Sonic hedgehog expression in zebrafish forebrain identifies the teleostean pallidal signaling center and shows preglomerular complex and posterior tubercular dopamine cells to arise from shh cells

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Abstract
Ventralization, a major patterning process in the developing vertebrate neural tube (central nervous system, CNS), depends on Sonic hedgehog (SHH) as a main signaling morphogen. We studied the CNS of late larval and young adult zebrafish in a transgenic shh-GFP line revealing increased neuroanatomical detail due to the progressed differentiation state compared to earlier stages. Some major findings emerge from the present study. (a) shh–GFP is still expressed along the adult zebrafish CNS neuraxis in most locations seen in larvae. (b) We newly identify a ventroposterior shh-pallidal domain representing the basal telencephalic signaling center important for basal ganglia development known in other vertebrates (i.e., the anterior entopeduncular area—basal medial ganglionic eminence of mammals). (c) We further show late-emerging shh-GFP positive radial glia cells in the medial zone of the dorsal telencephalon (i.e., the teleostan pallial amygdala). (d) Immunostains for tyrosine hydroxylase demonstrate that there is selective colocalization in adult dopamine cells with shh-GFP in the posterior tuberculum, including in projection cells to striatum, which represents a striking parallel to amniote mesodiencephalic dopamine cell origin from shh expressing floor plate cells. (e) There is no colocalization of shh and islet1 as shown by respective shh-GFP and islet1-GFP lines. (f) The only radially far migrated shh-GFP cells are located in the preglomerular area. (g) There are no adult cerebellar and tectal shh-GFP cells confirming their exclusive role during early development as previously reported by our laboratory.

KEYWORDS
floor plate, islet1, longitudinal gene expression, mesodiencephalic dopamine cells, pallium, posterior tuberculum, preglomerular complex, RRID: AB_2201528, RRID: AB_2340817, RRID: AB_10000240, RRID: AB_2340364, striatum, telencephalon, tyrosine hydroxylase, ventralization
1 | INTRODUCTION

Ventralization versus dorsalization represent major interdigitating patterning processes in the developing vertebrate neural tube (central nervous system, CNS). Hereby, morphogens are issued dorsally (roof plate) and ventrally (in two steps, first from notochord andprechordal mesoderm, later from floor plate and ventral forebrain cells; see section 4) in order to interact on neural cells of alar and basal plates with the result of a graded neurocellular fate along the doroventral CNS axis (Balasaks et al., 2012; Briscoe, 2009; Briscoe & Novitch, 2008; Briscoe & Small, 2015; Dessaud, McMahon, & Briscoe, 2008; Grossmann, Giraudin, Britz, Zhang, & Goulding, 2010; Marli & Bovolenta, 2002). Sonic hedgehog (SHH) is the main ventral signaling molecule (morphogen). We already summarized the hedgehog signaling pathway and the roles of three different zebrafish hedgehog genes, and also analyzed the larval expression of the sonic hedgehog gene (shh) using an established transgenic shh-GFP line (see section 2) in two recent previous papers (Baeuml, Biechl, & Wullimann, 2019; Biechl, Dorigo, Köster, Grothe, & Wullimann, 2016). These and other zebrafish brain expression studies (Ekker et al., 1995; Erter et al., 2007; Hagemann & Scholpp, 2012; Hauptmann & Gerster, 2000; Hauptmann, Söll, & Gerster, 2002; Holzschuh, Hauptmann, & Driever, 2003; Krauss, Concordet, & Ingham, 1993; Strähle, Blader, & Ingham, 1996; Wilson & Rubenstein, 2000) remain in line with general knowledge in vertebrates (see section 4).

However, there are two unresolved problems regarding sonic hedgehog expression in the zebrafish brain. One is the lack of a telencephalic (subpallial) shh expression domain comparable to what is described in amniotes as the anterior entopeduncular area (AEP) and medial ganglionic eminence (MGE; see section 4). These amniote telencephalic shh domains are crucial for correct ventral telencephalic gene expression (e.g., Dlx, Ascl1, Nkx2.1, Isl1, Lhx6/7) and, thus, for correct basal ganglia development as well as for repressing dorsal (i.e., pallial) gene expression ventrally (see section 4). A second issue is the developmental role of SHH in the generation of basal diencephalic dopamine cells. In mammals, midbrain substantia nigra/ventral tegmental dopamine cells are known to derive from sonic hedgehog expressing floor plate cells (Joksimovic et al., 2009; Blaess et al., 2011; Hayes, Zhang, Albert, Zervas, & Ahn, 2011; see section 4). Since teleosts lack midbrain dopamine cells and only possess basal diencephalic dopamine cells (posterior tuberculum)—which also contribute to the SN/VT in mammals (see section 4)—that nevertheless project to the fish basal ganglia (review Wullimann, 2014), we wanted to verify whether these zebrafish diencephalic ascending dopamine cells are produced by shh expressing cells.

Thus, we looked at early adult (3-month-old) transgenic shh-GFP zebrafish and described in neuroanatomical detail all GFP-positive CNS structures. Because the advanced differentiation state of the adult brain allows for a far more detailed identification of shh-GFP structures compared to larvae, we anticipated that the data will shed light onto both the recognition of a true pallidal shh domain as well as on the origin of dopaminergic posterior tubercular projection neurons to the teleostean striatum (see section 4.4.) from shh expressing cells from which part of the preglomerular area is also revealed to derive.

2 | MATERIALS AND METHODS

2.1 | Transgenic zebrafish strains

The transgenic line Tg(2.4shha-ABC-GFP)sb15 was originally published as Tg(2.2shhgfpABC#15) by Shkumatava, Fischer, Müller, Strähle, and Neumann (2004). The injected construct includes the sonic hedgehog promoter (SalI/XhoI fragment) upstream of gfp as well as intronic sequences for required enhancer regions (Müller et al., 1999). The line will be referred to in the following as shh-GFP line. Our lab has used it previously to study the larval expression of shh-GFP (Biechl et al., 2016). Here, we raised zebrafish shh-GFP specimens into larval stages and up to 3 months (early adults). Fish were maintained according to standard protocols (Westerfield, 2007).

The shh-GFP transgenic zebrafish line used here has previously been characterized to faithfully represent shh expression (Biechl et al., 2016; Shkumatava et al., 2004) in zebrafish retina and brain/spinal cord and the transgenic expression patterns are furthermore well in line with known shh expression patterns in other vertebrate species (reviewed in Biechl et al., 2016 and Baeuml et al., 2019).

The transgenic islet1-GFP line Tg(is1:GFP) was originally generated by Higashijima, Hotta, and Okamoto (2000) by fusing gfp sequences with islet-1 promoter sequences (ICP) to produce the core plasmid and adding enhancer elements (CM) for the construct that proved sufficient for specific neural expression. This line will be referred to here as islet1-GFP line. Details for the generation of these specimens, as well as the origin of brain sections depicted in this contribution, are given in a previous paper reporting on islet1-GFP expression (Baeuml et al., 2019).

All procedures involving live zebrafish were carried out according to EU guidelines and German legislation (EU Directive 2010_63, license number AZ 325.1.53/56.1-TU-BS). Transgenic animals used in this study were killed with an overdose of tricaine methanesulfonate (MS-222) and fixed in paraformaldehyde (4% PFA in Sörensen’s phosphate buffer, PB) at 4°C overnight. The raising and fixation of transgenic animals were performed in Prof. Reinhard Köster’s lab (Technical University Braunschweig, Germany) and kindly subsequently provided to us. Therefore, the present study only involved fixed animal tissue and needed no further approval.

2.2 | Cutting procedure

Following cryoprotection in sucrose solution (30% sucrose solution at 4°C overnight), the brains (heads) of adult shh-GFP zebrafish were embedded in TissueTek (tissue freezing medium, A. Hartenstein GmbH) and cryosectioned (Leica, CM 3050S) at 30 μm in the transverse or sagittal plane before thaw mounted onto Superfrost Plus glass slides (Thermo) and coverslipped after immunoprocedures. In total, 18 zebrafish specimens were used in this study, that is, one specimen each of 3–8 days postfertilization (dpf) larvae, four 13 dpf larvae, and eight 3-month-old specimens. Additionally, various 2, 3, 4, and 5 dpf shh-GFP specimens were available from a previous study (Biechl et al., 2016).
2.3 Immunohistochemical processing

Immunohistochemical incubations were done in a humid chamber. After washing off TissueTek in cryosections with phosphate-buffered saline (PBS) the sections were blocked with blocking buffer (2% normal goat serum, 2% bovine serum albumin, 0.2% Tween20, 0.2% TritonX-100 in PBS) for 1 hr at RT before exposure to a primary antibody against GFP diluted in blocking buffer at 4°C for 1–3 days (dilutions see Table 1). After washing in PBT (PBS + 0.1% Tween 20), the sections were incubated with the secondary antibody (see Table 1) diluted in blocking buffer solution overnight at 4°C. Subsequently, a second primary antibody against tyrosine hydroxylase (TH; see Table 1) was applied after intermittent washing in PBT and blocking (see above for details), followed by the application of the appropriate secondary antibody (see Table 1) diluted in blocking buffer overnight, after intermittent washing in PBT and blocking (see above). Finally, sections were washed in PBT and counterstained with DAPI (4′,6-diamidino-2-phenylindole; Carl Roth, 1:1000) and washed in PBS. Slides were then mounted with Vectashield (Vectorlabs) or ProLong Diamond (Invitrogen/Thermo Fisher) and coverslipped. Previously, various controls and Western blot analysis for the antibody against TH have been performed (Yamamoto, Ruuskanen, Wullimann, & Vernier, 2010, 2011).

Furthermore, there were no neuroanatomical differences between the intrinsic GFP signal with the one enhanced through the use of the anti-GFP antibody.

2.4 Photography

Cryostat sections of adult zebrafish heads were photographed using a light/fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Inc.) with a Nikon Digital Sight DSU1 Photomicrographic Camera (Nikon Instruments Inc.) and NIS-Elements F4.60.00 software. The microscope was equipped with Nikon Plan UW 0.06 (2x), Plan Fluor DIC L/N1, \( \times 0.17 \), WD 16.0 (10x/0.30)) and Plan Fluor DIC M/N2, \( \times 0.17 \), WD 2.1 (20x/0.50) objectives.

All images were eventually slightly adapted for brightness and contrast with Corel PHOTO-PAINT 9.0 and mounted into figures with Corel DRAW 9.0 (Corel Corporation, Ottawa, Canada).

2.5 Analysis of data

Most sections were photographed in three appropriate fluorescent spectral channels for the presence of the nuclear stain DAPI, shh-GFP or islet1-GFP, and TH. In cases where the GFP and TH label was in the same area, the ImageJ tool of synchronizing all windows was used to analyze cellular colocalization of shh-GFP with TH on a neuroanatomical background yielded by the DAPI pictures. Since the three microphotographs were identical in each case except for the fluorescence visualized, we could assign in detail to a cell nucleus seen in DAPI stain the associated cytoplasmic green GFP and red transmitter-related enzyme stain on a cell to cell basis.

3 RESULTS

The shh-GFP is generally still expressed in early adult zebrafish brains in most locations along the neuraxis as seen in larval zebrafish brains of 4/5 days postfertilization (dpf; see Baeuml et al., 2019; Biechl et al., 2016). These shh domains include classical floor plate cells defining the ventral midline of the neuraxis from spinal cord into the posterior diencephalon. However, the forebrain shows more complex shh-GFP expression patterns involving basal and alar plates. We will describe all shh-GFP expression domains from anterior to posterior levels, along with nuclear DAPI stains and, when necessary, with tyrosine hydroxylase (TH) immunostains. We use for identification of brain structures basically the Neuroanatomy of the Zebrafish Brain atlas (Wullimann, Rupp, & Reichert, 1996). In a recent study (Baeuml et al., 2019), we have detailed and justified some modifications from this atlas in the identification of the paraventricular organ, the intermediate hypothalamic nucleus and the posterior tuberal nucleus also applied here.

3.1 Analysis of shh-GFP and tyrosine hydroxylase in transverse plane

3.1.1 Telencephalon and preoptic region

Similar to the larval brain (4–8 dpf), there are no shh-GFP cell bodies in the adult subpallium and in the parenchyma of all adult pallial divisions. Different from the larval brain, however, radial glia cells (white arrows in Figure 1) in the adult medial zone of the dorsal telencephalon (Dm) stain for shh-GFP from precommissural (Figure 1a1–a2) via commissural (Figure 1b1–b2) to postcommissural levels (Figure 1c1–c2). These cells are identifiable by their soma location within the everted pallial ventricular surface and their long fibers extending towards the pial periphery (Figure 1d,d’). These fibers converge peripherally in the area identified as dorsal entopeduncular nucleus (ENd) and the ventral part of the posterior zone of the dorsal
telencephalon (Dp; Figure 1d) suggesting that this convergence area is a pial surface and not a ventricular surface in contrast to that of the medial (Dm), central (Dc) and lateral (Dl) zones of the dorsal telencephalon. The latter zone (Dl) shows no shh-GFP radial glia cells, but many other markers demonstrate the nature of its ventricular surface (see section 4).

In the postcommissural telencephalon, a strong shh-GFP expression domain is present in the most ventroposterior basal pallidal part of the subpallium (BP; Figure 1c,e). This area has not been identified as a separate entity before, including the Neuroanatomy of the Zebrafish Brain adult atlas (where it lies in the area between the anterior parvocellular preoptic nucleus, PPa, and the postcommissural nucleus of the ventral

FIGURE 1  Legend on next page.
The spot of shh-GFP cells seen at the base of the supraacommissural nucleus of the ventral telencephalon (Vs) is the most anterior extension of BP (arrowhead in Figure 1b2). We propose that this basal subpallial expression domain represents the zebrafish homolog of the mammalian palidal shh expressing domain (Mueller & Wullimann, 2009; Mueller, Wullimann, & Guo, 2008). The shh-GFP cell somata in BP mostly do not lie directly at the ventricular lining and do not exhibit long fibers towards the pial periphery. Thus, they likely are not radial glia cells.

A fair number of shh-GFP cells is seen in the anterior parvocellular preoptic nucleus (PPa; Figure 1b–c) and a few cells show up in the suprachiasmatic nucleus, but none are present in the anterior part of the posterior parvocellular preoptic and magnocellular preoptic nuclei (SC, PPp, PM; Figures 2a,b and 3i,j). Furthermore, neither subpallial nor preoptic dopamine cell somata ever colocalize with shh-GFP (Figure 1a–c).

3.1.2 Diencephalon

Transverse hind- and midbrain sections are leveled with respect to the caudorostral body axis and thus run horizontally through the forebrain because of the ventral bending of the neural tube’s front end. As suggested previously (Herget, Wolf, Wullimann, & Ryu, 2014; their figure 1), it is reasonable to identify in such forebrain sections dorsal as posterior and ventral as anterior to avoid confusion with body axes. Thus, forebrain sections may show from “dorsal” (i.e., posterior) to “ventral” (i.e., anterior) at the same time parts of the (most posterior) prosomere 1 (pretectum; P1), prosomere 2 (thalamus, previously dorsal thalamus; P2), and prosomere 3 (pretectal terminal field) as well as the (most anterior) hypothalamus (Figures 2–4). It has to be noted that these prosomeres include alar as well as basal plate components (see below). This clarification of neural tube axes is critical in the present investigation that focuses on a ventrally expressed marker (shh) in order to keep attention to the true ventral versus dorsal side of the forebrain.

Numerous shh-GFP cell bodies are seen in the zona limitans intrathalamic (ZLI; Figures 2c–e and 3k) and in cells in the prethalamus, the alar plate of prosomere 3 (Figures 2a,b and 3j). This somewhat unexpected alar plate shh-GFP expression is paralleled by islet1-GFP expression (see section 4). The thalamic (Th) and periventricular pretectum (Pr; alar plate of prosomeres 2 and 1, respectively) contain no shh-GFP cell bodies. However, the periventricular pretectum exhibits shh-GFP positivity in terminal visual projection fibers (pretectal terminal field) originating in shh-GFP retinal ganglion cells (prtf; Figures 2c–e, 3k, and 4b) and similar thalamic retinal terminal fields are seen lateral to the (anterior) thalamus and prethalamus (thtf; Figures 2a,b and 3).

Regarding diencephalic basal plate divisions, shh-GFP cell bodies clearly are present in both the parvocellular and magnocellular (pear-shaped) cells of the periventricular nucleus of the posterior tubercul (TPr-p, TPP-m) as well as in the paraventricular organ (PVO; Figures 2c–e and 4a,b). The shh-GFP label in the periventricular posterior tubercul is characterized by relatively far migrated cells of the TPr-m and peripherally leading labeled fibers. These fibers lead toward the preglomerular area where far migrated shh-GFP positive cell bodies are present (Figures 2e, 4b, and 5). Furthermore, shh-GFP cells are present in the dorsal and ventral periventricular hypothalamic zones (D, Hv; Figures 2d–f and 3k,l). In contrast, the adult caudal periventricular hypothalamic zone remains free of shh-GFP (Figures 3l, 4c, and 6a). However, in juvenile stages, Hc does express shh-GFP (see next section).

The shh-GFP cell bodies continue to be present in the basal plate domain of prosomere 1, which is the area of the nucleus of the medial longitudinal fascicle (Nmlf; Figures 2f and 6a). Most of these cells do not yet exhibit fibers typical for floor plate cells seen more posteriorly with the exception of some strongly stained midline cells (arrowhead in Figure 6) directly at the ventral midline ventricle. These shh-GFP cells have ventrally directed fibers as seen more posteriorly in the mid- and hindbrain and may be interpreted as most anterior floor plate cells. However, the remaining cells in the area of the Nmlf lie more distant to the ventricular lining and appear to lack such fibers. More “ventrally” (actually anteriorly because of the above mentioned neural tube bending) shh-GFP cells are seen in the TPr-m and the Hd (note inset in Figure 6a2).

3.1.3 Colocalization of adult dopamine cells with shh-GFP

Because dopamine cells are present in the posterior tubercular nuclei mentioned above (TPr-p, TPr-m, PVO) and the posterior tuberal...
FIGURE 2  Expression of shh-GFP in the adult zebrafish diencephalon. Six transverse sections showing habenular/prethalamic (a), anterior thalamic (b), posterior thalamic (c), posterior tubercular/paraventricular organ (d), posterior tuberal nuclear (PTN; e), and intermediate hypothalamic nuclear levels (f). The ventricle spot of the zona limitans intrathalamica is indicated with an asterisk. See text for details.

Abbreviations: ATN, anterior tuberal nucleus; DIL, diffuse nucleus of lobus inferior; DIV, diencephalic ventricle; Dm, medial zone of dorsal telencephalon; E, epiphysis; fr, fasciculus retroflexus; Ha, habenula; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; NmIf, nucleus of the medial longitudinal fascicle; ot, optic tract; pc, posterior commissure; PG, preglomerular complex; PGa/PGI/PGm, anterior/lateral/medial nucleus of PG; PM, magnocellular preoptic nucleus; PPr, posterior parvocellular preoptic nucleus; PPr, periventricular pretectum; prtf, pretectal retinal terminal field; PTh, prethalamus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SC, suprachiasmatic nucleus; TeO, optic tectum; thtf, thalamic retinal terminal field; TLa, torus lateralis; TLo, torus longitudinals; TPr, periventricular posterior tuberculum; ZLI, zona limitans intrathalamica [Color figure can be viewed at wileyonlinelibrary.com]
nucleus (PTN), an immunostain against tyrosine hydroxylase was applied to the same sections (Figure 4a3,b3,c3) and double-label with shh-GFP was indeed seen in three of four dopaminergic posterior tubercular systems (i.e., TPp-p, TPp-m, PVO; yellow arrows in Figure 4a,b; Table 2), but not in the adult PTN (Figure 4c). However, the juvenile PTN does stain for both shh-GFP and TH (see section 3.4). With the possible exception of the caudal zone of the periventricular hypothalamus, no other dopamine cells in the zebrafish brain show such double-label (i.e., in pretectum, prethalamus, or preoptic region). This is a stunning parallelism to the documented midbrain floor plate (i.e., shh expressing cells) origin of substantia nigra/ventral tegmental area in mammals and suggests that also diencephalic dopamine cells (including the striatal projecting cells) do originate from shh expressing cells (see section 4).

3.1.4 Mesencephalon, rhombencephalon, and spinal cord

Posterior to the Nmlf, shh-GFP cell bodies are restricted to mesencephalic, rhombencephalic, and spinal floor plate cells situated in the ventral midline of the ventricular lining (Figure 6b–g). These cell bodies typically extend long fibers ventrally into the neural parenchyma which reach with their endfeet (EF) the ventral pial brain or spinal cord surface. The midbrain floor plate cell fibers are seen to bypass the oculomotor nerve on their passage towards the ventral pia (Figure 6b). The interpeduncular nucleus is invaded and surrounded by such fibers (Figure 6c). Noradrenergic cells of the locus coeruleus colocalize with shh-GFP (yellow arrows, Figure 6d), but other hindbrain catecholaminergic cells, such as the area postrema (Figure 6f) do not.

3.2 Analysis of possible colocalization of shh-GFP and islet1-GFP

In the mid- and hindbrain, the shh-GFP cell bodies are restricted to floor plate cells and, thus, are never colocalized with islet1-GFP which is always localized in identifiable migrated brain nuclei, best studied for cholinergic motor neurons (see discussion in Baeuml et al., 2019). Because forebrain shh-GFP expression is more complex and includes many cell bodies beyond floor plate cells (sometimes peripherally migrated ones) we analyzed corresponding section levels of both shh-GFP (present study) and islet1-GFP (see also Baeuml et al., 2019) fish brains for the presence of possible co-localization of the two markers (Figure 3) in more detail. Generally, in brain structures positive for shh-GFP, these cell bodies are located directly at the ventricle with many more unstained cells peripheral to them (note dashed lines peripheral to shh-GFP cells in Figures 3g,h,k,l). In contrast, islet1-GFP cell bodies lie more peripheral leaving many unstained cells towards the ventricle in each area (note dashed lines towards ventricle respective to islet1-GFP cells in Figures 3a,b,e,f). Since we use two separate transgenic lines for this comparison, a double-label analysis is not directly possible in the same section. However, the distribution of the two transgenically expressed GFP markers is that mutually exclusive spatially as to make a colocalization extremely unlikely in the ventral telencephalon (Vs), preoptic region (PPa, SC), and dorsal and ventral zones of the periventricular hypothalamus (Hv, Hd). The more peripherally migrated magnocellular part of the periventricular posterior tubercular nucleus (TPp-m) and the paraventricular organ (PVO) are completely free of islet1-GFP cells (Baeuml, Biechl, & Wullimann, 2019). Although the (alar) prethalamic shh-GFP cells intermix with islet1-GFP cells, they are different cells because the former are never TH positive (this study) whereas islet1-cells are TH positive (Baeuml et al., 2019; see section 4). To decide about the very few islet1-GFP cells possibly positive for shh-GFP in the area of the Nmlf, a double transgenic line (shh-GFP and islet1-GFP) would be necessary. However, TH cells in the parvocellular part of the periventricular posterior tubercular nucleus (TPp-p) were found both to be shh-GFP positive (this study) and islet1-GFP positive. Thus, the TPp-p is the only nucleus with clear colocalization of both GFP markers.

3.3 Analysis of shh-GFP and tyrosine hydroxylase in sagittal plane

In order to provide additional means of verification and didactically improved visualization of data reported above, we also prepared sagittal sections of shh-GFP transgenic brains immunostained against tyrosine hydroxylase.

An overview of a shh-GFP zebrafish brain (Figure 7a1)—when compared to its DAPI stained alter ego (Figure 7a2)—illustrates the complete absence of shh-GFP cell bodies in all parts of the cerebellum (valvula, corpus and lobus caudalis cerebelli) as well as in the primary sensory lateral line area (cerebellar crest and underlying MON) and chemosensory (facial and vagal) lobes and, furthermore, such negativity is noted in the diffuse nucleus of the inferior lobe, the caudal periventricular hypothalamus and corpus mamillare (Figure 7c2). In contrast, the shh-GFP positive line of midline floor plate cells in the entire hindbrain up to midbrain and into Nmlf is nicely visualized in sagittal view, including the ventrally directed fibers of these cells (Figure 7a1,b1,d). More anteriorly the zona limitans intrathalamica (ZLI) forms a shh-GFP positive spear-shaped transverse barrier between thalamus and prethalamus (Figure 7a1,b1) extending into the alar plate. A more focused higher-power sagittal analysis of the diencephalon shows the ZLI and its relationship to surrounding structures at various parasagittal levels (Figure 8a2–d2). Anterior to the ZLI, again basally located shh-GFP cells in the basal plate diencephalon (posterior tuberculum) and hypothalamus, as well as in the alar plate preoptic region and basal subpallium follow (PPa, BP; Figures 7a1,a2 and 8a2–d2). Turning to the posterior tuberculum, the striking large cells of the periventricular posterior tuberculum (TPp-m) are visualized to be double-labeled for TH and shh-GFP (yellow arrows; Figures 7b1–b3 and 8b–d). The higher-power diencephalic pictures also show that other diencephalic dopamine cell groups, such as the periventricular pretectum (Pr) and the prethalamic/zona incerta) cells
(PTh; Wullimann & Rink, 2001) or the preoptic nuclei (PPa, PPp, and SC) and the posterior tuberal nucleus (PTN) as well as the caudal periventricular hypothalamic zone (Hc) are remote from shh-GFP cells (Figure 8). Double-label of shh-GFP and TH in the parvocellular division of the periventricular tubercular nucleus (TPp-P) and in the paraventricular organ (PVO) are hard to visualize in the sagittal plane, but sufficiently documented above in transverse sections (compare Figure 4).

Overall, this sagittal analysis delivered a highly consistent and corroborating picture but also showed that certain details are veiled.
(double-labeled in TPP-p and PVO) whereas other facts are better visualized in sagittal sections (longitudinal distribution of floor plate cells and relationship of ZLI to thalamus and prethalamus). Also, certain most laterally placed shh-GFP cell groups were better to be grasped in transverse sections (preglomerular area, locus coeruleus, dorsal zone of periventricular hypothalamus; see above).

3.4 | Colocalization of late larval dopamine cells with shh-GFP

Because of the broad shh-GFP expression in early larval zebrafish brains (Baeuml et al., 2019; Biechl et al., 2016) in the posterior tuberculum and hypothalamus, we wanted to check later larvae with a progressed brain differentiation state for the presence of shh-GFP and TH. We focused particularly on the fourth posterior tubercular division (i.e., the posterior tuberal nucleus; PTN) and the caudal division of the periventricular hypothalamus (Hc) both showing a clear absence of shh-GFP but the presence of TH in the present study in adult brain sections. Also, this larval investigation allows for checking on a possible early emergence of the subpallial shh-GFP positive pallidal domain (BP).

At 13 dpf, the zebrafish diencephalon is already differentiated into its major divisions visualized in DAPI stains (Figure 9a1–e1), namely, the pretectum (Pr), thalamus (Th), prethalamus (PTh), preoptic region (Po), the area of the nucleus of the medial longitudinal fascicle (N), the posterior tuberculum (PT), the preglomerular region (PG), and the rostral (adult: ventral), intermediate (adult: dorsal) and caudal periventricular hypothalamus (Hr, Hi, Hc). The shh-GFP stain (Figure 9a2–e2) corroborates these divisions, for example, the position of the ZLI between thalamus and prethalamus and the broad shh-GFP expression in the basal plate of the midbrain tegmentum leading into N and PT and more anteriorly into Hr, Hi, and Hc. Furthermore, all larval zebrafish diencephalic dopamine groups visualized by TH (previously described by Rink and Wullimann (2002) are clearly visible at this stage, starting with the pretectal and preoptic groups leading to the parvocellular (adult: TPP-p; larval 1) and magnoocular divisions (adult TPP-m; larval 2.4) of the periventricular posterior tuberculum, and to the paraventricular organ (PVO, larval 3), the posterior tuberal nucleus (PTN, larval 6) and the caudal zone of the periventricular hypothalamus (Hc, larval 7; Figure 9a3–e3). Upon closer inspection of the latter two (Figure 9c–e), clear colocalization of shh-GFP and TH is seen in single large cells (yellow arrows in Figure 9) in the PTN (and, as in the adult, in the TPP-m), whereas most cells in the Hc appear single labeled for either shh-GFP or TH (white arrows in Figure 9).

We do not see at this stage a subpallial shh-GFP positive pallidal population (BP) as in the adult brain, and, thus, the preoptic cells (Figure 9a2) are the most anterior shh-GFP positive cells.

4 | DISCUSSION

4.1 | General issues: Expectations meet surprises

A comparison of early adult zebrafish brain shh-GFP expression (see Results and Figure 10a) with early larval expression (Baeuml et al., 2019; Biechl et al., 2016) reveals that, generally, expression domains are retained into the adult stage, starting posteriorly with longitudinally arranged series of floor plate cells in spinal cord, hindbrain, midbrain, and, most anteriorly, in the area of the nucleus of the medial longitudinal fascicle (Nmlf), that is, the basal plate division of the most posterior diencephalic prosomere (P1) representing the most posterior forebrain. The only additional shh-GFP cells in the hindbrain are a few noradrenergic locus coeruleus cells. Some shh-GFP cells in the Nmlf are the most anterior floor plate cells unmistakably characterized by long radial fibers that extend from their cell bodies at the midline ventricular floor toward the pial periphery where they form endfeet. However, the amniote floor plate itself appears to extend further anteriorly into the mammillary (caudal) hypothalamus as indicated by longitudinally expressed marker genes, such as the LIM homeobox

**FIGURE 3** Analysis of colocalization of shh-GFP and islet1-GFP in the adult zebrafish brain. Transverse sections of an islet1-GFP brain are shown in two left columns (a–f) and of a shh-GFP brain in two right columns (g–l), both for GFP positivity and additionally shown in nuclear DAPI stain. The islet1-GFP data stem from our previous study (Baeuml et al., 2019), (a/g) supracommissural nucleus of ventral telencephalon (Vs). (b/h) anterior parvocellular preoptic nucleus (PPa). (c/i) suprachiasmatic nucleus (SC). (d/j) prethalamus (ventral thalamus; PTh). (e/k) periventricular nucleus of posterior tuberculum (TPp) and ventral zone of periventricular hypothalamus (Hv). (f/l) dorsal zone of periventricular hypothalamus (Hd). Generally, shh-GFP cells are located more ventrolaterally than islet1-GFP cells, as evidenced by dashed lines in Vs, PPa, Hv, and Hd where shh-GFP cells are always within the lining and islet1-GFP cells are on the outside (i.e., the latter are more distant from the ventricle than the former). For location of shh-GFP cells in Tpp see Figure 4. While such ventricually located shh-GFP cells are also seen in the prethalamus (tier 1 in d), both shh-GFP and islet1-GFP cells exist in tier 2 (or ventromedial thalamus; see section 4). In SC, the medial islet1-cells cells are far apart from the lateral shh-GFP signal. Inset in (i1) shows enlargement of shh-GFP cells in SC. Inset in (j1) shows enlargement of retinal terminal field lateral to the thalamus. See text for details. Abbreviations: ac, anterior commissure; DAO, dorsal accessory optic nucleus; DIL, diffuse nucleus of inferior lobe; DIV, diencephalic ventricle; fr, fasciculus retroflexus; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; mfb, medial forebrain bundle; pc, posterior commissure; PG, preglomerular complex; Pit, pituitary; PM, magnoocular preoptic nucleus; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; PPr, preglomerular pretectum; prtf, pretectal retinal terminal field; PTh, prethalamus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SC, suprachiasmatic nucleus; TeO, optic tectum; Th, thalamus; thtf, thalamic retinal terminal field; TLa, torus lateralis; TPP, periventricular nucleus of posterior tuberculum; Vs, supracommissural nucleus of ventral telencephalon; ZLI, zona limitans intrathalamica; 1, ventricular layer of PTh; 2, ventromedial thalamic layer of PTh; 3, ventrolateral thalamic layer of PTh [Color figure can be viewed at wileyonlinelibrary.com]
gene Lmx1b and the forkhead gene Fox1a (Martínez, Puelles, Puelles & Echevarría, 2012; Puelles & Martínez, 2013; Puelles, Martínez-de-la-Torre, Bardet, & Rubenstein, 2012) and this might well be so in all vertebrates, including zebrafish.

Anterior to the Nmi, adult shh-GFP cells continue to be present in zebrafish brain basal plate regions of thalamic and prethalamic prosomeres 2 and 3 (i.e., posterior tuberculum), as well as in the more anteriorly lying basal plate hypothalamus (dorsal and ventral zones of periventricular hypothalamus; Hd, Hv), and in the (alar plate) preoptic region. As in all vertebrates, the zebrafish zona limitans intrathalamica (ZLI) is visible as a transverse veil of cells forming a division between (dorsal) thalamus and prethalamus (ventral thalamus). The position of

**FIGURE 4** Legend on next page.
the ZLI is best seen in sagittal view in Figures 7b1 and 8a2–d2. Also the (alar) prethalamus contains shh-GFP cells (see section 4.3).

Transverse adult serial sections reveal that most zebrafish forebrain shh-GFP cell bodies lie close to the ventricle, yet they lack the typical cytological nature of floor plate cells seen more posteriorly. Only in a few forebrain regions are shh-GFP cell bodies observed in more migrated locations. This is expected for the zebrafish ZLI which forms a barrier between prosomeres 2 and 3 and, thus, its migrated cells are present in the diencephalic adult gray matter in this location (best seen in transverse view in Figure 2c, 2d). However, migrated shh-GFP cell bodies are also present in the area of the posterior tuberculum and, to a lesser degree, in the area of the nucleus of the medial longitudinal fascicle (NmLf) as well as within the (alar plate) prethalamus. We will focus below on ventricularly located adult forebrain shh-GFP cells, and also consider peripherally migrated shh-GFP cells. All adult shh-GFP positive regions just mentioned are present in larval shh-GFP zebrafish brains in a less differentiated state as will also be discussed below (see sections 4.2–4.4).

However, we will start by discussing three more surprising findings of shh expression in the young adult zebrafish brain. First, all TH positive (i.e., dopamine) cells of the posterior tuberculum and many noncatecholaminergic preglomerular complex cells arise from diencephalic shh-GFP cells (see section 4.2). Second, a shh-GFP positive basal pallidal population is identified in the zebrafish brain which almost certainly corresponds to the ventral telencephalic shh signaling center known in amniotes to guide telencephalic dorsoventral development (sections 4.3.1–4.3.3). Third, a population of shh-GFP positive radial glia cells is newly detected in the adult zebrafish telencephalon (see section 4.3.4).

4.2 Analysis of tyrosine hydroxylase and shh-GFP suggests that posterior tubercular dopamine cells and pregromelular cells arise from shh-GFP cells

It is widely accepted that a continuum of dopamine (i.e., tyrosine hydroxylase expressing) cells extends in embryonic amniote brains from the midbrain floor into basal diencephalic territories up to P3 (Puelles & Verney, 1998; Vitalis, Cases, Engelkamp, Verney, & Price, 2000; Smeets & González, 2000; Böjörklund & Dunnett, 2007; Smidt & Burbach, 2007; Smits, Burbach, & Smidt, 2006; Smits, von Oertel, Hoekstra, Burbach, & Smidt, 2013; review in Wullimann, 2014). These mesodiencephalic dopamine cells will form the adult substantia nigra pars compacta (SN) and the ventral and lateral segmental area (VTA/LTA) classically interpreted to be solely mesencephalic (Nieuwenhuys, 1985). Embryonically, these dopamine cells are described to arise mostly from midbrain floor plate (Verney, 1999).

Moreover, HNF3β, a floor plate marker induced by notochordal SHH (Echelard et al., 1993) is also seen in dopamine neurons of SN/VTA and, importantly, of basal diencephalon, as HNF3β coincides with TH expression there and the same is seen in adults (Thuret, Bhatt, O’Leary, & Simon, 2004). Furthermore, it has been demonstrated that mesodiencephalic SN/VTA dopamine cells arise from the ventralmost neuroepithelium positive only for shh (and not for Nkx2.2, a more dorsally longitudinally expressed marker gene; Puelles et al., 2004).

Finally, fate studies completed the picture by clearly showing that all divisions of the mesodiencephalic midbrain dopamine cells arise directly from shh expressing cells (Blaess et al., 2011; Hayes et al., 2011; Joksimovic et al., 2009).

Our findings in transgenic shh-GFP zebrafish brains reveal a striking parallel to this midbrain floor plate and ventral diencephalic shh cell origin of SN/VTA dopamine cells in amniotes. Teleosts lack midbrain dopamine cells, but exhibit such cells in the ventral diencephalon (posterior tuberculum; reviewed in Smeets & Reiner, 1994a, 1994b; Smeets & González, 2000; Wullimann, 2014). These ventral diencephalic dopamine cells include projection cells to the teleostean striatum (Mueller et al., 2008; Rink & Wullimann, 2001). Our shh-GFP data show that among all dopamine neurons in the adult zebrafish brain, exclusively those in the posterior tuberculum are shh-GFP positive (compare Figure 4 and Table 2). This is the case for the parvocellular (TPp-p) and magnocellular (TPp-m; i.e., the striatal projection neurons) nuclei of the periventricular posterior tuberculum and for the paraventricular organ (PVO), strongly suggesting that these dopaminergic posterior tubercular cells are direct derivatives of ventral diencephalic shh cells. Furthermore, the fourth posterior tubercular dopaminergic nucleus, the posterior tuberal nucleus (PTN), does also coexpress on the cellular level shh-GFP and TH at larval stages (although it loses shh-GFP in the adult brain). Other zebrafish dopamine cells in prethalamus, pretectum, preoptic region, telencephalon, and likely also hypothalamic ones (but see below), do not show such a colocalization.

**FIGURE 4** Expression of shh-GFP and tyrosine hydroxylase in the adult zebrafish posterior tuberculum. Two consecutive and one more posterior transverse section are shown each for nuclear DAPI stain (a1,b1,c1), shh-GFP (a2,b2,c2) and tyrosine hydroxylase (TH; a3,b3,c3) immunostains. Enlargements demonstrate neurons double-labeled for shh-GFP and TH in the parvocellular and magnocellular parts of the periventricular posterior tubercular nucleus (yellow arrows; TPp-p, TPp-m; a2’-a3’), as well as in the TPp-m and the paraventricular nucleus (PVO; yellow arrows; b2’-b3’), but such double-label is absent in the posterior tuberal nucleus (PTN) and the caudal periventricular hypothalamic zone (Hc; c1-c3). (b2’) Enlargement of shh-GFP signal in retinal projection field lateral to the periventricular preetectal nucleus. See text for details. Abbreviations: ATN, anterior tuberal nucleus; CM, corpus mamillare; CP, central posterior thalamic nucleus; DI, diffuse nucleus of lobi inferior; DIV, diencephalic ventricle; DP, dorsal posterior thalamic nucleus; fr, fasciculus retroflexus; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; pc, posterior commissure; PG, periglomerular complex; PPp, periventricular preetectum; PTN, posterior tuberal nucleus; PVO, paraventricular organ; TeO, optic tectum; TeV, tectal ventricle; TL, torus lateralis; TLo, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum; TPp-p, parvocellular part of TPp; TPp-m, magnocellular (pear-shaped) cell part of TPp [Color figure can be viewed at wileyonlinelibrary.com]
Thus, this coexpression of shh-GFP and TH in posterior tubercular nuclei is a striking developmental parallel to the amniote mesodiencephalic dopamine cells of SN/VTA and supports homology of the teleostean posterior tubercular striatal projecting neurons with the diencephalic portion of the amniote SN/VTA.

The finding that these posterior tubercular dopamine cells may derive directly from shh expressing cells is further consistent with the recently reported islet1-GFP patterns in the adult zebrafish brain (Baeuml et al., 2019). While islet1 is expressed in TPp-p, it is completely absent in TPp-m, PVO and PTN despite a strong larval shh
expression there, making it unlikely that dopamine cells there are induced there via islet1 expression. However, in Tpp-p, dopamine cells do colocalize with islet1-GFP (Baeuml et al., 2019) and with shh-GFP (this study) and, thus, these shh expressing cells might transform into dopamine cells which express islet1.

While dopamine cells are seen in the adult posterior tuberal nucleus (PTN) and caudal zone of periventricular hypothalamus (Hc), shh-GFP is absent there. However, as mentioned above already, larvae (Figure 9 (13dpf)) additionally show double-label of shh-GFP and TH in PTN. Although most cells in Hc were only single-labeled for either shh-GFP or TH (Figure 9) we cannot exclude double-label. Moreover, dopamine cells are not visualized by conventional antibodies in the intermediate nucleus of the periventricular zone of the dorsal periventricular hypothalamus and the anterior part of the caudal zone of the periventricular hypothalamus (Yamamoto et al., 2010, 2011). Therefore, an origin from shh expressing cells may be common for all divisions of zebrafish posterior tubercular and hypothalamic dopamine cells. Possibly, the exposure of shh-GFP fish to cyclopamine—which interferes with SHH function—would be a way to check on resulting absence of islet1-GFP cells and selective absence of certain dopamine groups (i.e., posterior tuberculum, but not prethalamicus or pretectum).

Finally, beyond these posterior tubercular dopamine groups, a second population of differentiated zebrafish brain neurons that expresses shh-GFP can clearly be identified in the preglomerular complex (Figure 5). This large migrated diencephalic area is specified for tel-estos and involved in processing ascending sensory information (reviews: Wullimann, 1998, Wullimann & Mueller, 2004; Wullimann & Grothe, 2013). It contains neither monoaminergic nor cholinergic cell bodies (review: Mueller & Wullimann, 2016) but contains few GABAergic and arguably many more glutamatergic cells (Mueller, Wullimann, & Guo, 2008; Mueller & Wullimann, 2016). The preglomerular shh-GFP cells are mostly located in the more anterior division of the PG and seem to have migrated out along radial fibers from the periventricular posterior tuberculum. Thus, the posterior tubercular dopamine cells together with these preglomerular neurons represent two clear cases in the zebrafish forebrain for which a direct origin from shh expressing cells is suggestive. In the adult zebrafish hypothalamus, we only see shh-GFP cells within the periventricular zones close to the ventricle and none in migrated areas. However, considerable numbers of mammalian hypothalamic (preoptic, tuberal, and mammillary) nuclei have been shown in elegant mouse brain fate studies to arise from Shh-expressing cells (Alvarez-Bolado, Paul, & Blaess, 2012).

FIGURE 5 Expression of shh-GFP in the preglomerular complex. Three transverse sections (levels shown in schema at bottom) from anterior (a) to caudal (c) preglomerular complex levels. Note that fibers (white arrows in b2) originating in posterior tuberculum (TP) extend towards the preglomerular complex (PG) and that numerous shh-GFP cell bodies in far peripherally migrated positions are contained in the PG as detailed in enlargements (a2’–c2’). Note that in these enlargements, the shh-GFP positive fibers in upper left corner are retinal fibers terminating in the optic tectum. See text for details. Abbreviations: fr, fasciculus retroflexus; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; LLN, lateral line nerves; mfb, medial forebrain bundle; Of, olfactory; PG, preglomerular complex; PGA, anterior nucleus of PG; PGI, lateral nucleus of PG; PPp, periventricular pretectum; prtf, pretectal retinal terminal field; PVO, paraventricular organ; SP, superficial pretectum; TeO, tectum opticum; Th, thalamus; TH, tuberal hypothalamus; TLa, lateral torus; Tpp, periventricular nucleus of posterior tuberculum; ZLI, zona limitans intrahalamic; I, olfactory nerve; II, optic nerve; IV, trochlear nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagal nerve [Color figure can be viewed at wileyonlinelibrary.com]

4.3 | Newly identified adult shh-GFP cells in zebrafish basal pallidal region (BP) and in pallial radial glia cells (Dm; pallial amygdala)

In tetrapods, as particularly well studied in amniotes, a basal telencephalic (pallidal) sonic hedgehog (shh) expressing center is known to be instrumental for correct basal ganglia development and telencephalic dorsoventral patterning in general (for citations see section 4.3.2 and 4.3.3). However, shh expression in the larval zebrafish telencephalon remains elusive in the literature. A fine spot of shh expression in the embryonic zebrafish telencephalon has sometimes been claimed (e.g., by Krauss et al., 1993, at 26 hr; their figure 2F; or Ertzer et al., 2007, at 42 hr; their figure 6A). However, Holzschuh et al. (2003), Ekker et al. (1995), and Strähle et al. (1996) all did not note a telencephalic shh expressing population, and it remained unclear in Hagemann and Scholpp (2012). Major reviews on the subject of a pallidal shh signaling center (e.g., Wilson & Rubenstein, 2000) only speak about amniotes. Thus, in the previous literature, there is no clear indication of a shh expression domain in the zebrafish ventral telencephalon. Therefore, we re-examined this issue in the established shh-GFP zebrafish line during larval stages between 2 and 13 days and in adult (3-month-old) brains. We definitely see no shh-GFP cell bodies in the telencephalon up to 13 dpf larvae. The most anterior shh-GFP cells of the larval zebrafish are located in the preoptic region, in line with in situ hybridization studies cited above.

However, in the early adult zebrafish brain, two additional telencephalic groups of shh-GFP cells are newly seen. The first is a most posterior basal subpallial (pallidal) population (BP) which somewhat extends into basal telencephalic ventral nuclei (supracommissural and postcommissural nuclei of the ventral telencephalon; Vs, Vp). The second population is represented by shh-GFP positive radial glial cells at the ventricular lining of the medial zone of the dorsal telencephalon (Dm, pallial amygdala; Portavella, Vargas, Torres, & Salas, 2002; Portavella, Torres, & Salas, 2004; Wullimann & Mueller, 2004; Lal et al., 2018).

4.3.1 | Basal pallidal shh-GFP population

We detect in early adult zebrafish brains in the intermediate area between the anterior parvo cellular preoptic nucleus (PPa) and the ventral telencephalic (subpallial) supracommissural (Vs)/posterior (Vp) nuclei an area which contains a distinct population of shh-GFP labeled cell bodies (BP; Figure 1). This area has remained
neuroanatomically uncharted in the Neuroanatomy of the Zebrafish Brain (see Wullimann et al., 1996; p. 40, cross-section 107). The shh-GFP cells continue somewhat into the basal posterior (Vp) and supracommissural (Vs) nuclei of the ventral telencephalon and maybe slightly even into the ventral part of the dorsal nucleus which represents the differentiated pallidum (Vd; Mueller et al., 2008; Mueller & Wullimann, 2009). In any case, this shh-GFP population is a basal telencephalic domain located in the ventroposterior subpallium, that is, basal pallidum (BP). This is the first time that the telencephalic shh signaling center in the basal pallidum common to

FIGURE 6 Legend on next page.
all tetrapods has unequivocally been visualized in the zebrafish brain.

4.3.2 | The comparative and developmental significance of the basal pallidal shh domain

Expression studies of longitudinally expressed genes are fundamental for the understanding of the vertebrate neural tube ventralization process (Figure 10b,c). In a seminal paper, Shimamura, Hartigan, Martinez, Puelles, and Rubenstein (1995) conceptually summarized and extended information on amniote Shh and related expression studies (Echelard et al., 1993; Ericson, Muhr, Jessel, & Edlund, 1995; Ericson, Muhr, Placzek, et al., 1995; Lazzaro, Price, de Felice, & di Lauro, 1991; Marti, Takada, Bumcrot, Sakaki, & McMahon, 1995; Placzek, Jessell, & Dodd, 1993; Placzek, Tessier-Lavigne, Yamada, Jessell, & Dodd, 1990; Price et al., 1992; Roelink et al., 1994; Van Straaten, Hekking, Wiertz-Hoessels, Thors, & Drukker, 1988; Yamada, Pfaff, Edlund, & Jessell, 1993) and described Shh expression in floor plate of spinal cord and hindbrain and its lateral expansion into the basal plate of the midbrain.

| TABLE 2 | Adult zebrafish brain nuclei with shh-GFP with/without tyrosine hydroxylase (TH). [Color table can be viewed at wileyonlinelibrary.com] |
| Structure | shh-GFP | TH | Colocalization shh-GFP/TH |
| Medial zone of dorsal telencephalon (Dm) (radial glia) | (+) | – | N.A. |
| Basal supracommisural nucleus of ventral telencephalon (Vs) (midline) | (+) | – | N.A. |
| Caudal subpallium (midline) | + | – | N.A. |
| Anterior parvocellular preoptic nucleus (PPa) | + | + | No |
| Posterior parvocellular preoptic nucleus (PPp) | – | + | N.A. |
| Magnocellular preoptic nucleus (PM) | – | (+) | N.A. |
| Suprachiasmatic nucleus (SC) | + | (+) | No |
| Zona limitans intrathalamic | + | – | N.A. |
| Ventral thalamus (VT,–Zona incerta) | + | + | No |
| Small cells of periventricular posterior tubercular nucleus (TPp-p) | + 1 | + | Yes |
| Large cells of periventricular posterior tubercular nucleus (TPp-m) | + | + | Yes |
| Paraventricular organ (PVO) | + | + | Yes |
| Posterior tuberal nucleus (PTN)a | +a | +a | Yes |
| Nucleus of medial longitudinal fascicle (Nmlf) | +2 | – | N.A. |
| Ventral zone of periventricular hypothalamus (Hv) | + | – | N.A. |
| Dorsal zone of periventricular hypothalamus (Hd) | + | – | N.A. |
| Intermediate hypothalamic nucleus (IN) | + | –b | N.A. |
| Posterior part of caudal zone of periventricular hypothalamus (Hc)a | +a | +a | May be |
| Locus coeruleus (LC) | + | + | Yes |

Notes: (+) few cells. Red: Dopamine/noradrenaline systems with suggested direct origin from shh expressing cells (see text and Figure 4) with 1 representing only case for TH cells colocalized with islet1-GFP (see text). Blue: Potential shh- and islet1-GFP colocalization ruled out (see text and Figure 4) with 2 being (unlikely) exception.
aobserved only in 13d zebrafish brains, where shh-GFP and TH are colocalized in PTN cells and maybe in posterior Hc.
bNote that these cells express TH2—not visualized with TH antibodies—and contain dopamine (Yamamoto, Ruuskanen, Wullimann, & Vernier, 2011) and may thus potentially be double-labeled with shh-GFP.

FIGURE 6 | Floor plate shh-GFP and tyrosine hydroxylase expression in adult zebrafish brain. Transverse sections run from posterior diencephalon (a: Prosomere 1, nucleus of the medial longitudinal fascicle; Nmlf) through midbrain (b: Oculomotor nerve nucleus; Nl; the corresponding nerve III is surrounded with dashed line) and hindbrain (c: Level of interpeduncular nucleus; Nl; d: Level of locus coeruleus; LC; e: Posterior hindbrain; f: Level of area postrema: AP) down to spinal levels (g). Note that fibers of the shh-GFP floor plate cells extend towards the pial periphery where they form endfeet (EF). In order to allow for identification of the magnocellular periventricular posterior tubercular nucleus, its tyrosine hydroxylase positive cells are shown in inset in (a2). Note that some noradrenergic locus coeruleus cells colocalize with shh-GFP label (yellow arrows), but not other hindbrain catecholaminergic cells (see text for details). Abbreviations: AP, area postrema; CC, central canal; CM, corpus mammillare; DH, dorsal horn; EF, radial glia endfeet; FP, floor plate; Iv, funiculus ventralis; Hv/Hd, caudal/dorsal zone of periventricular hypothalamus; LC, locus coeruleus; Ilf, lateral longitudinal fascicle; MA, Mauthner axon; nlf, medial longitudinal fascicle; NlNd/NlNv, dorsal/ventral interpeduncular nucleus; Nmlf, nucleus of the medial longitudinal fascicle; NLV, nucleus lateralis valvulae; PG, preglomerular complex; PTN, posterior tuberal nucleus; RIV, rhombencephalic ventricle; SGN, secondary gustatory nucleus; T, midbrain tegmentum; TpP-m, magnocellular (pear-shaped) cell part of TpP; Va, valvula cerebelli; VH, ventral horn; III, oculomotor nerve; IIIl, oculomotor nerve nucleus [Color figure can be viewed at wileyonlinelibrary.com]
Anterior to the midbrain, a morphologically defined floor plate is no longer seen, but Shh is still expressed in basal plate diencephalon (P1-P3) through the hypothalamus up into the (alar) preoptic area. A vertebrate typical deviation from this longitudinal course is seen at the interface of thalamus (P2) and prethalamus (P3) where Shh extends in transverse direction dorsally (Figure 10c). All along its neuraxial course from spinal cord up to preoptic levels, the Shh expression domain is closely accompanied dorsally by a thinner expression stripe of the homeobox gene Nkx2.2 (Price et al., 1992; Qiu et al., 1998; Shimamura et al., 1995). The related Nkx2.1 gene is exclusively expressed in the forebrain, largely overlapping with Shh from P3 into hypothalamus and preoptic region (Figure 10c; Lazzaro et al., 1991; Shimamura et al., 1995; Kimura et al., 1996; Qiu et al., 1998). Furthermore, ventral forebrain Islet1 expressing cells coexpress Nkx2.1 (Ericson, Muhr, Placzek, et al., 1995) in contrast to posterior islet1 cells (where Nkx2.1 is not expressed). In lateral views, the basal telencephalon appears to form an upper floor of Shh expression. However, transverse views reveal that Shh has a continuous expression in the neural wall from the preoptic area (POA) into the so-called anterior entopeduncular area (AEP) which in turn continues dorsally into the most basal division of the medial ganglionic eminence (Figure 10B2; MGE, i.e., the future pallidum; Asbreuk et al., 2002; Bulfone et al.,

**FIGURE 7** Sagittal analysis: Overview of shh-GFP and tyrosine hydroxylase expression in adult zebrafish brain. Parasagittal section shown for shh-GFP immunostain (a1) and shown for nuclear DAPI stain (a2). Enlargements (frames a through d in a1) show telencephalon (a), posterior tuberculum (b1–b3), hypothalamus (c1–c2) and hindbrain (d) in these two stains plus tyrosine hydroxylase (TH) immunostains when appropriate. Note that all shh-GFP cells in pallium are radial glia cells at the wrinkled medial and dorsal surface of the medial zone of the dorsal telencephalon (compare with transverse sections in Figure 1). Yellow arrows: Colocalization of shh-GFP and TH in magnocellular cells of periventricular posterior tuberculum (TPp-m). See text for details. Abbreviations: ac, anterior commissure; BP, basal pallidum; CC, crista cerebellaris; CCe, corpus cerebelli; CM, corpus mamillare; DIL, diffuse nucleus of lobus inferior; Dm, medial zone of dorsal telencephalon; EF, endfeet; FL, facial lobe; FP, floor plate; Hv/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; LCe, lobus caudalis cerebelli; MON, medial octavolateralis nucleus; Nmlf, nucleus of the medial longitudinal fascicle; OB, olfactory bulb; pc, posterior commissure; PPa/p, anterior/posterior parvocellular preoptic nucleus; PPt, periventricular pretectum; PT, posterior tuberculum; PTh, prethalamus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; T, midbrain tegmentum; Tel, telencephalon; TeO, optic tectum; TeV, tectal ventricle; th, thalamus; TLo, torus longitudinalis; TPp-p, parvocellular cell part of periventricular posterior tubercular nucleus; TPp-m, magnocellular (pear-shaped) cell part of periventricular posterior tubercular nucleus; Va, valvula cerebelli; Vp/Vs, postcommissural/supracommissural nucleus of ventral telencephalon; ZLI, zona limitans intrathalamica [Color figure can be viewed at wileyonlinelibrary.com]
1993; Ghanem et al., 2007; Marín & Rubenstein, 2001; Marín, Anderson, & Rubenstein, 2000; Qiu, Shimamura, Sussel, Chen, & Rubenstein, 1998; Sussel, Marín, Kimura, & Rubenstein, 1999). The Nkx2.2 expression does not follow this telencephalic shh domain dorsally. However, the telencephalic Nkx2.1 expression domain overlaps with that of Shh and extends even further dorsally throughout the entire MGE (Figure 10c; Puelles et al., 2000; Shimamura et al., 1995).
The Nkx6.1 gene codes for another longitudinally expressed homeobox transcription factor dorsal to the Shh domain (Figure 10c; Qiu et al., 1998). From spinal cord, up to hindbrain the Nkx6.1 stripe overlaps with that of Nkx2.2, but extends more dorsally than the latter. Many motoneurons coexpress Nkx6.1 and Islet1. In the midbrain and in P1/P2, Nkx6.1 overlaps with Shh expression, but Nkx2.2 expression is now dorsal to it. A thin double-positive Nkx6.1 and Nkx2.2 stripe extends from P3 into the hypothalamus, here dorsal to both shh and Nkx2.1 domains (Qiu et al., 1998). Expression of Nkx6.2 is similar to Nkx6.1 in hindbrain and midbrain, but absent in spinal cord.
and in P3 through hypothalamus (Qi et al., 1998). However, Nkx6.2 is again expressed in the most dorsal MGE (Fogarty et al., 2007).

The subpallial telencephalon includes the MGE (the future pallidum) and dorsal to it the lateral ganglionic eminence (LGE, the future striatum) and both are molecularly characterized by expression of homeodomain-containing genes such as various Dlx paralogs (i.e., Dlx1/2 and Dlx5/6; Panganiban & Rubenstein, 2002), Gsh2 and Islet1 genes, as well as the basic-Helix–Loop–Helix (bHLH) gene Ascl1 (mouse: Mash1), followed more dorsally by expression of pallial genes, such as Pax6, Enx, or Tbr genes, as well as the bHLH genes Ngn1/2 and Nrd (Figure 10B1,B2; Casarosa, Fode, & Guillemot, 1999; Corbin, Gaiano, Machold, Langston, & Fishell, 2000; Enl gland et al., 2005; Fode et al., 2000; Horton, Meredith, Richardson, & Johnson, 1999; Ma, Sommer, Cserjesi, & Anderson, 1997; Muzio et al., 2002; Osório, Mueller, Rétaux, Vernier, & Wullimann, 2010; Parras et al., 2002, 2004; Price et al., 1992; Puelles et al., 2000; Schuurmans & Guillemot, 2002; Sommer, Ma, & Anderson, 1996; Stoykova, Treichel, Hallonet, & Gruss, 2000; Toresson & Campbell, 2001; Toresson, Potter, & Campbell, 2000; Torii et al., 1999; Yun, Garel, Fischman, & Rubenstein, 2003). Islet1 expressing Dlx2 positive cells are dynamically emerging over time both in MGE and LGE (Toresson et al., 2000; Wang & Liu, 2001; Yu, Fotaki, Mason, & Price, 2009) with those of LGE developing into GABAergic striatal projection neurons and those of MGE into cholinergic striatal interneurons (Flames et al., 2007; Marín et al., 2000; Olsson, Björklund, & Campbell, 1998; Pliz et al., 2013; Stenman, Toresson, & Campbell, 2003).

Dlx 1/2 and Dlx5/6 genes have indispensable overlapping and sequential roles in the differentiation of GABAergic cells in all subpallial divisions (i.e., striatal LGE, pallidal MGE, and CGE, the caudal ganglionic eminence, the future subpallial amygdala; Panganiban & Rubenstein, 2002; Wonders & Anderson, 2006). However, some genes, such as Nkx2.1 (see above), Gsh1 (Corbin et al., 2000; Toresson & Campbell, 2001; Yung et al., 2003) and the LIM/homeodomain genes Lhx6 and Lhx7 as well as the homeobox gene Gbx1 are exclusively expressed in MGE and septum, but not in LGE (Asbreuk et al., 2002; Grigoriou, Tucker, Sharpe, & Pachnis, 1998; Marín et al., 2000; Stoykova et al., 2000). Nkx2.1 and Lhx6 are essential for tangential migration of MGE cells into LGE or cortex (Allfrangs, Liapi, & Parnavelas, 2004; Marín et al., 2000; Wonders & Anderson, 2006), whereas Lhx7 (synonymous to Lhx8; Zhao et al., 2003) has an additional role in conferring the transmitter phenotype to cholinergic-GABAergic striatal and basal forebrain neurons (Asbreuk et al., 2002; Fragkouli et al., 2005; Fragkouli, Pachnis, & Stylianopoulos, 2006; Manabe et al., 2005; Marín et al., 2000; Mori et al., 2004; Wonders & Anderson, 2006). Dlx1/2 genes act upstream of these MGE expressed genes, because—when mutant—the generation of all cortical GABAergic interneurons is suppressed (Anderson, Eisenstat, Shi, & Rubenstein, 1997; Nery, Corbin, & Fishell, 2003). While the MGE is the predominant provider of tangentially migrating interneurons into striatum (cholinergic) and cortex (GABAergic), the LGE and CGE also participate later in this tangential migration process. This follows from Nkx2.1 mutants where most cortical interneurons are gone, but some persist (Anderson, Marín, Horn, Jennings, & Rubenstein, 2001; Corbin, Nery, & Fishell, 2001; Nery et al., 2003; Nery, Fishell, & Corbin, 2002; Tamamaki, Fujimori, & Takaui, 1997; Wonders & Anderson, 2006). Regarding these cortical interneurons, the MGE and CGE (Lhx6 cells) provide somato-statin, parvalbumin or calbindin positive inhibitory interneurons which are physiologically different, whereas the dLGE (which expresses Nkx6.2) produces double calretinin/somatostatin positive ones (Butt et al., 2005; Fogarty et al., 2007; Nery et al., 2002; Wonders & Anderson, 2006). The CGE is heterogeneous and also provides calretinin cells, originating from its Nkx2.1/Lhx6 negative domain as these interneurons remain present in Nkx2.1 mutants (Nery et al., 2002; Xu, Cobos, De La Cruz, Rubenstein, & Anderson, 2004). The POA and AEP share much of pallidal-type gene expression (Ascl1, Gsh1/2, Dlx1/2/5/6, Lhx6/7) (Asbreuk et al., 2002).

Two conclusions follow from these studies. First, the differential ventrodorsal gene expression patterns along the neuraxis share similarities into the telencephalon (e.g., Shh; nkh genes) suggesting ventrodorsal induction also there. Second, local differences in longitudinal gene expression (e.g., Nkox2.1 vs. Nkox6.1) suggest that the mechanisms of induction of ventral phenotypes along the anteroposterior axis differ (Balaskas et al., 2012; Litingtung & Chiang, 2000; Placek & Briscoe, 2005). We will focus on data directly relevant to forebrain ventralization, in particular, the telencephalon, for which we report new results in the zebrafish.

4.3.3 | What is the role of SHH in telencephalic gene expression induction and repression?

The differential transcription factor expression described above for amniotes presents a distinctly nested anteroposterior and ventrodorsal

FIGURE 9 Expression of shh-GFP and tyrosine hydroxylase in the late larval/juvenile (13d) zebrafish brain. Transverse sections run from alar diencephalon (a; pretectum/thalamus/prethalamus), through posterior tuberculum (b), into three levels of hypothalamus (c–e). Designations and Arabic numbers are used as established for larval zebrafish brain by Rink and Wullimann (2002). White arrows in (b1) point to tectal ventricle. Note that unlike in the adult zebrafish brain, the posterior tuberal nucleus (PTN) contains shh-GFP cells, which colocalize with tyrosine hydroxylase (TH) and, maybe also the caudal zona of the periventricular hypothalamus (Hc, around posterior recess). In magnifications shown in most right column, single cells double-labeled for TH and shh-GFP are indicated by yellow arrows and labeled only for TH by white arrows. See text for details. Abbreviations: Cce, corpus cerebelli; EG, eminentia granularis; FP, floor plate; Hr/Hi/Hc, rostral/intermediate/caudal hypothalamus; LC, locus coeruleus; N, area of the nucleus of the medial longitudinal fascicle; NII, interpeduncular nucleus; oc, optic chiasma; PG, pregglomerular complex; Po, preoptic region; Pr, pretectum; PT, periventricular posterior tuberculum; PTH, prethalamus (O); PTH, posterior tuberal nucleus (d); T, midbrain tegmentum; TeO, optic tectum; Th, (dorsal) thalamus; Va, valvula cerebelli; ZLI, zona limitans intrathalamica; 0, prethalamic dopamine cells (zona incerta); 2, 4, anterior and posterior magnocellular (pear-shaped) dopamine cells of periventricular posterior tuberculum; 3, paraventricular organ dopamine cells; 6, posterior tuberal nucleus dopamine cells; 7, caudal hypothalamic dopamine cells [Color figure can be viewed at wileyonlinelibrary.com]
forebrain patterning. These patterns principally result from the combined spatiotemporally dynamic activity of inductive signals (morphogens) from various sources (signaling centers), followed by cross-regulatory interactions of homeodomain and bHLH gene activity (Campbell, 2003; Marín & Rubenstein, 2001; Schuurmans & Guillemot, 2002). The signaling centers include in addition to the ventral notochord/prechordal mesoderm and later ventral neural tube (SHH), also the anterior neural ridge (fibroblast growth factor, FGF8), the dorsal neural tube midline/cortical hem (bone morphogenetic protein 4-BMP4 and Wnt3a), the lateral mesoderm (retinoic acid, RA) and the transverse zona limitans intrathalamica (SHH) as

**FIGURE 10** Legend on next page.
well as more caudally the midbrain–hindbrain boundary (FGF8). The dor- 
sal BMP and Wnt signals have a dorsalizing effect (Lupo et al., 2006; 
Stort et al., 2006; but see BMP7 below) counteracted by ventralizing 
SHH effect (see above). This activity results in ventral forebrain neurones 
and, more caudally, in motor neurones both apparent by Isl1 expressing 
cells laterally adjacent to the longitudinal Shh domain (Ericson, Muhr, 
Jessel, et al., 1995; Ericson, Muhr, Placzek, et al., 1995). Except for the 
telencephalon, Shh expression precedes that of Isl1 (Echelard et al., 
1993; Ericson, Muhr, Placzek, et al., 1995). Neural plate explants from 
different anteroposterior levels exposed to Isl1-1 source are induced to 
coexpress different gene markers with Isl1 (Nkx2.1 only in forebrain, 
including telencephalon; LIM1 in basal diencephalon; SC1 and Nkx6.1 in 
midbrain, hindbrain, spinal cord; Roelink et al., 1994; Ericson, Muhr, 
Placzek, et al., 1995; Qiu et al., 1998). Additionally, non-Isl1 positive 
populations (such as serotonin and dopamine cells; Yamada, Placzek, 
Tanaka, Dodd, & Jessell, 1991; Hynes, Poulsen, Tessler-Lavigne, & 
Rosenthal, 1995) are induced by SHH in midbrain/hindbrain explants. 
Also, Nkx2.2 is induced in neural tube explants by SHH (Qiu et al., 1998), 
but not Nkx6.1 which is induced by additional signals from the notochord 
(Qiu et al., 1998).

Although various studies show that Nkx2.1 expression is induced in forebrain neural plate explants exposed to SHH (see above), the pallidal shh domain is downstream of Nkx2.1 (Qiu et al., 1998; Shim-
amura & Rubenstein, 1997). This follows from studies on Nkx2.1 mutants, 
where Shh expression is absent in MGE and hypothalamus 
(except for POA), but present from midbrain to spinal cord (Sussel et al., 1999). 
Thus, initial pallidal Nkx2.1 expression is induced by 
earlier SHH influence from prechordal mesoderm (Dale et al., 1997; 
Shimamura & Rubenstein, 1997) together with FGF8 from the ante-
rior neural ridge (Stort et al., 2006; Wonders & Anderson, 2006). Fur-
thermore, in the forebrain, high concentrations of SHH induce BMP7 
(from prechordal mesoderm) which in turn suppresses Nkx6.1 and pro-
motes Nkx2.1 expression (Anderson, Lawrence, Stottmann, Bachiller, & 
Klingensmith, 2002; Dale et al., 1997; Pera & Kessel, 1997; Qiu et al., 1998). 
Also, different types of tangentially migrating MGE neurones 
are dependent on differential SHH levels (Xu et al., 2010).

Beyond the early SHH influence on ventralization of the telen-
cephalon through Nkx2.1 (i.e., inducing MGE features), later various 
Dlx and Islet1/2 gene expression is also induced in LGE (Kohtz, Baker, 
Corte, & Fishell, 1998). For example, SHH induces different LGE cell 
populations to express singly or combined Dlx and Mash1 as well as 
Dlx and Islet1/2, with Isl1 always only in postmitotic cells (Kohtz 
et al., 2001). In contrast, the expression of certain dorsal (pallial) genes 
is prohibited by SHH in the subpallium. For example, Gsh2 expression 
in LGE/MGE is SHH dependent and has a particularly strong role for 
correct striatal development. In Gsh2 (but not in MGE-specific Gsh1) 
mutants, typical striatal gene expression is absent (Mash/Dlx/Islet1) 
and pallial genes (Pax6, Ngn1/2) expand ventrally into striatum (Corbin 
et al., 2000; Toresson & Campbell, 2001; Toresson, Potter, & Camp-
bell, 2000; Yun et al., 2003). Furthermore, in Shh mutants, pallial Emx1 
gene expression expands into the ventral (striatal) area (Chiang 
et al., 1996).

In addition to this information in amniotes, a similar situation is 
present in basal amniote saccoptrygians (frogs, salamanders, and
lungfishes) regarding forebrain expression patterns in general (Domínguez, González, & Moreno, 2014; Domínguez, Morona, González, & Moreno, 2013; González, Morona, Moreno, Bandin, & López, 2014; Medina, Brox, Legaz, García-López, & Puelles, 2005; Moreno & González, 2011), as well as for Shh (exhibiting a small basal pallial domain; Domínguez, González, & Moreno, 2010), Nkx2.1 (pallium; Domínguez, López, & Marín, 2002; González, López, Sánchez-Camacho, & Marín, 2002; Van den Akker, Brox, Puelles, Durston, & Medina, 2008; Moreno et al., 2018), Lhx2 (pallidum; Moreno, Bachy, Rétaux, & González, 2004)) and Islet1 (striatum/pallidum; Moreno, Domínguez, Rétaux, & González, 2008; Moreno et al., 2018), as well as regarding the process of tangential migration (Moreno, González, & Rétaux, 2008).

The basic message from these studies is that SHH (from mesodermal and ventral neural tube sources) has a pivotal role in ventralization of the tetrapod/sarcopterygian forebrain including the telencephalon. As mentioned above, an early pallidal shh domain is elusive in zebrafish. However, in a new finding of a zebrafish pallidal shh-GFP expression domain shows that this domain is also present in zebrafish. Its late developmental emergence, however, has prohibited a functional investigation (see section 1).

In teleosts, general forebrain expression patterns also agree with those in tetrapods, for example regarding pallial versus subpallial gene expression (reviewed in Wullimann, 2009; Mueller & Wullimann, 2009, 2016), as well as regarding the process of tangential migration (Mueller et al., 2008; Mueller, Vernier, & Wullimann, 2006). Two paralogs of Nkx2.1 (a/b) exist in zebrafish (Manoli & Driever, 2014; Rohr, Barth, Varga, & Wilson, 2001), with Nkx2.1b expressed in the embryonic/larval pallidum and Nkx2.1a in hypothalamus. This is confirmed by larval expression of both "pallial" genes Lhx6/7 (Menuet, Alunni, Joly, Jefferey, & Rétaux, 2007). Our new finding of a zebrafish pallidal shh-GFP expression domain shows that this domain is also present in zebrafish. Its late developmental emergence, however, has prohibited a functional investigation (see section 1).

In tetrapods, general forebrain expression patterns also agree with those in tetrapods, for example regarding pallial versus subpallial gene expression (reviewed in Wullimann, 2009; Mueller & Wullimann, 2009, 2016), as well as regarding the process of tangential migration (Mueller et al., 2008; Mueller, Vernier, & Wullimann, 2006). Two paralogs of Nkx2.1 (a/b) exist in zebrafish (Manoli & Driever, 2014; Rohr, Barth, Varga, & Wilson, 2001), with Nkx2.1b expressed in the embryonic/larval pallidum and Nkx2.1a in hypothalamus. This is confirmed by larval expression of both "pallial" genes Lhx6/7 which are expressed in a ventral subdivision of the dorsal nucleus of the zebrafish ventral telencephalon (Mueller et al., 2008) and by corresponding adult pallidal islet1 expression (Vdv; Baeuml et al., 2019). As discussed in this previous paper, we interpret the adult islet1 expression extending into Vdv as defining the zebrafish pallidum, as also applies to basal telencephalic Nkx2.1b domain, both of which are erroneously assigned to the ventral nucleus of the ventral telencephalon by Ganz et al. (2012).

4.3.4 | Pallial shh-GFP radial glia cells in medial zone of dorsal telencephalon (Dm)

In addition to the long-known basal longitudinal expression of shh in the mesodermal notochord/prechordal plate and floor plate/ventral forebrain (see above), evidence later arose in anniiotes for previously unknown early shh expression in dorsal (i.e., alar) CNS regions (Dahmne et al., 2001; Dahmne & Ruiz i Altaba, 1999; Kriegstein & Alvarez-Buylla, 2009; Ruiz i Altaba, Palma, & Dahmne, 2002) such as the mammalian isocortex, the superior colliculus, and the cerebellum. In the cerebellum, shh expressing Purkinje cells act in transit amplification in the external granular layer. In the early mammalian isocortex, shh expression was reported in radial glia cells (Wang, Hou, & Han, 2016) and other cortical cells in intermediate zone, subplate, and deep cortical plate cells (Radonicj et al., 2016). Also, shh is more strongly expressed in gyrencephalic species (primates) than lissencephalic mammalian brains (rodents) in the developing cortical ventricular zone and apparently plays a role in the multiplication of progenitors (outer radial glia/intermediate progenitors; Han, 2016).

We demonstrated recently that shh expressing cells are also found in the larval zebrafish optic tectum and cerebellum (Biechel et al., 2016), but no such cells are seen in the larval pallial telencephalon. However, here we show newly a shh-GFP expressing population in the adult zebrafish pallial telencephalon, that is, pallial radial glia cells. Various studies have shown that mitotic stem cells (radial glia) exist in the adult zebrafish telencephalon along the subpallial and pallial ventricular lining (e.g., Chapouton, Jagasia, & Bally-Cuif, 2007; Diotel et al., 2015; Kaslin, Ganz, & Brand, 2007; Lillessar, Stigloher, Tannhäuser, Wullimann, & Bally-Cuif, 2009; Lindsey, Darabie, & Tropepe, 2012; März, Schmidt, Rastegar, & Strähle, 2010; Than-Trong & Bally-Cuif, 2015). However, to the best of our knowledge, a role for shh has not been shown in adult telencephalic stem cells. Recently, zebrafish telencephalic stem cells were investigated with a transcriptomic approach (Cosacak et al., 2019) and shown to be organized into molecularly separable populations that are clearly closely correlated with earlier established neuroanatomical divisions (Wullimann et al., 1996). One of these stem cell populations is in the medial zone of the pallial telencephalon (Dm; considered the pallial amygdala; Portavella et al., 2002, 2004; Wullimann & Mueller, 2004; Lal et al., 2018) which is characterized by marker genes Pou3f1 and DmrtA2 (Cosacak et al., 2019). It appears that we show here with shh-GFP specifically this population of molecularly defined radial glia cells within the medial zone of the dorsal (pallial) telencephalon (Dm; Figure 1). Thus, while there is no support for an early role for shh in the developing zebrafish pallium in the literature, a later role for shh in the adult pallium is suggested by our finding of shh-GFP positive radial glia cells in Dm and their function will be interesting to be studied in the future in the context of the known continuing proliferative activity in the zebrafish pallium (see citations above).

4.4 | Analysis of shh-GFP in comparison to islet1-GFP suggests that most forebrain shh cells remain at the ventricle and are not integrated into parenchymal tissue

The analysis of transverse zebrafish brain sections reveals that shh-GFP positive cells are generally located close to the ventricular lining. This is evident for the floor plate cells of spinal cord and hindbrain which remain the only shh-GFP cells there also in the adult brain with the exception of a few locus coeruleus cells. Floor plate cells are also seen in the adult midbrain tegmentum (T; Figures 6 and 7), although in larvae many more cells appear to be present there compared to the
hindbrain (Figure 9; Biechl et al., 2016; Baeuml et al., 2019). A position close to the ventricle is also seen for most shh-GFP cells in the forebrain, starting with the most caudal ones in the basal plate of P1 which partly are clearly identifiable as floor plate cells (see above). However, there are a number of more migrated shh-GFP cells seen in this area of the medial longitudinal fascicle (NmIF). Such peripherally migrated shh-GFP positive cells become more abundant at the level of the ZLI and anterior to it in the area of the posterior tuberculum (Figure 2). Because dopamine cells in this area form various well-known brain nuclei, we consider sections additionally stained for tyrosine hydroxylase (TH) in detail in section 4.2. However, in the hypothalamus, shh-GFP cells remain again rather close to the ventricle and this is also true for the telencephalon (see above).

We have recently analyzed in detail the expression of islet1 using a transgenic islet1-GFP line (Baeuml et al., 2019) and since islet1 expressing cells are generally considered to be influenced by ventricularly located SHH secreting cells (see above), we looked at comparable levels of islet1-GFP and shh-GFP transverse zebrafish brain sections at 3 months and evaluated qualitatively their positions with respect to the ventricle. Clearly, at telencephalic and preoptic into hypothalamic levels, islet1-GFP cells are always located more peripherally remote from the ventricle than shh-GFP cells (see Figure 3, where dashed lines enclose more ventriculally located shh-GFP cells in Figure 3g through Figure 3l and exclude more migrated islet1-GFP cells in Figure 3a through Figure 3f). Thus, the zebrafish diencephalon is largely similar compared to the more posterior brain ventricularly located SHH secreting cells (see above), we looked at comparable levels of islet1-GFP and shh-GFP transverse zebrafish brain sections at 3 months and evaluated qualitatively their positions with respect to the ventricle. Clearly, at telencephalic and preoptic into hypothalamic levels, islet1-GFP cells are always located more peripherally remote from the ventricle than shh-GFP cells (see above). However, because TH is colocalized only with islet1-GFP (Baeuml et al., 2019), but never with shh-GFP (this study), these are not the same cells. Thus, generally shh-GFP and islet1-GFP label in adult zebrafish brain structures do not colocalize on the cellular level (for the only possible exception see section 3.2 and Table 2).

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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