysciences, Warrington, PA). No staining was observed after omission of the primary antibodies (data not shown). Table 1 summarizes the antibodies used in this study.

Antibodies specificity

PCNA and GFAP antibodies have been previously documented for their use and their specificity in zebrafish (Pellegrini et al., 2007; Lam et al., 2009; Diotel et al., 2010b, 2016; März et al., 2011; Baumgart et al., 2012).

Consistent with previous studies, PCNA-labeling was observed in cell nuclei across the neurogenic niches lining the ventricles as shown by Pellegrini et al. (2007). In addition, its distribution is similar to BrdU proliferative assay (Zupanc et al., 2005). GFAP-positive cells correspond to radial glial cell, as they display a soma localized along the ventricular layer and a long radial process that crosses the brain parenchyma and reaches the pial surface. Its expression and its distribution along the ventricles correspond to previous data obtained with the same antibody in zebrafish (Baumgart et al., 2012) or with GFAP:GFP transgenic line (Lam et al., 2009; März et al., 2010).

Microscopy

Sections were analyzed with an Eclipse 80i Nikon fluorescence microscope equipped with a Hamamatsu ORCA-ER digital camera (Life Sciences, Japan) or with a confocal microscope (Nikon D Eclipse C1), both of which

TABLE 2.

Target	Forward primer	Reverse primer
ef1α	AGCAGCAGCTGAGGAGTGAT	CCGCATTTGTAGATCAGATGG
il8	GTCGCTGCATTGAAACAGAA	AGGGGTCCAGACAGATCTCC
ilβ	GCTGGAGATCCAAACGGATA	ATACGCGGTGCTGATAAACC
tnfα	GCGCTTTTCTGAATCCTACG	TGCCCAGTCTGTCTCCTTCT
il6	TCAACTTCTCCAGCGTGATG	TCTTTCCCTCTTTTCCTCCTG
neurod4	GGACAGGTGCTTCAATAACAGA	TTAAGTCTTCGTCCATCCATCC
zo1a	GAACCCATCAACCGCATC	CGGGGCCCCTACATTTAC
zo1b	GAGGTCAAAGGGAAAGCTGA	CACGGAGTGGATGTCTGAAA
claudin5a	TCCTGGGTCTGATCCTGTG	CTCGATGAAGGCGGTGAC
nestin	TCGACAGACCAACACCAAAC	TGAGGCGATTGATCTGTGAC

were connected to a computerized image analysis system (NIS-Elements software; RRID: SCR_014329).

Cell counting

For analyzing constitutive neurogenesis, proliferative activity was determined by quantification of PCNA-labeled cells on images captured from at least three consecutive paraffin sections representative of a region of interest.

For analyzing injury-induced neurogenesis, counting was performed on 3–4 vibratome sections of 50 μm thickness each in the injured versus uninjured telencephalon. Images were analyzed for detection of PCNA-positive nuclei using ImageJ software (National Institutes of Health, Bethesda, MD; RRID: SCR_003070) by adjusting parameters (threshold, binary, and watershed) and running an analysis on "particles" taking into consideration the size and the circularity. Briefly, the parameters were set up as follows for each picture: threshold = 30–100, particle size = 20–1,000, circularity = 0.3–1.00. Minor modifications in these parameters could be slightly adjusted according to the experiments. In addition, ImageJ automated selection of PCNA-positive nuclei was manually double-checked and adjusted if necessary for each picture. No significant difference was observed concerning the average size of counted particles in control versus hyperglycemic conditions (data not shown).

Neuroanatomical structures were identified with DAPI counterstaining according to the zebrafish brain atlas (Wullimann et al., 1996). Cell counting was performed in blind conditions by two different people on at least 2–4 independent experiments on a total of n fish.

Brain RNA extraction and reverse transcription

Two whole mixed zebrafish (from a mixed-sex sampling zebrafish) brains were immediately removed from the skull, pooled, and stored at –80°C prior to RNA extraction. Pools of brains were next grinded with TissueLyser II (Qiagen, Chatsworth, CA) for RNA extraction using TRIzol reagent (Invitrogen, France). A total of 2 μg of RNA was reverse transcribed to cDNA using random hexamer primers (Life Technologies) and M-MLV reverse transcriptase (Invitrogen).

Gene expression analysis by quantitative real-time polymerase chain reaction (qPCR)

PCR experiments were performed with the AB 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using the SYBR green master-mix (Eurogentec) and specific zebrafish primers (Table 2). Each PCR cycle was conducted for 15 seconds at 95°C and 1 minute at 60°C. Melting curve analyses were performed to confirm correct amplification. Results were analyzed using the 7500 system SDS software (Applied Biosystems), and the relative expression of the claudin 5a, zo1a, zo1b, il1β, il6, il8, tnfα, neurod4, and nestin genes was normalized against the expression level of the ef1α gene.

Statistical analysis

Comparisons between groups were performed using a statistical Student's t-test. If more than two groups were analyzed, multiple testing was corrected by Benjamini-Hochberg. Error bars correspond to the standard error of the mean (SEM), and n values correspond to the number of animals for all experiments. $P < 0.05$ was considered statistically significant; *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

RESULTS

Development of a new model of acute hyperglycemia in adult zebrafish

We first investigated the effect of acute hyperglycemia (acute HG) on brain cell proliferation. For this purpose, we established a model of acute HG by i.p. injection of D-glucose (2.5 g/kg of body weight). At 1 hour 30 minutes (1h30) post-injection (p.i.), zebrafish injected with D-glucose exhibited significantly higher

Acute HG	Blood glucose levels (mg/dl)	
	1h 30 p.i. (2.5g D-glucose/kg of body weight)	24h p.i. (2.5g D-glucose/kg of body weight)
PBS	149.8 ± 27.8 ($n = 4$)	97.8 ± 16.3 ($n = 21$)
AHG	502.8 ± 33.8 ($n = 4$)***	94.2 ± 13.1 ($n = 26$)
Chronic HG	Blood glucose levels (mg/dl) (14 days in 111mM D-glucose)	
CTRL	66.7 ± 6.6 ($n = 21$)	
CHG	281.6 ± 30.1 ($n = 24$)***	

For acute HG experiments, adult zebrafish were intraperitoneally injected with PBS or with D-glucose. Glycemia was significantly increased in D-glucose injected fish at 1h30 p.i. compared to controls (P-value = 0.0002), while no significant differences were observed between PBS and D-glucose-injected fish at 24h p.i.

For chronic HG experiments, zebrafish were maintained in normal fish water (CTRL) while other zebrafish (chronic HG) were maintained in fish water supplemented with D-glucose (111mM) for 14 days. After the treatment, blood glucose levels are significantly higher in chronic HG fish. ***$P \leq 0.0001$. n refers to the total number of fish.

blood glucose levels compared to the controls injected with the PBS vehicle only (Table 3; 502.8 mg/dl ± 33.8 vs. 149.8 mg/dl ± 27.8; $n = 4$; $P < 0.0002$; $n = 4$). Importantly, blood glucose levels were similar between the two groups at 24 hours p.i. (Table 3; 97.8 mg/dl ± 16.3 vs. 94.2 mg/dl ± 13.1; $n = 21$ for PBS and $n = 26$ for acute HG), showing that hyperglycemia was transient.

Brain cell proliferation is not impaired by acute hyperglycemia in adult zebrafish

To test our hypothesis that hyperglycemia may impact neural stem cell activity, we assessed brain proliferation after 24-hour acute HG p.i. by performing PCNA immunohistochemistry (Fig. 1). We thus focused our analysis on the ventricular regions that contain neural progenitors. We counted the number of PCNA-positive cells at the junction between the olfactory bulbs (OB) and the telencephalon (TEL) (Fig. 1A, OB/TEL); in the ventral nucleus of the ventral telencephalic area (Fig. 1B, Vv); in the dorsomedian telencephalon (Fig. 1C, Dm); in the periventricular pretectal nucleus (Fig. 1D, PPv); in the mediobasal hypothalamus, where the lateral recess starts to open (Fig. 1E, Hv LR); and in the caudal hypothalamus around the lateral and posterior recess of the diencephalic ventricle (Fig. 1F, LR PR). Acute HG did not impact neural stem cell proliferation in our experimental conditions, as further evidenced by PCNA and GFAP labeling at the OB/TEL junction, the Vv/Vd, and also in the PPv (Fig. 1G–L), even though a slight tendency for increased cell proliferation was observed in these regions except in the PPv (Fig. 1). In line with these observations, the gene expression of the neuronal marker *neurod4* and the progenitors marker

nestin was not altered in the brain of control (PBS) vs. acute HG fish at 24 hours p.i. (Fig. 2A).

Acute hyperglycemia induces brain inflammation

Hyperglycemia has been reported to induce the expression of proinflammatory cytokines and to increase BBB permeability in mammals (humans and rodents) (Esposito et al., 2002; Gyurko et al., 2006; Hawkins et al., 2007; Prasad et al., 2014). We thus tested the effects of acute HG on gene expression of inflammatory cytokines and proteins involved in the maintenance of BBB integrity. Our transcriptional analysis shows that acute HG resulted in a significant increased expression of *il1β* ($P = 0.0042$), *il8* ($P = 0.019$), and *tnfα* ($P = 0.015$) (Fig. 2B). However, we did not observe any transcriptional change in the expression of genes encoding for proteins involved in BBB integrity, namely, *claudin 5a* or *zona occludens 1a* and *1b*, under acute HG conditions (Fig. 2C).

Chronic hyperglycemia impairs brain cell proliferation in adult zebrafish

We next investigated the impact of chronic hyperglycemia on brain cell proliferation. For this purpose, zebrafish were kept in fish water supplemented with D-glucose (111 mM) for 14 days. This protocol was previously shown to increase blood glucose levels from a baseline of about 3 mM (~54 mg/dl) to about 12 mM (~216 mg/dl) and to induce a wide variety of metabolic changes such as insulin resistance (Capiotti et al., 2014a). In our experimental conditions, after 14 days of this protocol, a significant proportion (17%) of fish displayed normal glycemia (<100 mg/dl) and were consequently excluded, while most zebrafish (83%) exhibited
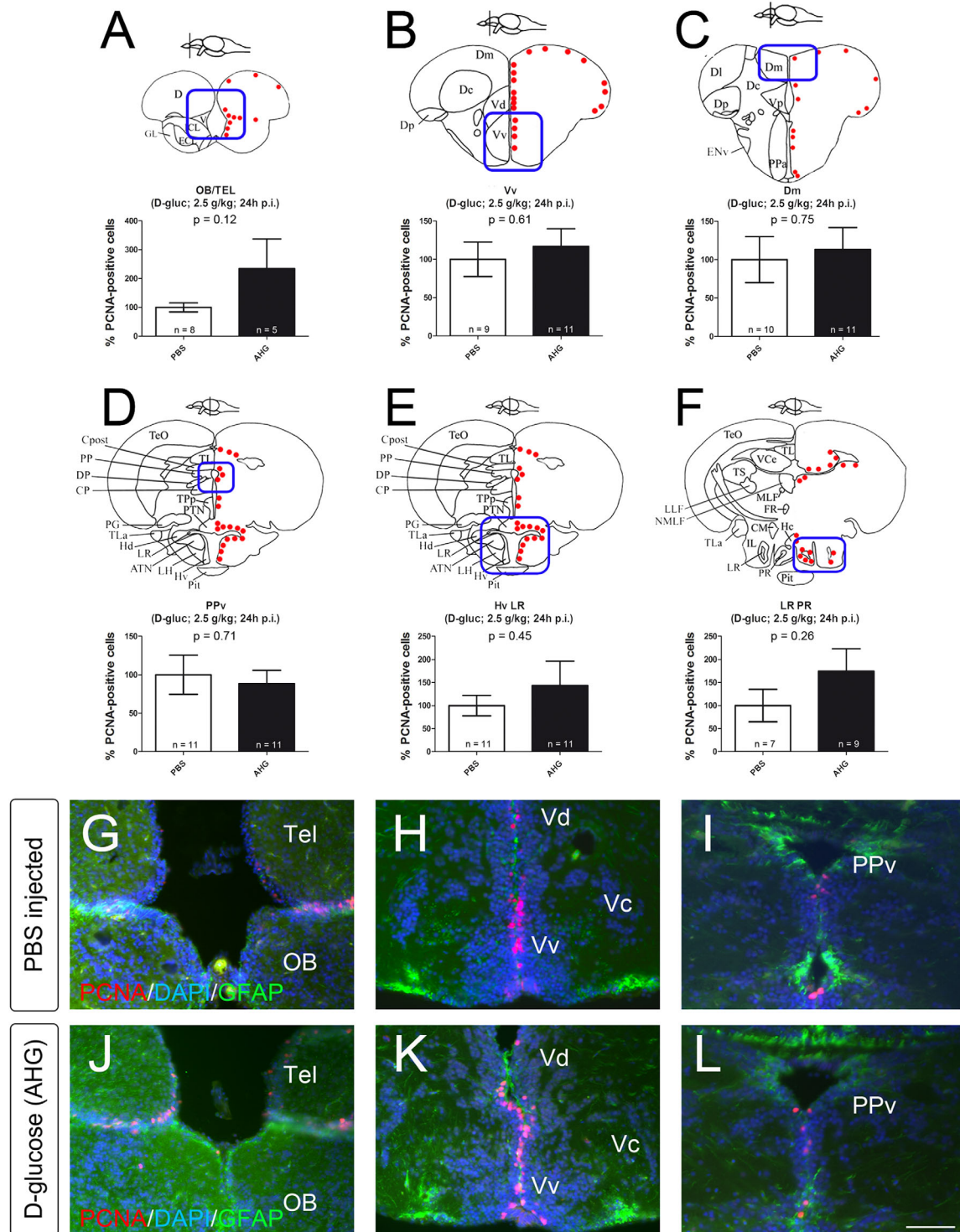
Figure 1. Acute hyperglycemia does not impair brain cell proliferation. **A–F:** Statistical analysis of proliferative cells in control (PBS) and acute HG (AHG) zebrafish at 24 hours p.i. by performing PCNA immunohistochemistry. Schematic representations taken from the zebrafish brain atlas (Wullimann et al., 1996) show the different regions of interest and the proliferative cells (red dots) as previously described in the literature (Zupanc et al., 2005; Pellegrini et al., 2007). Blue boxes highlight regions where the number of proliferative cells (PCNA-positive) was counted. No significant difference in proliferative activity was observed between PBS- and D-glucose-injected zebrafish in the OB/TEL (A), Vv (B), Dm (C), PPv (D), mediobasal hypothalamus (E), and caudal hypothalamus (F). **G–L:** Representative digital pictures of PCNA (red) and GFAP (green, a radial glial marker) immunohistochemistry on paraffin brain sections of PBS- and D-glucose-injected zebrafish (24 hours p.i.) counterstained with DAPI. Pictures were taken with an epifluorescence microscope. Error bars correspond to the standard error of the mean (SEM) and *n* refers to the total number of fish. Scale bar = 70 μm.

significantly higher blood glucose levels (>100 mg/dl) compared to control fish (Table 3; 281.6 mg/dl ± 30.1 [$n = 24$] vs. 66. 7 mg/dl ± 6.6 [$n = 21$]). We then examined the effect of chronic HG on brain cell proliferation by performing PCNA immunohistochemistry in the above-described regions (Fig. 3). In contrast to acute HG, chronic HG resulted in a significant decrease in

ventricular proliferation in most of the examined regions (Fig. 3). Indeed, chronic HG zebrafish displayed an almost 50% reduction in PCNA-positive cells in the anterior part of the brain, at the OB/TEL junction (Fig. 3A, A1, $P = 0.0008$), in the ventral nucleus of the ventral telencephalic area (Fig. 3B, B1, $P = 0.0009$), and in the dorsomedian telencephalon (Fig. 3C, C1, $P = 0.001$). In more posterior regions, such as in the periventricular pretectal nucleus (PPv) localized just below the posterior commissure (Cpost), we observed a 63% reduction in the number of PCNA-positive cells in chronic HG-treated fish relative to control (Fig. 3D, D1, $P = 0.0011$). In the diencephalon, including the anterior part of the preoptic area (data not shown) and the mediobasal and caudal hypothalamus (Fig. 3E, E1, 3F, F1), we observed no significant difference of proliferation between the two groups (33% decrease for Hv LR, $P = 0.08$; and 24% for LR PR, $P = 0.16$). Similar to the acute HG condition, *neurod4* and *nestin* gene expression was unaltered in chronic HG fish compared to control (Fig. 4A).

Chronic hyperglycemia increased *zo1a* and *zo1b* gene expression

Expression of *il1β, il6, il8,* and *tnfα* was not modulated after a 14-day chronic hyperglycemic treatment (Fig. 4B). However, chronic HG-treated fish displayed a significant upregulation of *zona occludens 1a* and *1b,* suggesting that chronic hyperglycemia may have an impact on the physiology of the BBB (Fig. 4C).

Chronic hyperglycemia impairs the regenerative neurogenesis after stab wounding in the adult telencephalon

In the last part of our study, we aimed at investigating the impact of acute and chronic hyperglycemia on
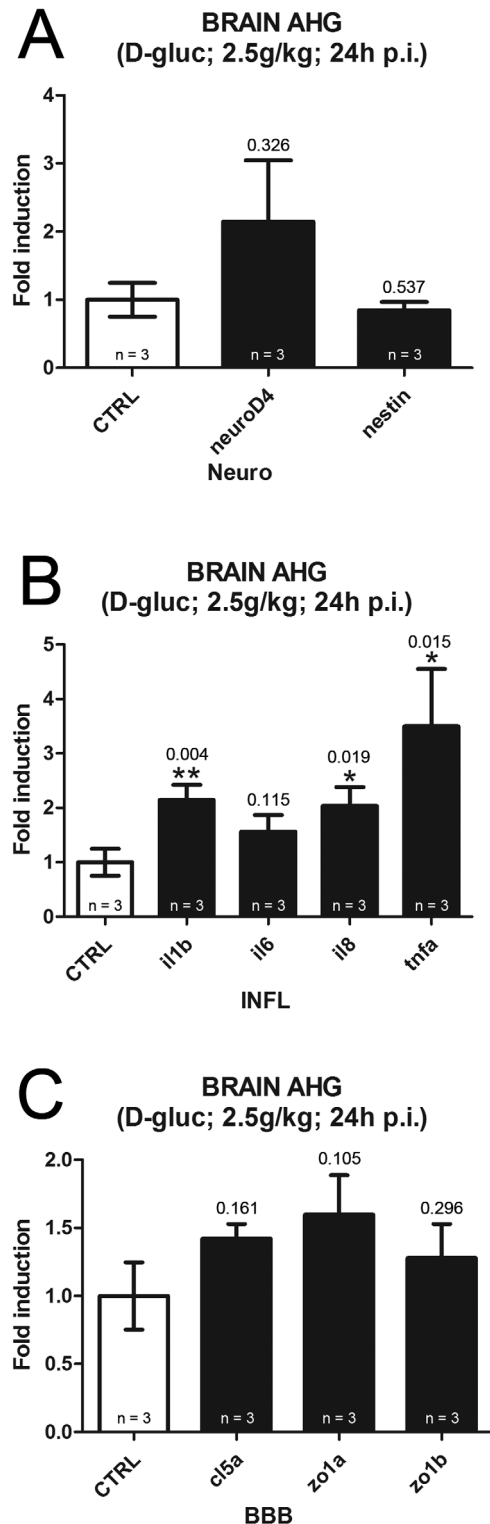
Figure 2. Gene expression of proinflammatory cytokines and tight junctions in the brain of fish following acute hyperglycemia. Semiquantitative PCR analysis in the brain of adult zebrafish following acute hyperglycemia (24 hours p.i.). **A:** The neuronal and progenitor marker expression, *neurod4* and *nestin,* is not significantly modulated at 24 hours p.i. **B:** Acute hyperglycemia upregulates proinflammatory cytokines gene expression of *il1β, il6, il8,* and *tnfα* at 24 hours p.i. This increase is statistically significant for *il1β, il8,* and *tnfα* ($P = 0.0042$ for *il1β*, 0.019 for *il8*, 0.015 for *tnfα*). **C:** Acute hyperglycemia does not significantly modulate gene expression of *claudin 5a* (*cl5a*) or *zona occludens 1a* and *1b* (*zo1a* and *zo1b*), encoding for proteins involved in the maintenance of the blood-brain barrier. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$. Error bars correspond to the standard error of the mean (SEM) and *n* refers to the total number of two pooled zebrafish brains.
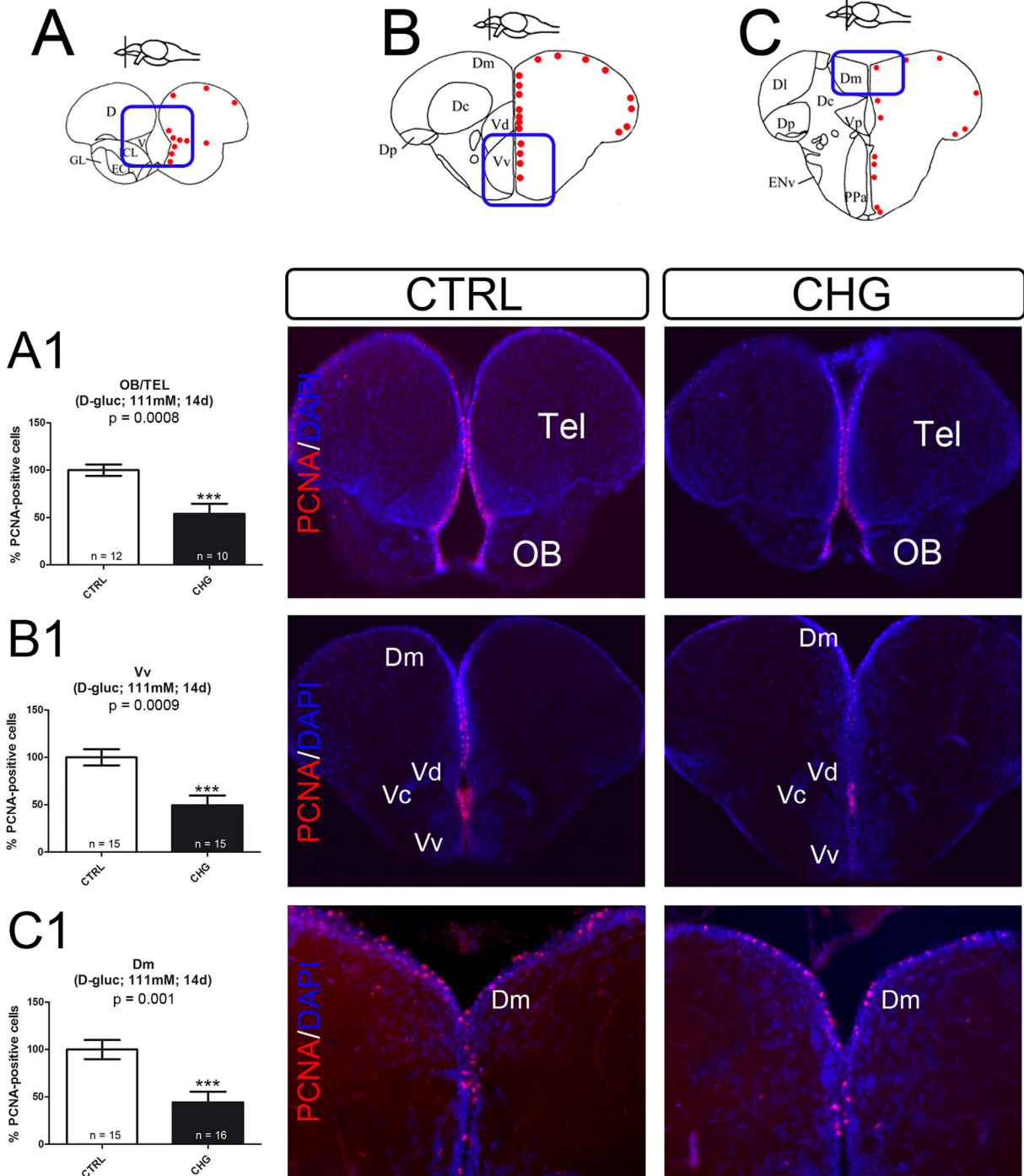
Figure 3. Chronic hyperglycemia results in a significant decrease in neural progenitor proliferation. **A–F:** Schematic representations taken from the zebrafish brain atlas (Wullimann et al., 1996) show the different regions of interest and the proliferative cells (red dots) as previously described in the literature. Blue boxes highlight the regions where the number of proliferative cells (PCNA-positive) was counted. **A1–F1:** Statistical analysis of proliferative cells (PCNA-positive) in the forebrain of control (CTRL) and CHG (D-glucose; 111 mM; 14d) zebrafish. The number of proliferative cells was decreased by around 50% at the OB/TEL junction (A1, $P = 0.0008$) and in the Vv (B1, $P = 0.0009$), Dm (C1, $P = 0.001$), and PPv (D1, $P = 0.0011$). In the hypothalamus, brain cell proliferation was also decreased, but not significantly, at the opening of the lateral recess (E1, $P = 0.0861$) and around the lateral and posterior recess (F1, $P = 0.1687$). The representative digital pictures of PCNA (red) immunohistochemistry staining at the OB/TEL junction, the Vv, the Dm, the PPv, the Hv LR, and the LR PR regions with DAPI (blue) counterstaining show a decrease in proliferation under chronic HG. Error bars correspond to the standard error of the mean (SEM). These data were obtained from four independent experiments, with n referring to the total number of fish. Pictures of vibratome sections were taken with an epifluorescence microscope. $**P \leq 0.01$ and $***P \leq 0.001$. Scale bar = 70 μm (H); 100 μm (L); 140 μm (F); 220 μm (B,D,J).
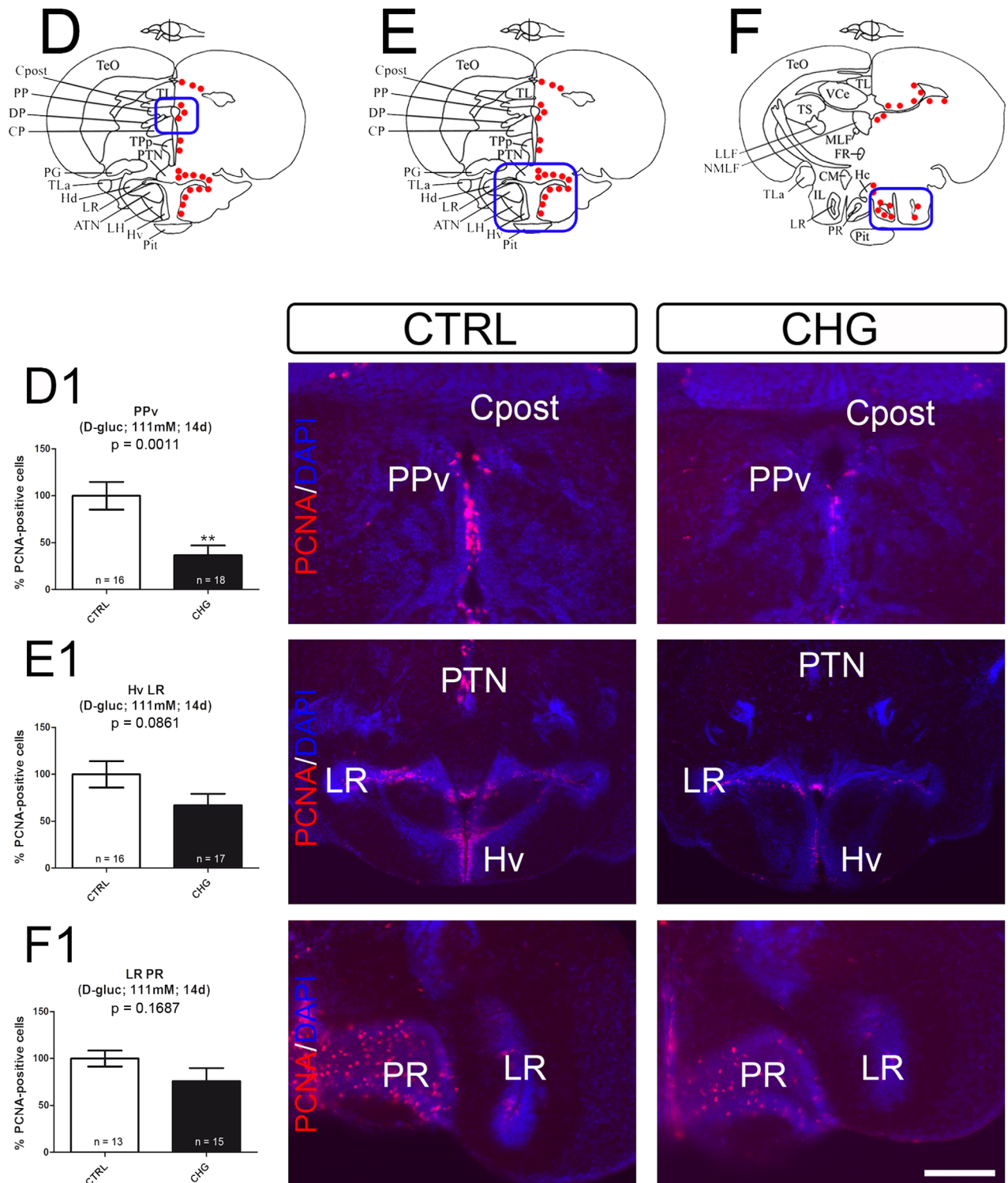
Figure 3. Continued.

brain cell proliferation during brain repair. For this purpose, the telencephalon was submitted to a mechanical injury, following an established procedure (Ayari et al., 2010; Diotel et al., 2010a). It has been reported that at 1 day postlesion (dpl), the first proliferating cells are microglial cells and oligodendrocytes, which are localized in the brain parenchyma (März et al., 2011), whereas from 3 to 7 dpl, proliferative cells are localized

along the brain ventricles and can be identified as radial glia cells that give rise to new neurons (März et al., 2011; Baumgart et al., 2012). We thus investigated the effects of acute hyperglycemia on proliferation in the brain parenchyma and the effects of chronic hyperglycemia on proliferation along the ventricular layer. In

both normoglycemic and acute HG conditions (24 hours p.i. and 1 dpl), the number of PCNA-positive cells increased by almost two times in the injured telencephalon (Fig. 5A,C). This observation indicates that acute HG does not impair the initial step of parenchymal brain cell proliferation.

We next analyzed the effect of chronic HG on the proliferation of ventricular cells mainly corresponding to radial glial cells (type 1 and 2 progenitors; type 1 corresponding to quiescent radial glia and type 2 to actively dividing radial glia). Therefore, we stab wounded fish after 7 days of chronic HG and allowed them to survive for 1 week in chronic HG conditions. In control and chronic HG conditions, we observed a significant increase in cell proliferation in the ipsilateral hemisphere compared to the contralateral one (Fig. 5B,D). Interestingly, injury-induced ventricular proliferation was significantly reduced in chronic HG-treated fish compared to control fish (Fig. 5B,D; $P = 0.0127$). Consequently, our results clearly show that hyperglycemia impairs recruitment and/or activation of radial glial cells following stab wound injury.

DISCUSSION

In the central nervous system, dysglycemia is known for impairing synaptic transmission, neural plasticity, and cognition (Mooradian, 1997a,b; Kodl and Seaquist, 2008; Wang et al., 2012; Ho et al., 2013; Prasad et al., 2014). It has been reported that more than 50% of patients admitted for ischemic stroke experience hyperglycemia (Scott et al., 1999; Capes et al., 2001), whether due to stress or type 2 diabetes, and that hyperglycemia is associated with poor clinical outcome after thrombolysis (Desilles et al., 2013).

In the present study, we investigated the impact of acute and chronic hyperglycemia on adult zebrafish neurogenesis and brain homeostasis. Our results showed that brain homeostasis was differently affected in acute HG and chronic HG conditions. Indeed, acute HG led to brain
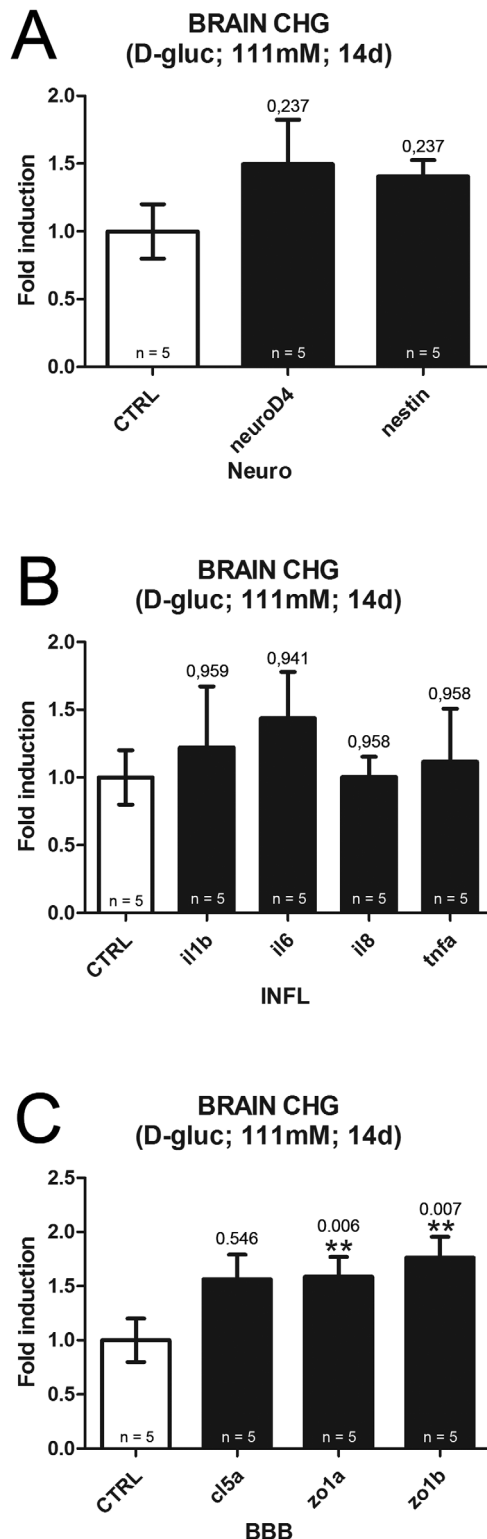
Figure 4. Proinflammatory cytokines and BBB gene expression following chronic hyperglycemia. Semiquantitative PCR analysis in the brain of adult zebrafish following chronic hyperglycemia (D-glucose; 111 mM; 14d). **A,B:** Chronic hyperglycemia treatment did not modulate expression of the neuronal and progenitor markers, *neurod4* and *nestin*, respectively, or those of the proinflammatory cytokines *il1β, il6, il8,* and *tnfα*. **C:** Chronic hyperglycemia treatment results in a significant upregulation of *zona occludens 1a* and *1b* (*zo1a* and *zo1b*, $P = 0.006$ and 0.007, respectively), involved in the maintenance of the BBB. Although *claudin 5a* gene expression is not significantly modulated, it shows an increasing trend. ** $P \leq 0.01$. Error bars correspond to the standard error of the mean (SEM) and *n* refers to the total number of two pooled zebrafish brains.
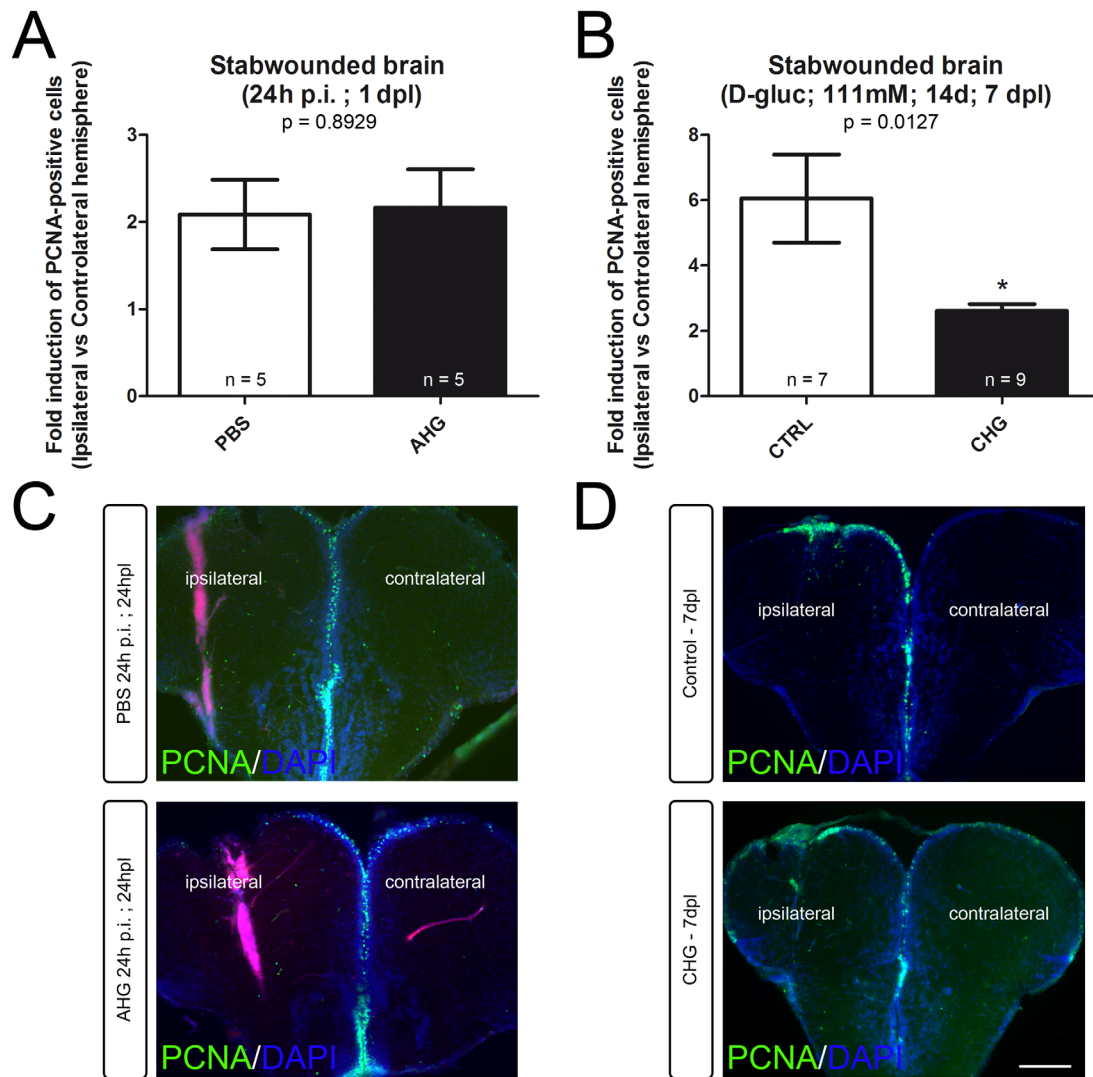
Figure 5. Chronic hyperglycemia impairs brain cell proliferation after stab wounding injury of the telencephalon. **A:** Fold induction of PCNA-positive cells (ipsilateral vs. contralateral) in PBS- and D-glucose- (2.5 g/kg of body weight) injected fish at 24 hours post-injection (p.i.) and 1 day post-lesion (dpl). **B:** Fold induction of PCNA-positive cells (ipsilateral vs. contralateral) in control fish and chronic HG-treated fish (111 mM D-glucose; 14 days) with stab wounding at day 7. Ventricular cell proliferation induction after stab wounding is significantly decreased in chronic HG-treated fish ($P = 0.0127$). **C,D:** PCNA immunohistochemistry (green) and DAPI counterstaining (blue) on vibratome brain sections of 24 hours stab-wounded telencephalon of PBS- and D-glucose- (2.5 g/kg of body weight) injected fish (C), and of chronic HG-treated fish (111 mM D-glucose; 14 days) stab-wounded at 7 days of treatment and allowed to survive for 7 days in chronic HG conditions. A decrease in ventricular cell proliferation is observed under chronic HG conditions. Pictures were taken with an epifluorescence microscope. *$P \leq 0.05$. Error bars correspond to the standard error of the mean (SEM) and n refers to the total number of fish. Scale bar = 220 μm.

inflammation without impairing adult neurogenesis, whereas chronic HG resulted in a significant decrease in cell proliferation along the brain ventricle, where neural stem cells and further committed progenitors are localized. Finally, we showed that chronic HG markedly reduced neurogenesis following stab wound injury in the brain of adult zebrafish.

Hyperglycemia

As shown in our experimental conditions, both acute i.p. injection and chronic exposure to D-glucose in the fish water induced hyperglycemia (Table 3). We observed significantly higher blood glucose levels for acute HG zebrafish 1h30 after D-glucose injection compared to control fish (Table 3). We also observed an increasing trend in blood sugar level in PBS-injected fish at 1h30 p.i. compared to baseline glycemia, which could be attributed to stress induced by the injection and/or the use of the anesthetic that is known to induce a wide variation of blood glucose levels (Chavin and Young, 1970; Eames et al., 2010). As expected, at 24 hours p.i., D-glucose-injected fish displayed

normoglycemic values (Table 3). Therefore, blood glucose levels appear to be dynamically regulated in zebrafish, as previously shown (Chavin and Young, 1970; Eames et al., 2010).

To our knowledge, this is the first description of an acute hyperglycemia model in zebrafish. We also validated the chronic HG model, as zebrafish immersed in D-glucose water (111 mM for 14 days) became hyperglycemic. This is consistent with previous studies from Capiotti et al. (2014) showing an increase in blood glucose level that correlates with the time of immersion in D-glucose supplemented water (Capiotti et al., 2014a). Under such conditions, it was found that hyperglycemia had already been induced starting from 3 days of treatment (Capiotti et al., 2014a). Interestingly, i.p. D-glucose injection induced a peak of hyperglycemia at ∼500 mg/dL, whereas acute exposure to D-glucose led to a glycemia of ∼280 mg/dl. Taken together, these data further strengthen the utility of our procedures as relevant models for studying acute HG and chronic HG. Importantly, we noticed that, in some experiments, not all the fish displayed hyperglycemia after the chronic immersion in D-glucose (data not shown; 17% nonhyperglycemic vs. 83% hyperglycemic). This might be due to different susceptibilities of the individual fish to hyperglycemia according to age or compensatory mechanisms (Sarras and Intine, 2013; Connaughton et al., 2016).

Inflammation and BBB

In the brain of acute HG fish, we noticed an increase in proinflammatory cytokine expression (*il1β*, *il6*, *il8*, and *tnfα*), reaching statistical significance for *il1β*, *il8*, and *tnfα* (Fig. 2B). Such proinflammatory effects of hyperglycemia are already well-characterized in mammals in a wide variety of tissues, including tissues of the central nervous system (Esposito et al., 2002; Lin et al., 2005; Gyurko et al., 2006). Proinflammatory cytokines are important mediators of the early immune response and display pleiotropic effects such as promoting immune cell proliferation (Randelli et al., 2008) and modulating neuronal survival and neurogenesis (Fuster-Matanzo et al., 2013; Tobin et al., 2014; Borsini et al., 2015). In our experimental conditions, proinflammatory cytokine gene expression was unchanged in the brain of chronic HG zebrafish after 14 days (Fig. 4A). This finding does not preclude the possibility that the expression of these genes is modulated within the first days of chronic HG, but that an adaptive response may take place to counteract chronic inflammation, particularly in light of the zebrafish's high capacity for adaptation and regeneration (Randelli et al., 2008). Such presumed compensatory mechanisms will require further investigation.

Regarding the BBB, *claudin 5a* and *zona occludens 1a* or *1b* gene expression was not modulated under acute HG at 24 hours p.i. (Fig. 2B), but *zona occludens 1a* and *1b* gene expression was significantly upregulated under chronic HG (Fig. 4B). Our results show that chronic hyperglycemia affects the expression of genes involved in BBB establishment. However, our attempts at testing for BBB leakage using Evans blue dye were not conclusive due to a wide variability in staining (data not shown). Numerous *in vitro* and *in vivo* studies have demonstrated that hyperglycemia increases BBB leakage by downregulating tight junction protein expression (e.g., occludin and zona occludens-1) (Chehade et al., 2002; Hawkins et al., 2007; Ergul et al., 2009; Chao et al., 2016). Indeed, type 2 diabetes rats were reported to display an impaired hippocampal BBB (Yoo et al., 2016). In zebrafish, the blood–retinal barrier was equally impacted by hyperglycemia during development, as shown by abnormalities in blood vessels morphology and decreased levels of ZO-1 protein expression (Alvarez et al., 2010; Jung et al., 2016). We hypothesized that the *zona occludens 1a* and *1b* upregulation could be due to compensatory mechanisms in response to BBB aggression.

Neurogenesis

In our experiments, acute HG did not impair neural stem cell proliferation in the different brain regions or nuclei studied (Fig. 1); however, chronic HG-treated fish exhibited a significant decrease in ventricular proliferation in all regions studied (OB/TEL, Vv, Dm, PPv) except the mediobasal (Hv LR) and caudal hypothalamus (LR PR). Nevertheless, the hypothalamus displays a consistent decrease in cell proliferation (Fig. 3). Our observations are in line with previous reports in mammals showing that type 2 diabetes-associated oxidative stress and/or inflammation inhibit hippocampal proliferation and neuronal migration and promote neuronal death (Beauquis et al., 2006; Wiltrout et al., 2007; Jakubs et al., 2008; Stranahan et al., 2008; Yi et al., 2009; Ho et al., 2013). High glucose levels were also shown to induce deleterious effects on embryonic quail neurogenesis, by decreasing neurite length and neuronal migration, and by increasing reactive oxidative species generation (Chen et al., 2013). In both acute HG and chronic HG conditions, *neurod4* and *nestin* gene expression remained unchanged, suggesting no influence of either acute or chronic hyperglycemia on neuronal differentiation and neural progenitor levels (Figs. 2A, 4A). However, further investigations should elucidate how hyperglycemia impacts neurogenic processes.

Brain regeneration in zebrafish has been well characterized. It starts with an initial parenchymal proliferation of microglial cells and oligodendrocytes (day 1 to 3) that is followed by a highly sustained proliferation of

radial glial cells in the ventricular layer from 3 days post-injury, allowing the production of new neurons (Kaslin et al., 2008; Kroehne et al., 2011; März et al., 2011; Diotel et al., 2013; Alunni and Bally-Cuif, 2016). By performing stab wound injury of the telencephalon, we showed that acute HG did not impair the initial step of parenchymal brain cell proliferation but that chronic HG disrupted the ventricular cell proliferation at 7 days post-injury. Such a deleterious effect of hyperglycemia in regenerative mechanisms is consistent with the well-known complications of type 2 diabetes relating to impaired wound healing (Rosenberg, 1990; Lioupis, 2005). In zebrafish, hyperglycemia was also shown to affect the healing process during fin regeneration (Olsen et al., 2010). Additionally, numerous studies have documented the adverse effects of hyperglycemia/diabetes on brain stoke and traumatic brain injury (Cherian et al., 1997; Huang et al., 2013; Jauch-Chara and Oltmanns, 2014).

It is interesting to note that diabetes is also known for impairing brain memory (Whitmer, 2007; Ho et al., 2013). Recently, using the same experimental conditions, Capiotti and colleagues demonstrated that hyperglycemia induces memory impairment in adult zebrafish (Capiotti et al., 2014b), similar to the mammalian situation. Taken together, these data demonstrate that hyperglycemia in zebrafish impairs brain functions such as neurogenesis and memory.

Such adverse effects of hyperglycemia on brain cell proliferation have probably multiple direct and/or indirect origins. By increasing brain inflammation and by disrupting the BBB, hyperglycemia could favor the central effects of peripheral factors such as proinflammatory cytokines and adipokines that have been documented to disrupt neural stem cell activity (Arnoldussen et al., 2014; Letra et al., 2014; Parimisetty et al., 2016). Hyperglycemia is also known for inducing an increase in advanced glycation endproducts that is correlated with decreased hippocampal neurogenesis in STZ-induced diabetic rats (Ho et al., 2013). Variation of insulin and/or IGF-1 levels resulting from hyperglycemia could also be factors modulating the activity of neural stem cells (Rafalski and Brunet, 2011; Ziegler et al., 2015). Taken together, these data highlight the necessity of further investigations in order to understand the mechanistic behind the decreased proliferation observed in our model.

CONCLUSION

In the present study, we demonstrate that hyperglycemia disturbs brain homeostasis, influencing brain inflammation and neurogenesis in homeostatic and in injury-induced conditions. Taken together, our data and those reported by Capiotti and colleagues highlight an evolutionary-conserved impact of hyperglycemia on brain homeostasis and plasticity. Finally, this work confirms the value of the zebrafish model in studying the effects of hyperglycemia on the central nervous system, particularly in pathological situations such as stroke.

CONFLICT OF INTEREST

The authors declare that they have no competing interest.

AUTHOR CONTRIBUTIONS

Conception and design of the experiments: ND, OM, CLDH, ACD, MW, EB. Acquisition of data: ACD, ND, SS. Analysis of the data: ND, ACD, SS, CLDH, OM, MW, EB.

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