Check for updates

Received: 1 December 2021 | Revised: 9 May 2022 | Accepted: 11 May 2022

DOI: 10.1002/cne.25351

RESEARCH ARTICLE

JCN RESEARCH IN SYSTEMS NEUROSCIENCE THE JOURNAL OF COMPARATIVE NEUROLOGY WILEY

A versatile transcription factor: Multiple roles of *orthopedia a* (*otpa*) beyond its restricted localization in dopaminergic systems of developing and adult zebrafish (*Danio rerio*) brains

Jaime Eugenin von Bernhardi[1,2] | Daniela Biechl[1] | Laura Miek[1] | Ulrich Herget[3] | Soojin Ryu[4,5] | Mario F. Wullimann[1,6]

[1]Faculty of Biology, Division of Neurobiology, Ludwig-Maximilians-Universität Munich, München, Bavaria, Germany

[2]The Solomon Snyder Department of Neuroscience, Johns Hopkins Univeristy, Baltimore, Maryland, USA

[3]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California, USA

[4]Living Systems Institute University of Exeter, Exeter, Devon, UK

[5]College of Medicine and Health, University of Exeter, Exeter, Devon, UK

[6]Max-Planck-Institute of Neurobiology, Planegg-Martinsried, Germany

Correspondence
Mario F. Wullimann, Max-Planck-Institute of Neurobiology, 82152 Planegg-Martinsried, Germany.
Email: wullimann@bio.lmu.de

Funding information
Ludwig-Maximilians Universität München (LMU Munich); Graduate School for Systemic Neurosciences (GSN)

Abstract

Many transcription factors boost neural development and differentiation in specific directions and serve for identifying similar or homologous structures across species. The expression of Orthopedia (Otp) is critical for the development of certain cell groups along the vertebrate neuraxis, for example, the medial amygdala or hypothalamic neurosecretory neurons. Therefore, the primary focus of the present study is the distribution of Orthopedia a (Otpa) in the larval and adult zebrafish (*Danio rerio*) brain. Since Otpa is also critical for the development of zebrafish basal diencephalic dopaminergic cells, colocalization of Otpa with the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) is studied. Cellular colocalization of Otpa and dopamine is only seen in magnocellular neurons of the periventricular posterior tubercular nucleus and in the posterior tuberal nucleus. Otpa-positive cells occur in many additional structures along the zebrafish neuraxis, from the secondary prosencephalon down to the hindbrain. Furthermore, Otpa expression is studied in *shh*-GFP and *islet1*-GFP transgenic zebrafish. Otpa-positive cells only express *shh* in dopaminergic magnocellular periventricular posterior tubercular cells, and only colocalize with *islet1*-GFP in the ventral zone and prerecess caudal periventricular hypothalamic zone and the perilemniscal nucleus. The scarcity of cellular colocalization of Otpa in *islet1*-GFP cells indicates that the Shh-*islet1* neurogenetic pathway is not active in most Otpa-expressing domains. Our analysis reveals detailed correspondences between mouse and zebrafish forebrain territories including the zebrafish intermediate nucleus of the ventral telencephalon and the mouse medial amygdala. The zebrafish preoptic Otpa-positive domain represents the neuropeptidergic supraopto-paraventricular region of all tetrapods. Otpa domains in the zebrafish basal plate hypothalamus suggest that the ventral periventricular hypothalamic zone corresponds to the *otp*-expressing basal hypothalamic tuberal field in the mouse. Furthermore, the mouse *otp* domain in the mammillary hypothalamus

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2022 The Authors. *The Journal of Comparative Neurology* published by Wiley Periodicals LLC.

compares partly to our Otpa-positive domain in the prerecess caudal periventricular hypothalamic zone (Hc-a).

KEYWORDS
catecholamines, dopamine, hypothalamus, islet1, medial amygdala, mesodiencephalic complex, orthopedia, preoptic region, sonic hedgehog, tyrosine hydroxylase, zebrafish

1 | INTRODUCTION

The transcription factor Orthopedia (Otp; Drosophila *orthopedia* gene, *otp*) has distinct expression domains along the entire neuraxis in the tetrapod vertebrate brain (Simeone et al., 1994; Wang & Lufkin, 2000; Caqueret et al., 2005; Bardet et al., 2008; Morales-Delgado et al., 2011; Puelles et al., 2012; Domínguez et al., 2015). In mouse, chick, turtle, frog, and axolotl, there is one expression domain in the telencephalon, and various ones in the hypothalamus, posterior tuberculum, and hindbrain, as well as in the spinal cord (Bardet et al., 2008). In the larval zebrafish *Danio rerio*, some *otp* expression domains have been well studied. A recent example is the neurosecretory oxytocinergic and vasopressinergic preoptic-hypothalamic supraopto-paraventricular (SPV) area (PO in Figure 1a; adapted from Herget et al., 2014; Del Giacco et al., 2008), where *otp* is critically involved in the proper development of these neuropeptidergic neurons. Another studied domain is the medial amygdala (amniotes: Bupesh et al., 2011; Medina et al., 2011; Abellán et al., 2013) or intermediate nucleus of area ventralis telencephali in teleosts (Vi; Figure 1b; adapted from Biechl et al., 2017). The Vi is the only telencephalic expression site of *otp* in teleosts and it was confirmed that the Vi receives olfactory bulb projections which relay input from olfactory epithelial microvillous and crypt cells to the Vi in the zebrafish (Biechl et al., 2017). In turn, the Vi projects to the tuberal hypothalamus (Biechl et al., 2017). Thus, the Vi clearly represents part of the accessory olfactory system which is observed as an anatomically separate system from the main olfactory system in land vertebrates and is apparently also present in teleosts, albeit morphologically less obvious (Biechl et al., 2017). A third *otp*-positive population is located in the zebrafish posterior tuberculum, namely in the periventricular nucleus of the posterior tuberculum (TPp; Figure 1c/c1). These posterior tubercular (PT) Otpa cells (Figure 1 d/d1) are dopaminergic, as opposed to the first two mentioned ones, and projections of these PT dopaminergic cells reach the subpallial telencephalon (i.e., striatum) and the spinal cord (Rink & Wullimann, 2001; Ryu et al., 2007; Tay et al., 2011).

In contrast, more posterior *otp*-expressing domains remain to be defined in greater neurobiological detail. The zebrafish has two paralogues, *otpa* and *otpb* (Wolf & Ryu, 2013; Fernandes et al., 2013; Herget et al., 2014). A survey of *otpa/b* expression domains in the larval zebrafish shows that, particularly, additional PT and hypothalamic (beyond preoptic ones), and rhombencephalic domains lack reliable neuroanatomical description (see Figure 1a,b for larval domains shown but not analyzed further in Herget et al., 2014). Furthermore, the role and neuroanatomy of zebrafish hindbrain *otp* domains has hardly been addressed satisfactorily.

Therefore, the primary focus of the present article is to demonstrate in the adult zebrafish brain the exact location of all Otpa-expressing domains. There is a striking continuity of Otpa expression in the adult zebrafish brain in the same general areas seen in primordial form in the zebrafish larval brain (for example, in the posterior tuberculum and hypothalamus, Figure 1c/c1). Adult zebrafish brain Otpa expression domains correspond well with larval domains and also with embryonic mouse *otp* expression domains (see Section 4).

The present analysis of Otpa domains in the fully differentiated adult zebrafish brain, therefore, reveals their definitive neuroanatomical allocations, which will allow for further focused functional investigation. We will show in the present contribution exactly which regions of the zebrafish posterior tuberculum and hypothalamus express Otpa and, in addition, define hindbrain Otpa domains. Otpa is the third in a series of three genes active in the zebrafish brain which we have studied with molecular neuroanatomical means, the previous ones being *sonic hedgehog* (Wullimann & Umeasalugo, 2020) and *islet1* (Baeuml et al., 2019). Therefore, we additionally made a survey of Otpa domains in the brains of transgenic *shh*-GFP and *islet1*-GFP zebrafish to check for possible cellular colocalization of Otpa with *shh*-GFP or with *islet1*-GFP.

A second main focus is the detailed analysis of adult Otpa colocalization with tyrosine hydroxylase (TH). Dopaminergic posterior tubercular projection cells express Otpa, and molecular genetic knockdown studies in the zebrafish have shown that TH groups in the posterior tuberculum (TPp-p, TPp-m, PTN; see Results) are diminished in the absence of *otp* function (Filippi et al., 2012). We wondered whether additional catecholaminergic cells in the zebrafish brain are also expressing Otpa. Therefore, we used confocal microscopy in addition to epifluorescence microscopy to demonstrate or refute the detailed cellular colocalization of Otpa in all adult catecholaminergic (dopaminergic and noradrenergic) zebrafish neuronal groups.

The exact roles of Otp in many brain areas beyond the medial amygdala, preoptic region, and posterior tuberculum projection cells have yet to be established. To this aim, the present article serves as a guide for future experiments in clearly defined *otp*-expressing domains to elucidate the role of Otp in these regions.

2 | MATERIALS AND METHODS

2.1 | Animals and cutting procedure

A total of 11 adult zebrafish (*D. rerio*) specimens (nine wild-type and two transgenic specimens, see Section 2.2) were used in the

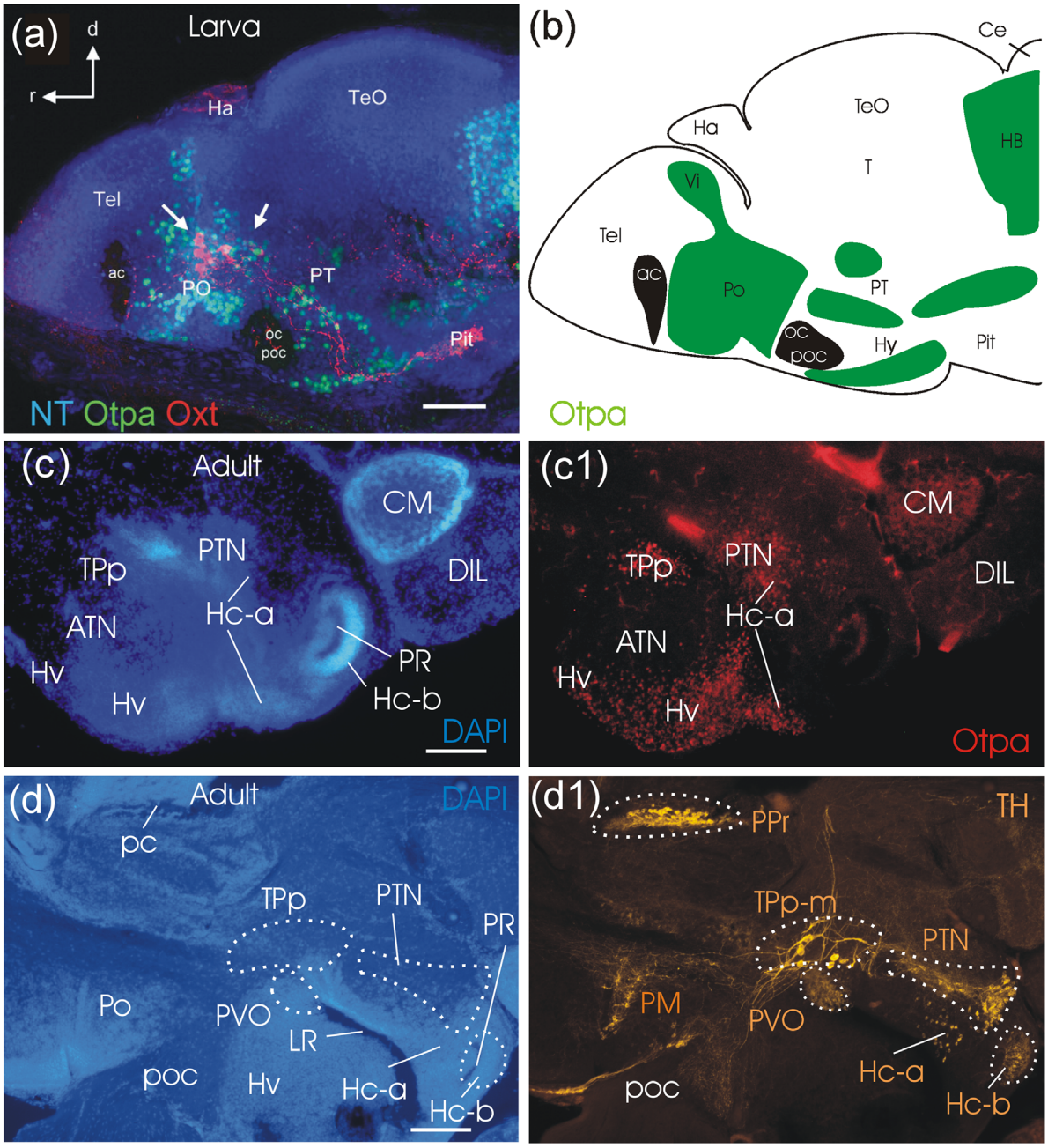

FIGURE 1 Sagittal overviews of the larval zebrafish brain (5 dpf; a,b) and the adult zebrafish basal diencephalon and hypothalamus (c-d). (a) is adapted from Herget et al. (2014) and highlights oxytocin cells (white arrows pointing to red cells) in the preoptic region among Otpa (green) immunopositive cells. (b) Drawing of this brain section shows additional Otpa domains (indicated in green, brain commissures in black), for example, in the intermediate nucleus of the ventral telencephalic area (Vi, interpreted as medial amygdala; adapted from Biechl et al., 2017), in the posterior tuberculum (PT), hypothalamus(Hy), and hindbrain (HB). (c/c1) shows a parasagittal adult section close to the midline stained for DAPI (c) and Otpa (c1) identifying some posterior tubercular and hypothalamic Otpa domains. (d/d1) shows a slightly more lateral parasagittal section (d; DAPI stained) highlighting posterior tubercular and hypothalamic catecholaminergic domains (d1; TH immunostained). Scale bars = 50 µm (a), 250 µm (c/d). Abbreviations: ac, anterior commissure; ATN, anterior tuberal nucleus; Ce, cerebellum; CM, corpus mamillare; d, dorsal; DIL, diffuse nucleus of hypothalamic inferior lobe; Ha, habenula; Hc-a, caudal zone of periventricular hypothalamus in front of a posterior recess; Hc-b, caudal zone of periventricular hypothalamus around posterior recess; Hv, ventral zone of periventricular hypothalamus; Hy, hypothalamus; LH, lateral hypothalamic nucleus; LR, lateral hypothalamic recess; NT, NeuroTrace; oc, optic chiasma; Oxt, oxytocin; pc, posterior commissure; Pit, pituitary; PM, magnocellular preoptic nucleus; Po (PO in panel A), preoptic region; poc, postoptic commissure; PPr, periventricular pretectum, PR, posterior hypothalamic recess; PT, posterior tuberculum; PTN, posterior tuberal nucleus; PVO, paraventricular organ; r, rostral; T, midbrain tegmentum; Tel, telencephalon; TeO, optic tectum; TPp, periventricular nucleus of posterior tuberculum; TPp-m, magnocellular cells of TPp; TPp-p, parvocellular cells of TPp; Vi, intermediate nucleus of ventral telencephalon

present study. Fish were maintained according to standard protocols (Westerfield, 2007). After cryoprotection in 30% sucrose solution at 4°C overnight, brains were embedded in TissueTek (tissue freezing medium; A. Hartenstein GmbH) and cryosectioned (Leica, CM 3050 S) at 30 µm. Sections were thaw mounted onto Superfrost Plus glass slides (Thermo) and coverslipped after immunostaining (see Section 2.3).

All procedures regarding live zebrafish followed EU guidelines and German legislation (EU directive 2010_63, license number AZ 325.1.53.56.-TU-BS). Transgenic and wild-type zebrafish used were killed with an overdose of tricaine methanesulfonate (MS-222) before fixation in paraformaldehyde (4% PFA in Sörensen's phosphate buffer, PB) at 4°C overnight. Raising and fixation of transgenic animals was done in Prof. Reinhard Köster's lab (Technical University Braunschweig, Germany) and kindly subsequently provided for this study. Therefore, we exclusively used fixed animal tissue in this study and needed no further approval.

2.2 | Transgenic lines

Some adult brains used stem from transgenic line specimens, that is, construct (ZFIN) *Tg*(*-2.4shha-ABC:GFP*) generated by Shkumatava et al. (2004); originally published as *Tg(2.2shh:gfp:ABC#15)*, and Tg(*isl1:GFP*) (ZFIN name: *Tg*(*isl1a:GFP*), generated by Higashijima et al. (2000), and will be referred to respectively as *shh*-GFP and *islet1*-GFP lines, and transgene expressing cells as *shh*-GFP and *islet1*-GFP cells below. For details on the characterization of these *shh*-GFP and *islet1*-GFP lines, see our two respective previous articles (Baeuml et al., 2019; Wullimann & Umeasalugo, 2020).

2.3 | Immunohistochemistry

Immunohistochemical incubations of cryosections were performed on the slide in a humid chamber. After washing off TissueTek with phosphate buffered saline (PBS), blocking buffer (2% normal goat serum, 2% bovine serum albumin, 0.2% Tween20, 0.2% TritonX-100 in PBS) was applied for 1 h at room temperature (RT). Then, a primary antibody (see Table 1 for list of antibodies and dilutions used) was applied (either anti-GFP, three specimens, or anti-TH, seven specimens; one sagittally cut additional specimen was only incubated with anti-Otpa) diluted in blocking buffer at 4°C for 1–3 days. After washing in PBT (PBS + 0.1% Tween20), cryosections were exposed to the appropriate secondary antibody (see Table 1), diluted in blocking buffer solution overnight at 4°C. After PBT washing and blocking buffer solution (1 h at RT; see above), a second primary antibody (against Otpa), followed by the appropriate secondary antibody—each applied overnight—with PBT washing and blocking buffer solution (1 h at RT; see above) in between. Finally, cryosections were washed in PBT and counterstained in addition with fluorescent DAPI (4'-6-diamino2-phenylindole; Carl Roth, 1:1000) and then washed in PBS before mounting 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 et al., 2010, 2011). The Otpa antibody has been created in the Driever laboratory (Ryu et al., 2007) and has been further characterized by Herget et al. (2014).

TABLE 1 Antibodies

Antibody against	Host	Company	Dilution
Green Fluorescent Protein (GFP)	Chicken	Aves Labs #GFP-1020 RRID:AB_10000240	1:500
Chicken IgY(-FITC)	Donkey, polyclonal	Dianova (Mol. Probes) #703-095155 RRID:AB_2340356	1:100
Tyrosine hydroxylase (TH)	Mouse, monoclonal	Millipore (AbCys), #MAB318 RRID:AB_2201528	1:100
Mouse IgG(-Alexa488)	Donkey, polyclonal	Molecular Probes, #21202 RRID:AB_141607	1:200
Orthopedia a (Otpa) Synthetic peptide CKKPVHPGDLAPVSDA*	Rat, polyclonal	Covance (USA) RRID:AB_2905490	1:500
Rat IgG(-Cy3)	Donkey, polyclonal	Dianova, #712-165-153 RRID:AB_2340667	1:200

**Note* that this sequence used for otpa antibody production occurs in the otpa sequence (described in Herget et al., 2014).

2.4 | Neuroanatomy

The neuroanatomical analysis and nomenclature is according to Wullimann et al. (1996) with updates described in Baeuml et al. (2019).

2.5 | Photography

Microphotographs of zebrafish brain cryosections were taken using a light or epifluorescence 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. Objectives used included Nikon Plan UW 0.06 (2×), Plan Fluor DIC L/N1, ∞/0.17, WD 16.0 (10×/0.30), and Plan Fluor DIC M/N2, ∞/0.17, WD 2.1 (20×/0.50). Further, a Leica TCS SP-5 confocal laser-scanning microscope (Leica Microsystems) was used, and microphotograph stacks of optical sections were processed using ImageJ. For final confocal pictures presented, between one and four single optical sections were used. Additionally, brightness and

contrast were slightly adapted using Corel-Photo-Paint and final photographic (and other) plates were generated using CorelDraw 12.0 (Corel Corporation).

3 | RESULTS

3.1 | Otpa expression in the larval zebrafish brain

Inspection of Otpa domains in the larval zebrafish brain previously revealed various additional Otpa domains beyond the well-investigated neurosecretory preoptic region (PO; Figure 1a, adapted from Herget et al., 2014). These additional domains include one in the subpallium (intermediate nucleus of the ventral telencephalon, Vi) and various posterior tubercular (PT), hypothalamic (Hy), and hindbrain (HB) domains (Figure 1b, adapted from Biechl et al., 2017). In the following, we examine adult zebrafish brains, which yield optimal neuroanatomical resolution for determining the exact location of Otpa domains. A sagittal zebrafish brain section stained for DAPI and Otpa shows an overview of posterior tubercular and hypothalamic domains (Figure 1c/c1) which will be analyzed below in detail in more informative transverse sections. This approach was already used in previous expression studies of *shh* (Wullimann & Umeasalugo, 2020) and *islet1* (Baeuml et al., 2019), two genes with predominant expression in the ventral zebrafish brain. Similarly, *otpa* is also expressed mostly in ventral brain areas and appears to show continued expression from larval into adult stages (Figure 1).

3.2 | Otpa domains in the adult zebrafish brain

An overview of adult zebrafish brain Otpa domains may be gained from epifluorescence microphotographs shown in Figures 2 through 5.

***Telencephalon* (*Vi*)**. The zebrafish telencephalon shows only one Otpa-positive domain, that is, the subpallial intermediate nucleus of the ventral telencephalon (Vi; Figure 2c/c1; Biechl et al., 2017; see Discussion for its identification as medial amygdala). A chain of loosely distributed Otpa-positive cells (Figure 2c1) extends between the (also strongly Otpa-positive) preoptic region and the Vi, indicating a migratory activity of Otpa-positive cells from the preoptic region into Vi, as similarly observed for the amniote medial amygdala (García-Moreno et al., 2010; Medina et al., 2011; Bupesh et al., 2011; Abellán et al., 2013; Morales et al., 2022; see Discussion in Gerlach & Wullimann, 2021).

***Preoptic region* (*PPa, PPp, PM, SC*)**. All zebrafish preoptic nuclei, that is, the anterior and posterior parvocellular (PPa, PPp) and magnocellular (PM, the latter includes some gigantocellular neurons, Figure 2c/c1, white arrow) preoptic nuclei, as well as the suprachiasmatic nucleus (SC; Figure 2b/b1, c/c1, d/d1; 3a/a1) contain cells which express Otpa.

***Ventral/Dorsal thalamus* (*VM, VL, A, DP, CP*)**. Zebrafish dorsal (A, DP, CP) and ventral thalamic nuclei (VM, VL; Figure 2d/d1; 3a/a1, b/b1) are free of Otpa cells.

***Periventricular pretectum* (*PPr*)**. Similarly, the periventricular pretectum (PPr; Figure 3a/a1, b/b1) does not contain Otpa-positive cells (Figures 3a, b and 6).

***Posterior tuberculum* (*TPp, PVO, PTN*)**. In contrast to zebrafish alar dorsal and ventral thalamic and pretectal nuclei, the nuclei of the posterior tuberculum, that is, the periventricular posterior tuberculum nucleus, including parvocellular and (pear-shaped) magnocellular neurons (TPp-p, TPp-m; Figure 3c/c1), as well as the posterior tuberal nucleus (PTN; Figure 3d/d1, e/e1), contain many Otpa-positive cells, but the paraventricular organ (PVO) does not (Figure 3c/c1; see also Figure 6).

***Hypothalamus* (*Hv, ATN, LH, Hd, IN, Hc*)**. The remainder of the zebrafish hypothalamus beyond the preoptic region contains many Otpa-positive cells in the ventral zone of the periventricular hypothalamus (Hv), as well as some Otpa-positive cells in the anterior tuberal and lateral hypothalamic nuclei (ATN, LH) (Figure 3a/a1-c/c1). The dorsal zone of the periventricular hypothalamus (Hd) lacks Otpa-positive cells, except for a distinct population in its intermediate hypothalamic nucleus (IN, Figure 3d/d1; see also Figure 7a-b; nucleus defined by Rink & Wullimann, 2001; see Discussion). The caudal zone of the periventricular hypothalamus exhibits a midline division (i.e., below: Hc-a: prerecess caudal periventricular hypothalamic zone; Figure 3d/d1; see also Figures 7b-d; 8d-d2) in front of the posterior recess ventricle. A second division of Hc (i.e., Hc-b) follows which extends laterally around the PR (Figure 3e/e1; see also Figure 8e-e2). Only the Hc-a, but not the Hc-b, contains Otpa-positive cells.

***Midbrain* (*PL, DTN*)**. Within the zebrafish midbrain, Otpa-positive cells are present in the perilemniscal nucleus (PL) and the dorsal tegmental nucleus (DTN) (Figure 4a/a1-c/c1). Large alar midbrain divisions, like the optic tectum, torus longitudinalis, and torus semicircularis (Figure 4a/a1-d/d1), are completely free of Otpa-positive cells.

***Hindbrain* (*NLV, GC, SRF, NIn, SR, IRF, MA, AP*)**. In the zebrafish hindbrain, Otpa domains occur in the nucleus lateralis valvulae (NLV, Figure 4b/b1, d/d1), the rim of the dorsal part of the interpeduncular nucleus (NIn; Figure 4d/d1), the central gray (GC; Figure 4d/d1-f/f1, 5a/a1), as well as the superior, intermediate (IMRF, not shown), and inferior reticular formation (SRF, IRF; Figures 4c/c1-f/f1; 5a/a1-c/c1) and the area postrema (AP) (Figure 5c/c1). Very few Otpa-positive cells are located in the superior raphe (Figure 5a/a1). The Mauthner axon and some large axons within the medial longitudinal fascicle (mlf) show Otpa stain. This is noteworthy because otherwise the Otpa stain typically covers the cell nucleus, as expected for a transcription factor. The DAPI stain confirms that there is no cell nucleus in the center of the stained large diameter axon (see inset in Figure 5c/c1). However, we could not identify in the Otpa and DAPI stainings the Mauthner cell body among the numerous Otpa-positive cells labeled dorsal to the reticular formation (including the IMRF where the Mauthner cell body lies; Wullimann et al., 1996). The Otpa cell populations of NLV, GC, and DTN apparently extend beyond the boundaries of these brain nuclei defined by Nissl or DAPI stains. Furthermore, all parts of the cerebellum are free of Otpa-positive cells. Finally, all Otpa-positive zebrafish brain populations are listed in Table 2.

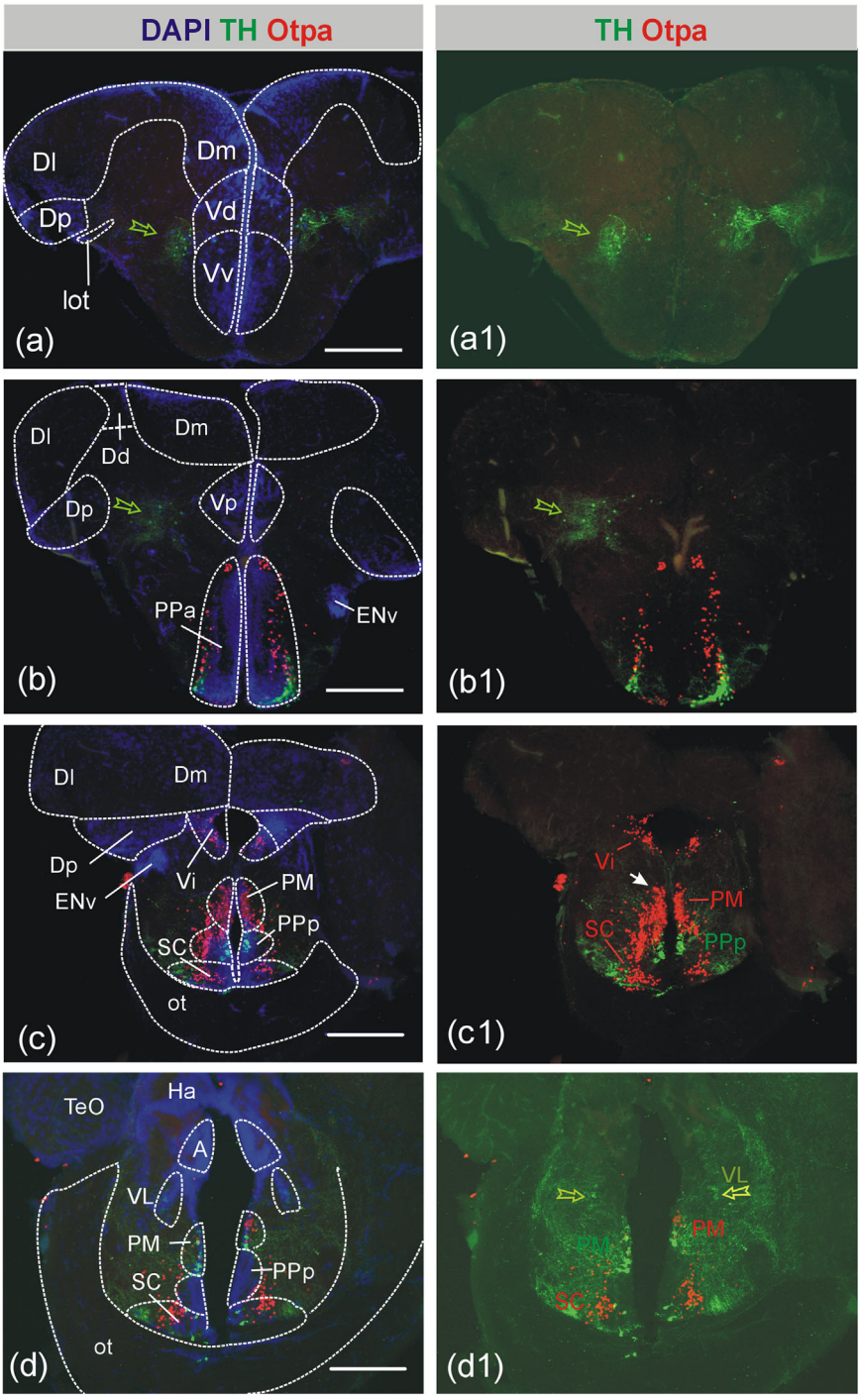

FIGURE 2 Distribution of catecholaminergic (TH) and Otpa-positive cells in the adult zebrafish forebrain I. Based on DAPI stains, epifluorescence microscopic analysis of immunostained transverse sections shows no cellular colocalization of TH and Otpa in these forebrain catecholaminergic sites, that is, in the ventral telencephalon (a/a1), in the preoptic area (b/b1-d/d1), or in the alar ventral thalamus (interpreted as zona incerta cells in the prethalamus, d/d1; Rink & Wullimann, 2002a). Open green arrows point to TH-positive cells, white arrow to gigantocellular preoptic neuron. Scale bars = 250 µm. Abbreviations: A, anterior thalamic nucleus; Dd/Dl/Dm/Dp, dorsal/lateral/medial/posterior zone of dorsal telencephalon; ENv, ventral entopeduncular nucleus; Ha, habenula; lot, lateral olfactory tract; ot, optic tract; PM, magnocellular preoptic nucleus; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; SC, suprachiasmatic nucleus; TeO, optic tectum; Vd/Vi/Vp/Vv, dorsal/intermediate/posterior/ventral nucleus of ventral telencephalon; VL, ventrolateral thalamic nucleus

3.3 | Comparison of catecholaminergic cells and Otpa domains in the adult zebrafish brain

A first overview of the adult distribution of tyrosine hydroxylase (TH, marker for catecholaminergic cells) in comparison to Otpa is provided by epifluorescence microphotographs (Figures 2 through 5). Additionally, confocal pictures were generated (Figures 6 through 8) in order to reject or confirm cellular colocalization in various brain nuclei which asked for scrutinized inspection.

Telencephalon. The zebrafish telencephalic TH-positive cell populations somewhat lateral to the subpallial nuclei (open green arrows in Figure 2a/a1, b/b1) are remote from the only Otpa-positive telencephalic structure, that is, the intermediate nucleus of the ventral telencephalon (Vi; Figure 2c/c1).

Preoptic region. Like Otpa, TH is also present in all zebrafish preoptic nuclei, but they are never cellularly colocalized (Figures 2b/b1-c/c2; 3a/a1). Otpa- and TH-positive cells form nonoverlapping clusters within each of the preoptic nuclei. This is confirmed in confocal pictures (e.g., white arrows in Figure 8a-a2).

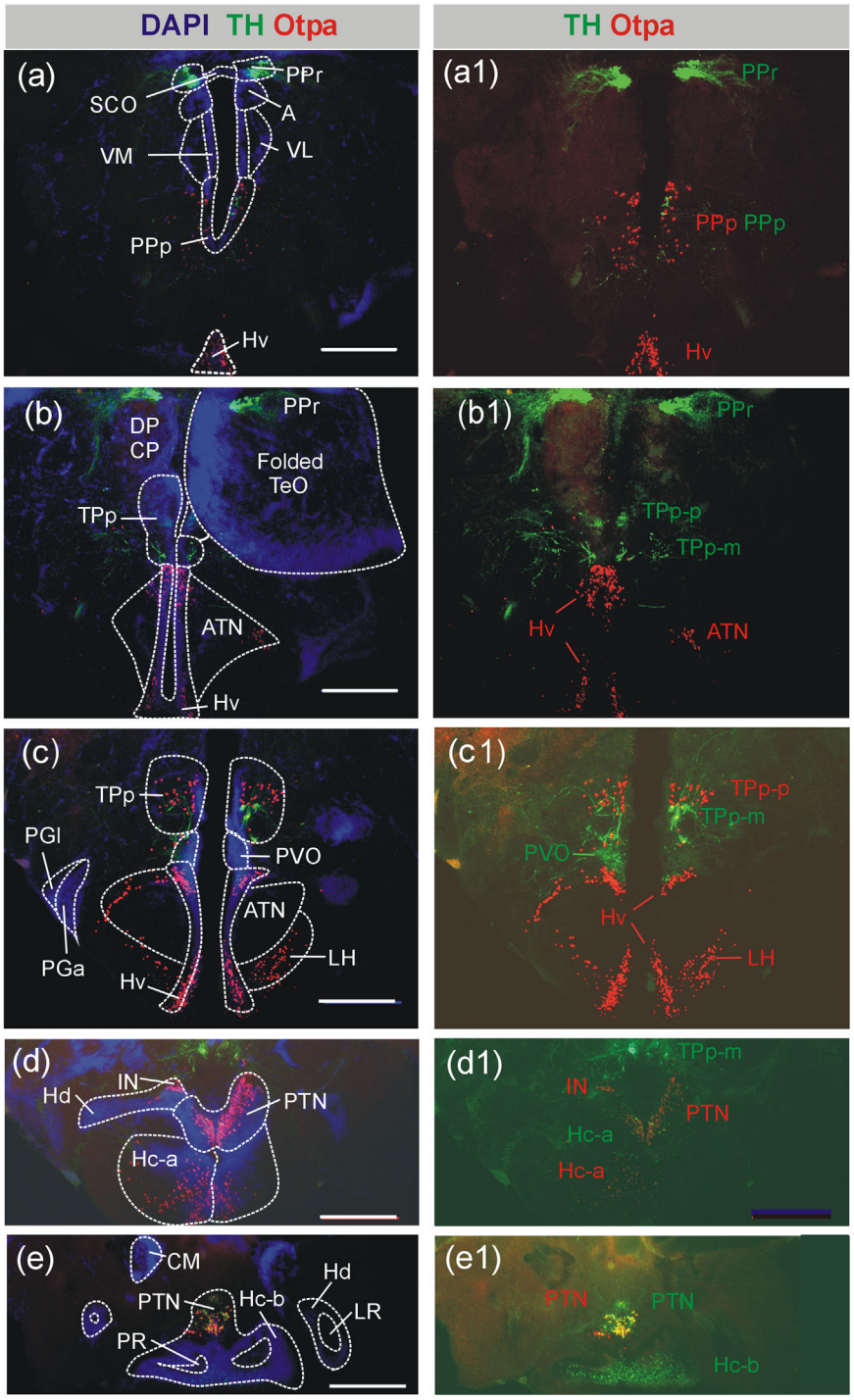

FIGURE 3 Distribution of catecholaminergic (TH) and Otpa-positive cells in the adult zebrafish forebrain II. Based on DAPI stains, epifluorescence microscopic analysis of immunostained transverse sections shows no cellular colocalization of TH and Otpa in most of these catecholaminergic forebrain sites, that is, neither in the periventricular pretectum (PPr; a/a1-b/b1), the paraventricular organ (PVO; c,c1), the small-celled (parvocellular) part of the periventricular posterior tuberculum (TPp-p; c/c1), nor in the caudal zone of the periventricular hypothalamus (Hc-a/b; d/d1–e/e1). Furthermore, the ventral zone of the periventricular hypothalamus (Hv), and the anterior tuberal and lateral hypothalamic nuclei (ATN, LH) as well as the intermediate hypothalamic nucleus only contain Otpa-positive cells. However, cellular colocalization of Otpa and TH is seen in the pear-shaped magnocellular part of the posterior tuberculum (TPp-m; b/b1) and the posterior tuberal nucleus (PTN; see also Figures 7; 8). Scale bars = 250 µm. Abbreviations: A, anterior thalamic nucleus; ATN, anterior tuberal nucleus; CM, corpus mamillare; CP, central posterior thalamic nucleus; DP, dorsal posterior thalamic nucleus; Hc-a, caudal zone of periventricular hypothalamus in front of posterior recess, Hc-b, caudal zone of periventricular hypothalamus around posterior recess; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; LH, lateral hypothalamic nucleus; LR, lateral hypothalamic recess; PGl/PGm, lateral/medial preglomerular nucleus; PPp, posterior parvocellular preoptic nucleus; PPr, periventricular pretectum; PR, posterior hypothalamic recess; PTN, posterior tuberal nucleus; PVO, paraventricular organ; TPp, periventricular nucleus of posterior tuberculum; SCO, subcommissural organ; VL/VM, ventrolateral/ventromedial thalamic nucleus

Ventral/Dorsal thalamus. The catecholaminergic TH-positive cells in the zebrafish ventral thalamus (Figure 2d1, open green arrows; zona incerta homolog) are remote from the basally located Otpa-positive cells in the preoptic region (Figure 2d/d1). There are no further catecholaminergic cells in the ventral and dorsal thalamus (Figures 3a,b; 6–8a).

Periventricular pretectum. As mentioned already, the (TH-positive) periventricular pretectum (PPr) does not exhibit Otpa cells at all (Figures 3a, b; 6).

Posterior tuberculum. As previously described (see Section 4), catecholaminergic TH-positive cells are abundant in the zebrafish periventricular posterior tuberculum (TPp), both in the form of parvocellular and magnocellular (pear-shaped) projection cells (i.e., TPp-p, TPp-m; Figure 3b/b1-c/c1), bipolar liquor-contacting (cerebrospinal fluid-contacting) cells in the PVO (Figure 3c/c1), and small round cells in the PTN (Figure 3d/d1-e/e1). Confocal analysis reveals in more detail that catecholaminergic cells in the TPp-p and PVO are never Otpa-positive (Figures 6; 8c-c2). Of note, the ventricularly directed neurites

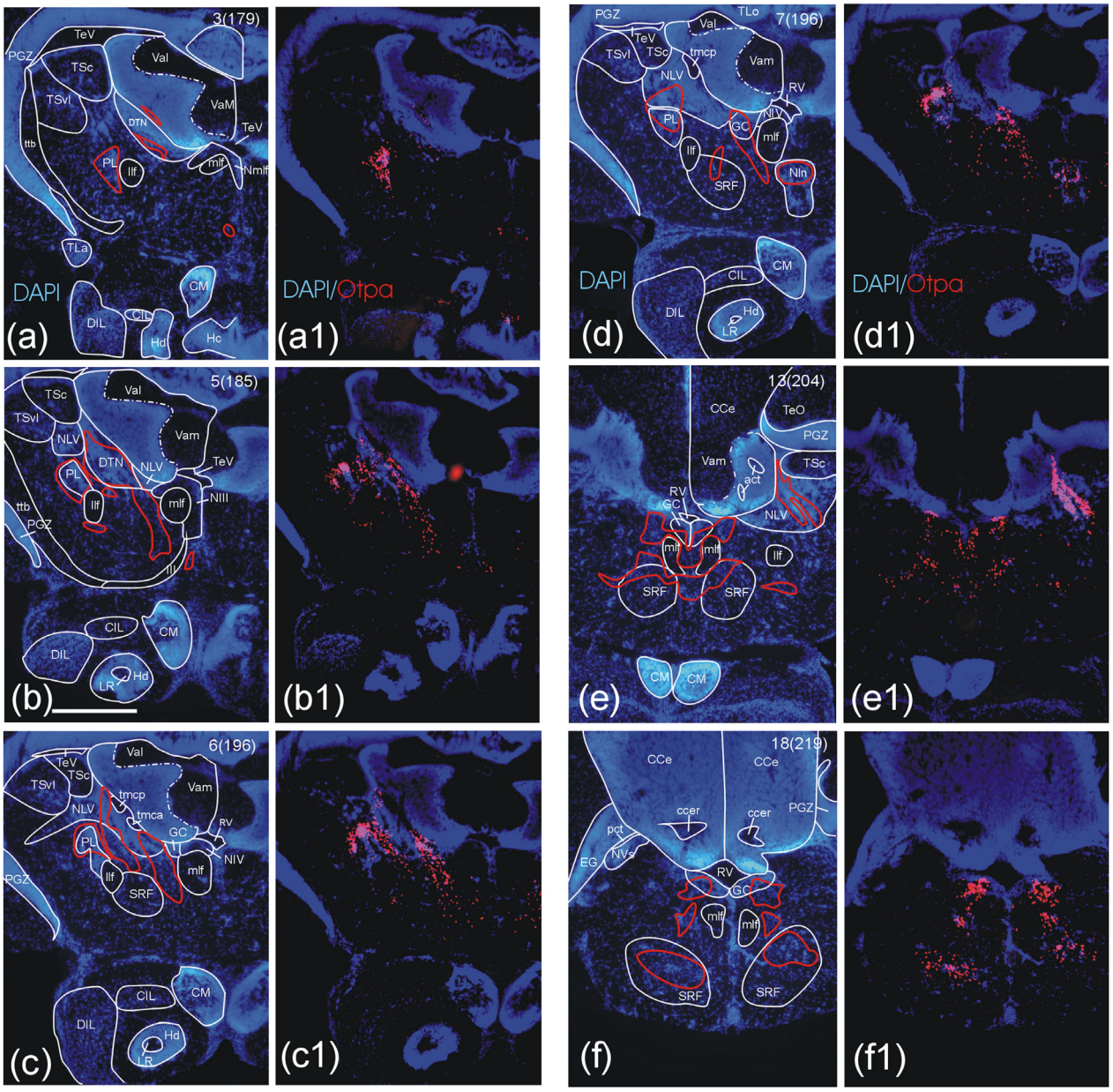

FIGURE 4 Distribution of Otpa-positive cells in the adult zebrafish midbrain and anterior hindbrain. Epifluorescent microscopic analysis shows Otpa domains in anterior midbrain and hindbrain regions. (a–f) A series of sequential transverse sections shows DAPI stain-based analysis of brain nuclei and tracts with red outlines indicating major Otpa-positive cell populations. Immunohistochemical detection of Otpa-positive cells is shown in the same sections in panels (a1) through (f1). Otpa-positive cells are seen in the perilemniscal (a-d) and the dorsal tegmental nucleus (a, b), in the nucleus lateralis valvulae (c-e), in the interpeduncular nucleus (d), as well as in the superior reticular formation (c-f) and the central gray (d-f; see text for details). Note that the Otpa immunostain does not adhere strictly to boundaries of brain nuclei as defined by the DAPI stain. Note complete absence of Otpa-positive cells in optic tectum (a), cerebellum (a-f), and dorsal/caudal zone of periventricular hypothalamus around lateral/posterior recess ventricle, respectively (a-c) and all nuclei of the inferior lobe (e.g., diffuse and central nuclei, corpus mamillare; a1-e1). Slide and section numbers are indicated in upper right corner for giving an estimate of relative anteroposterior distance between levels shown. Scale bar in B = 250 μm (applies to all panels). Abbreviations: act, anterior cerebellar tract; CCe, corpus cerebelli; ccer, cerebellar commissure; CIL, central nucleus of the hypothalamic inferior lobe; CM, corpus mamillare; DIL, diffuse nucleus of the hypothalamic inferior lobe; DTN, dorsal tegmental nucleus; EG, eminentia granularis; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; llf, lateral longitudinal fascicle; LR, lateral hypothalamic recess; mlf, medial longitudinal fascicle; NIII, oculomotor nucleus; NIV, trochlear motor nucleus; NIn, interpeduncular nucleus; NLV, nucleus lateralis valvulae; Nmlf, nucleus of mlf; NVs, primary sensory trigeminal nucleus; pct, posterior cerebellar tract; PGZ, periventricular gray zone of optic tectum; PL, perilemniscal nucleus; RV, rhombencephalic ventricle; TeV, tectal ventricle; TLo, torus longitudinalis; tmca/p, tractus mesencephalocerebellaris anterior/posterior; TSc, central nucleus of torus semicircularis; TSvl, ventrolateral nucleus of torus semicircularis; ttb, tractus tectobulbaris; Val, lateral division of valvula cerebelli; Vam, medial division of valvula cerebelli; Vas. vascular lacuna of area postrema, III oculomotor nerve

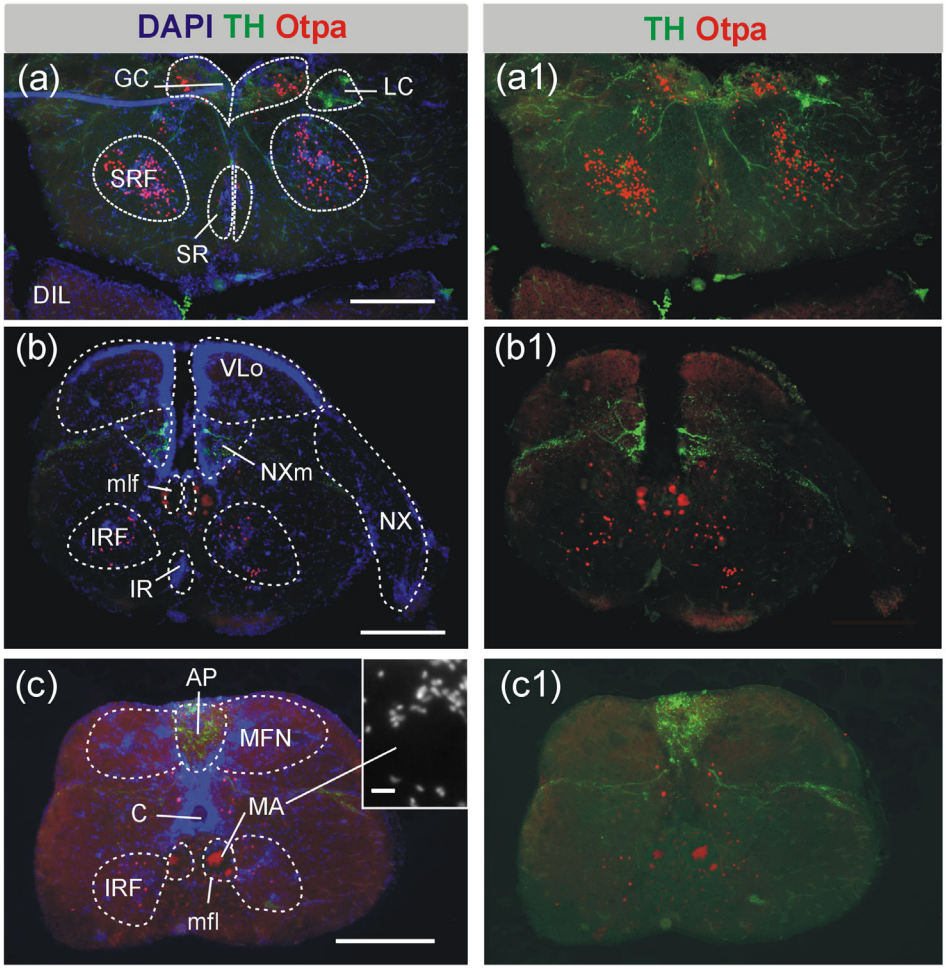

FIGURE 5 Distribution of catecholaminergic (TH) and Otpa-positive cells in the adult zebrafish posterior hindbrain. Based on DAPI stains, epifluorescence microscopic analysis of transverse sections shows no cellular colocalization in the locus coeruleus (a/a1) and in the vagal catecholamine group (b/b1). In the area postrema, catecholaminergic (TH-positive) and Otpa-positive cells intermingle, but with no apparent double-labeled cells (c/c1). Otpa-positive cells are furthermore seen in the central gray, superior reticular formation, and superior raphe (very few, a/a1), as well as in the inferior reticular formation (b/b1-c/c1). Scale bars = 250 μm (a-c), 10 μm, inset in (c). Abbreviations: AP, area postrema; C, central canal; DIL, diffuse nucleus of hypothalamic inferior lobe; GC, central gray; IRF, inferior reticular formation; LC, locus coeruleus; MA, Mauthner axon; MFN, medial funicular nucleus; mlf, medial longitudinal fascicle; NX, vagal nerve; NXm, vagal motor nucleus; SR, superior raphe; SRF, superior reticular formation; VLo, vagal lobe

of the PVO (but not of the TPp-p) catecholaminergic cells contact the ventricular lining (Figure 6a3). In contrast, many—but not all—catecholaminergic TPp-m and PTN cells are indeed double-labeled for Otpa and TH (Figures 7b-d; 8b-e).

Hypothalamus. Zebrafish catecholaminergic (TH-positive) cells were neither detected in the Otpa-positive ventral zone of the periventricular hypothalamus (Hv) or in the ATN and LH nuclei, nor in the intermediate hypothalamic nucleus (IN). However, the prerecess part of the caudal periventricular hypothalamic zone (Hc-a; Figures 3d/d1; 7b-d; 8d) shows many TH-positive cells dorsal to its Otpa-positive cells. Unlike in the adjacent PTN, the catecholaminergic cells in Hc-a are never cellularly colabeled with Otpa, and they are liquor-contacting bipolar cells (clearly visible in Figure 7b2-2, white arrow in right lower corner), not small round cells as in the PTN. Furthermore, TH is also absent in the Otpa-free hypothalamic structures, such as the dorsal zone of the periventricular hypothalamus (Hd), the corpus mamillare (CM), and the diffuse and central nuclei of the inferior lobe (DiL, CIL; Figure 4; but see Section 4 for both the possibility of colocalization of *th2* with *otpa* or *otpb* in Hd and IN and some other structures). Furthermore, the caudal periventricular hypothalamic zone lining the posterior recess (Hc-b) exhibits (ventrally only) bipolar liquor-contacting catecholaminergic (TH-positive) cells (Figures 3e1; 8e1), but the entire Hc-b is completely free of Otpa (Figures 3e1; 8e1).

Midbrain. There are no catecholaminergic cells in the zebrafish midbrain.

Hindbrain. The zebrafish hindbrain is mostly free of TH (Figures 4; 5). The catecholaminergic locus coeruleus (LC) cells, some TH-positive cells in the vagal motor nucleus (NXm) and in the AP all do not

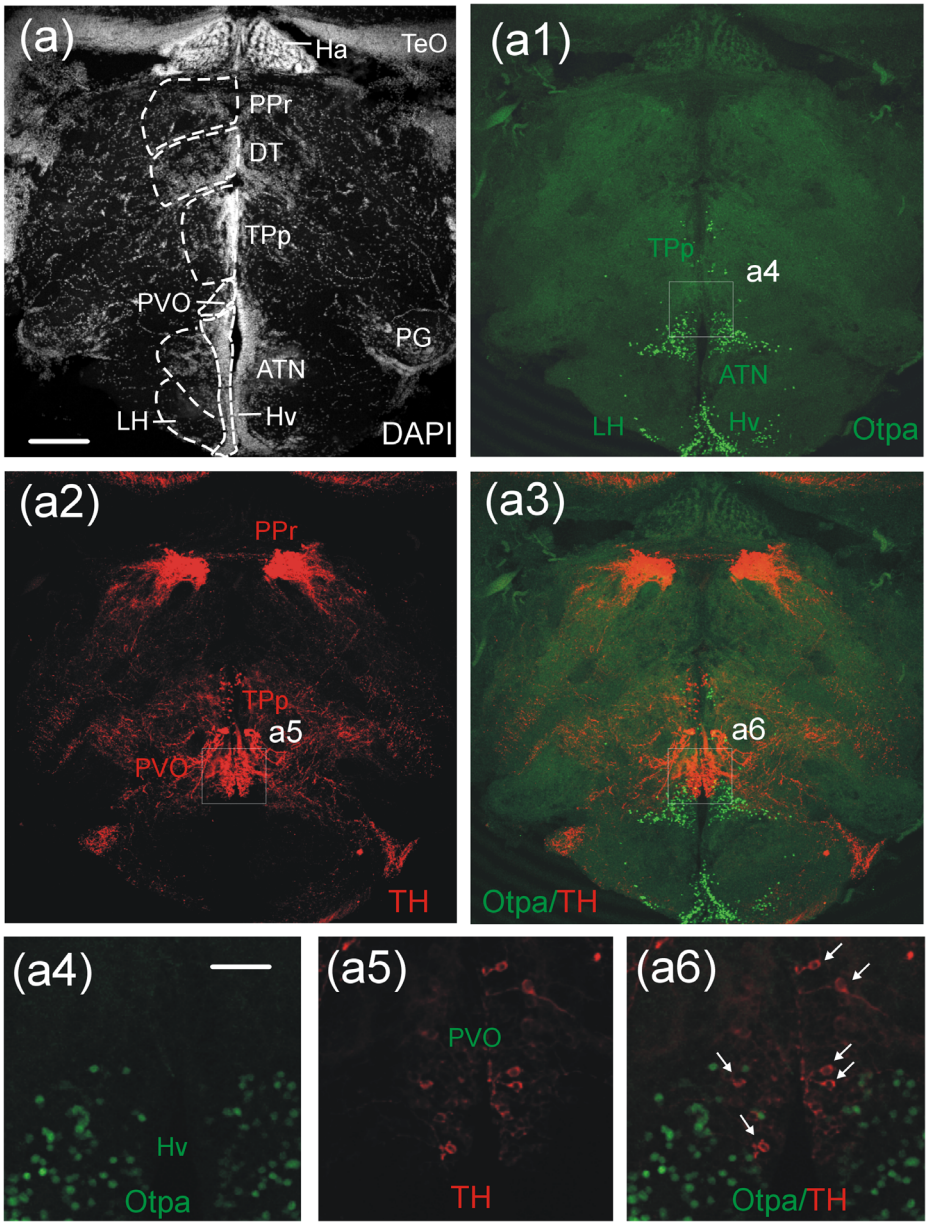

FIGURE 6 Confocal microscopic analysis of catecholaminergic (TH) and Otpa-positive cells in the adult zebrafish diencephalon at the level of the paraventricular organ. An ideal transverse section for displaying the periventricular pretectum (PPr), dorsal thalamus (DT), periventricular nucleus of the posterior tuberculum (TPp), ventral periventricular hypothalamic zone (Hv), as well as anterior tuberal and lateral hypothalamic nuclei (ATN/LH) is shown for DAPI stain (a), and Otpa (a1), TH (a2) and merged Otpa/TH immunostains (a3). Separate enlarged microphotographs show Otpa (a4), TH (a5) and merged Otpa/TH (a6) immunostained cells and demonstrate that no cellular colocalization occurs in the paraventricular organ (PVO; white arrows), as is obviously also the case for the periventricular nucleus of the posterior tuberculum (TPp), the periventricular pretectum (PPr), the ventral zone of the periventricular hypothalamus (Hv), and the anterior tuberal and lateral hypothalamic nuclei (ATN/LH). Scale bars = 250 μm (a), 125 μm (a4), applies also to (a5/a6). Abbreviations: ATN, anterior tuberal nucleus; DT ,dorsal thalamus; Ha, habenula; Hv, ventral zone of periventricular hypothalamus; LH, lateral hypothalamic nucleus; PG, preglomerular complex; PPr, periventricular pretectum; PVO, paraventricular organ; TeO, optic tectum; TPp, periventricular nucleus of posterior tuberculum

colocalize cellularly with the Otpa signal (Figure 5). All catecholaminergic zebrafish brain structures are listed in Table 2, as is the presence or absence of cellular colocalization with Otpa.

Summary of catecholaminergic and Otpa-positive brain structures in adult zebrafish. An overview of previously established catecholaminergic (i.e., noradrenergic and dopaminergic; see Figure 12a and Section 4) and Otpa-positive structures (Figure 12b) in the zebrafish brain reveals that the only two structures with cellular colocalization of Otpa and dopamine are magnocellular periventricular posterior tubercular cells (TPp-m) and cells in the posterior tuberal nucleus (PTN).

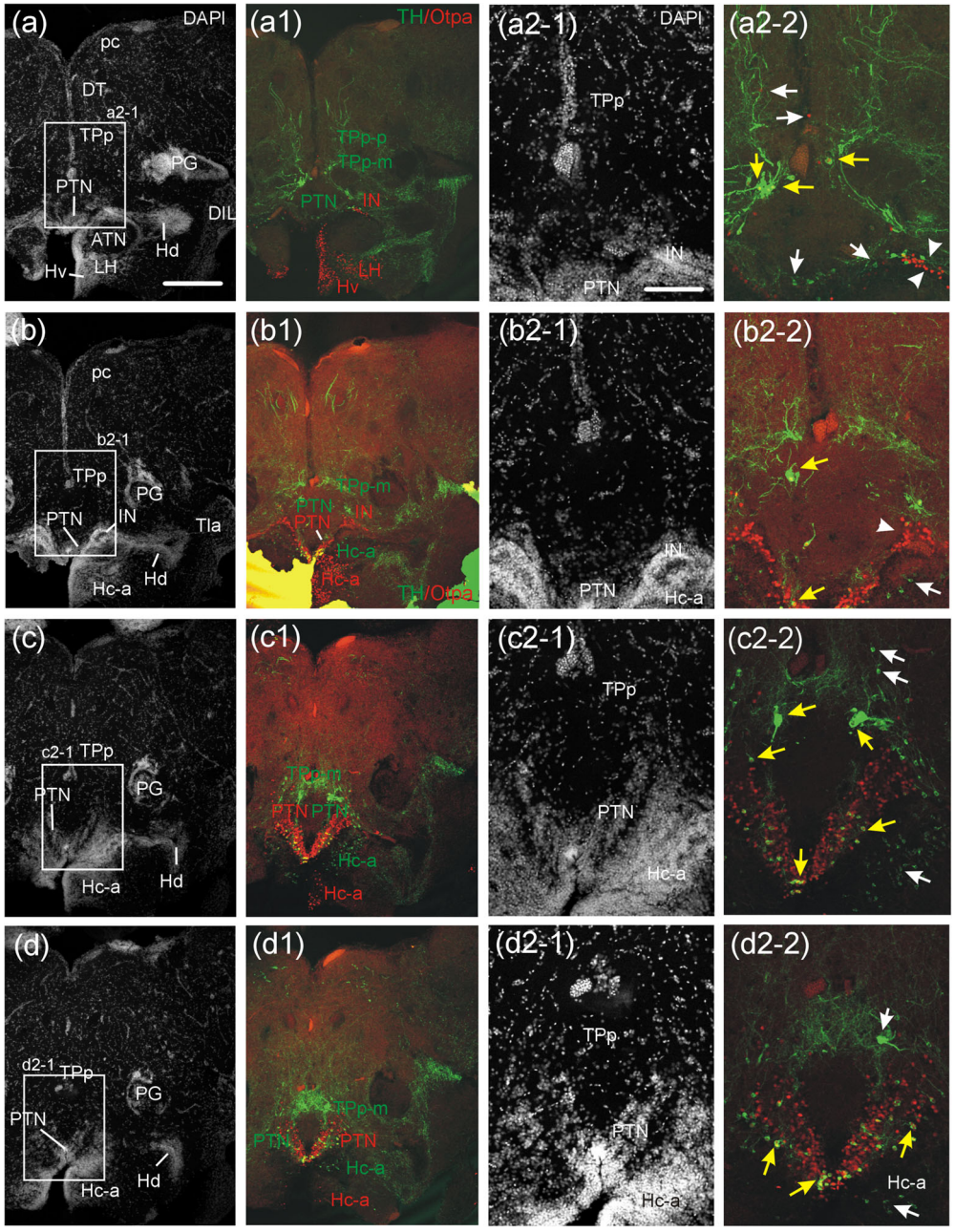

FIGURE 7 Confocal microscopic analysis of catecholaminergic (TH) and Otpa-positive cells in the adult zebrafish diencephalon at levels of the posterior tuberal nucleus, intermediate hypothalamic nucleus, as well as dorsal and prerecess caudal periventricular hypothalamic zones. Four transverse levels (a-d/a2-1 to d2-1): DAPI stain; (a1-d1/a2-2 to d2-2): merged TH/Otpa immunostain) cover the rostrocaudal extent of the posterior tuberal nucleus (PTN). Note that (a2-1/a2-2) to (d2-1/d2-2) are separate enlarged microphotographs. The PTN shows rostrally TH cells (two lower white arrows in a2-2) and few Otpa-positive cells. Increasingly more Otpa-positive cells in PTN occur caudally (b2-1/b2-2 to d2-1/d2-2) where also the majority of TH/Otpa double-labeled cells in PTN sit (respective yellow arrows in b2-2 to d2-2). These three levels also show presence of TH/Otpa colabeled magnocellular cells of the periventricular posterior tuberculum (TPp-m; a2-2 to c2-2; respective yellow arrows, white arrow in [d2-2] shows a TH-only positive TPp-m cell). The parvocellular TPp-p is not double-labeled (two upper white arrows in a2-2/c2-2). The intermediate hypothalamic nucleus (IN) is characterized by a distinct Otpa-positive cell cluster, two white arrowheads in (a2-2); white arrowhead in (b2-2). Some TH-positive cells of PTN sit at the medial edge of IN, one of them double-labeled with Otpa. The caudal periventricular hypothalamic zone in front of the posterior recess (Hc-a) contains many Otpa-positive cells basal to its TH-positive cells (b1-d1). TH-positive cells in Hc-a are indicated by a white arrow in the right lower corner of (b2-2 to d2-2), note their bipolar liquor-contacting nature in (b2-2). Scale bars = 250 μm (a) applies also to (b-d) and (a1-d1), 125 μm (a2-1) applies also to (b2-1 to d2-1) and (a2-2 to d2-2). Abbreviations: ATN, anterior tuberal nucleus; DIL, diffuse nucleus of hypothalamic inferior lobe; DT, dorsal thalamus; Hc-a, caudal zone of periventricular hypothalamus in front of posterior recess; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; LH, lateral hypothalamic nucleus; pc, posterior commissure; PG, preglomerular complex; PTN, posterior tuberal nucleus; TLa, torus lateralis; TPp, periventricular nucleus of posterior tuberculum; TPp-m, magnocellular cells of TPp; TPp-p, parvocellular cells of TPp

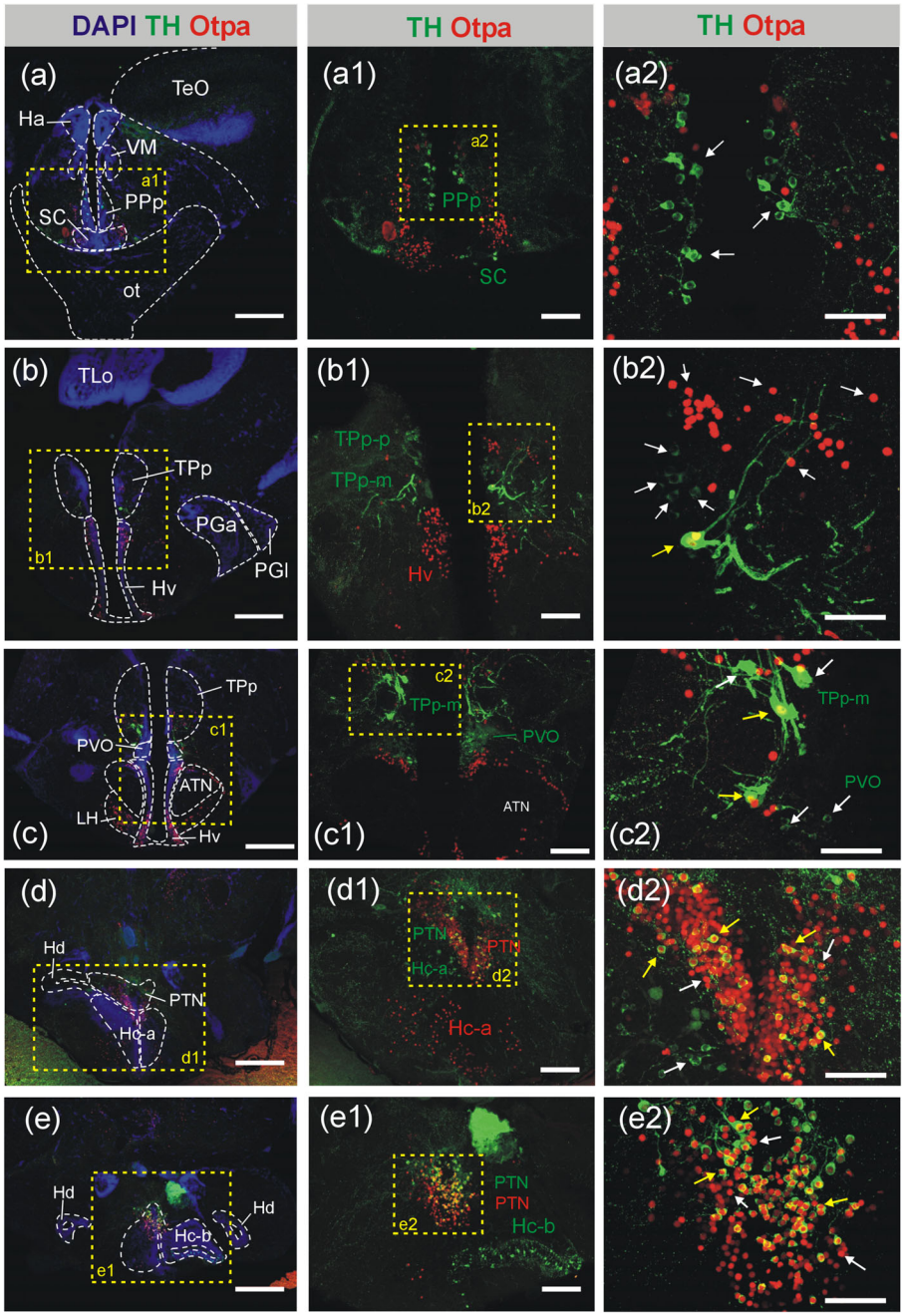

FIGURE 8 Confocal microscopic analysis of TH- and Otpa-positive cells in the adult zebrafish preoptic region and diencephalon at levels of the periventricular posterior tuberculum, posterior tuberal nucleus, and caudal periventricular hypothalamic zone. Note that a1/a2-e1/e2 are separate enlarged microphotographs of these transverse sections. The entire preoptic complex shows no cellular co-localization of TH and Otpa (a-b) as demonstrated for the posterior parvocellular preoptic (PPp) and suprachiasmatic nucleus (SC; a-a2; white arrows). Only the pear-shaped magnocellular part of the periventricular posterior tuberculum (TPp-m; b-b2/c-c2) shows cellular colocalization of TH and Otpa, but not the parvocellular nucleus of the periventricular posterior tuberculum (TPp-p), as demonstrated in magnifications (b1-c1/b2-c2, white and yellow arrows, respectively). Similarly, in the posterior tuberal nucleus (PTN) many cells show co-localization of TH and Otpa (d1-d2/e1-e2, white versus yellow arrows). Note complete absence of such overlap in the paraventricular organ (PVO; c1-c2) and the entire caudal hypothalamus (Hc; d1/e1), but TH immunostain in liquor-contacting cells of the ventral posterior recess lining of the Hc-b (e1). In both, TPp-m and PTN many–but not all—cells show colocalization of TH and Otpa. Scale bars = 250 μm (a-e), 100 μm (a1-e1), 50 μm (a2-e2). Abbreviations: ATN, anterior tuberal nucleus; Ha, habenula; Hc-a, caudal zone of periventricular hypothalamus in front of posterior recess; Hc-b, caudal zone of periventricular hypothalamus around posterior recess; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; LH, lateral hypothalamic nucleus; ot, optic tract; PGa/PGm, anterior/medial preglomerular nucleus; PPp, posterior parvocellular preoptic nucleus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SC, suprachiasmatic nucleus; TeO, optic tectum; TLo, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum; TPp-m, magnocellular cells of TPp; TPp-p, parvocellular cells of TPp; VM, ventromedial thalamic nucleus

TABLE 2 Catecholaminergic systems and otpa expression

Structure	TH	Otpa	Double-label
Telencephalon			
Dm/Dl/Dd/Dp	-	-	-
Vd/Vv/Vs/Vp	+*	-	-
Vi	-	+	-
Preoptic region			
PPa	+	+	-
PM	+	+	-
SC	+	+	-
PPp	+	+	-
Diencephalon			
VL/VM	+	-	-
TPp-p	+	+	-
TPp-m	+	+	+/-
PTN	+	+	+/-
PVO	+(+)	-	-
PPr	+	-	-
Hv	-	+	-
Hd	-(+)	-	-
Hc-a	+(+)	+	-
Hc-b	+(+)	-	-
IN	-(+)	+	-
LH	-	+	-
ATN	-	+	-
Hindbrain			
LC	+	-	-
PL	-	+	-
DTN	-	+	-
NLV	-	+	-
GC	-	+	-
NIn	-	+	-
SR	-	+	-
SRF	-	+	-
NXm	+	-	-
IRF	-	+	-
AP	+	+	-

*TH cells are lateral to these subpallial nuclei, (+) contain TH2 enzyme not visible with TH antibody (see Section 4). Right column: in addition to double-labeled cells (+), also single labeled TH cells (-) occur in these two regions.

Furthermore, Otpa-positive cells occur in many additional structures along the entire zebrafish neuraxis, from the alar secondary prosencephalon, that is, telencephalon and preoptic region, to basal plate structures, that is, remaining hypothalamus, posterior tuberculum, midbrain tegmentum, as well as hindbrain structures (Figure 12b; Table 2).

3.4 | Distribution of Otpa in comparison to *shh*-GFP and *islet1*-GFP

Otpa is predominantly expressed in ventral zebrafish brain divisions as are the signaling factor-coding gene *sonic hedgehog* (*shh*) and one of the transcription factor-coding genes whose expression is promoted in nearby cells, *islet1*. Therefore, we prepared 3-month-old adult zebrafish brain sections coming from relevant transgenic line specimens, that is, *Tg(-2.4shha-ABC:GFP)*, generated by Shkumatava et al. (2004), and *Tg(isl1a:GFP)*, generated by Higashijima et al. (2000), respectively (see Baeuml et al., 2019 and Wullimann & Umeasalugo, 2020, for details on these transgenic lines). These sections were costained for GFP and Otpa. Epifluorescence microphotographs of this material were analyzed using ImageJ and allowed for identifying locations where colocalization of *shh*-GFP or *islet1*-GFP with Otpa is evident or can be ruled out. In addition, confocal microphotographic analysis served to confirm or extend such cellular colocalizations.

3.4.1 | Otpa and *shh*-GFP

Telencephalon. As reported before (Wullimann & Umeasalugo, 2020), *shh*-GFP radial glia cells are present in the zebrafish medial division of the dorsal telencephalon (Dm) and very few *shh*-GFP-positive cell bodies extend ventrally along the ventricle into the intermediate nucleus of the ventral telencephalon, but never overlap cellularly with Otpa-positive cells there (Figure 9a-a3).

Preoptic region. Some *shh*-GFP cells are present directly at the ventricular lining, extending into the magnocellular preoptic nucleus (PM), but never come close to its distinctly more peripherally located Otpa cells (Figure 9a-a3).

Posterior tuberculum and Nmlf. On the anterior side of the *shh*-GFP-positive zona limitans intrathalamica (ZLI; Figure 9b2), the zebrafish periventricular posterior tubercular nucleus (TPp) exhibits many *shh*-GFP-positive cells located somewhat migrated away from the ventricle (Figure 9b2). These represent parvo- and magnocellular cells (TPp-p/TPp-m) of the periventricular PT nucleus, which are colocalized with TH (Wullimann & Umeasalugo, 2020). In contrast, there are no Otpa cells in the PVO (see confocal analysis in Figure 6). As expected, *shh*-GFP cells in the TPp-m are sometimes colocalized with Otpa (yellow arrows in Figures 9b1-b3; 11a-a2). Many *shh*-GFP cells are furthermore seen in the basal part of prosomere 3, that is, the nucleus of the medial longitudinal fascicle (Nmlf; Figure 9c2/d2), but no Otpa cells are present there. Finally, the Otpa-positive cells in the PTN have no *shh*-GFP cells in their vicinity (Figure 9d-d3; unlike in the larva, see Section 4).

Hypothalamus. There are *shh*-GFP cells close to the ventricle extending into the ventral zone of the zebrafish periventricular hypothalamus (Hv) without apparent colocalization with Otpa (Figure 9b/c). However, the dorsal zone of the periventricular hypothalamus (Hd), including

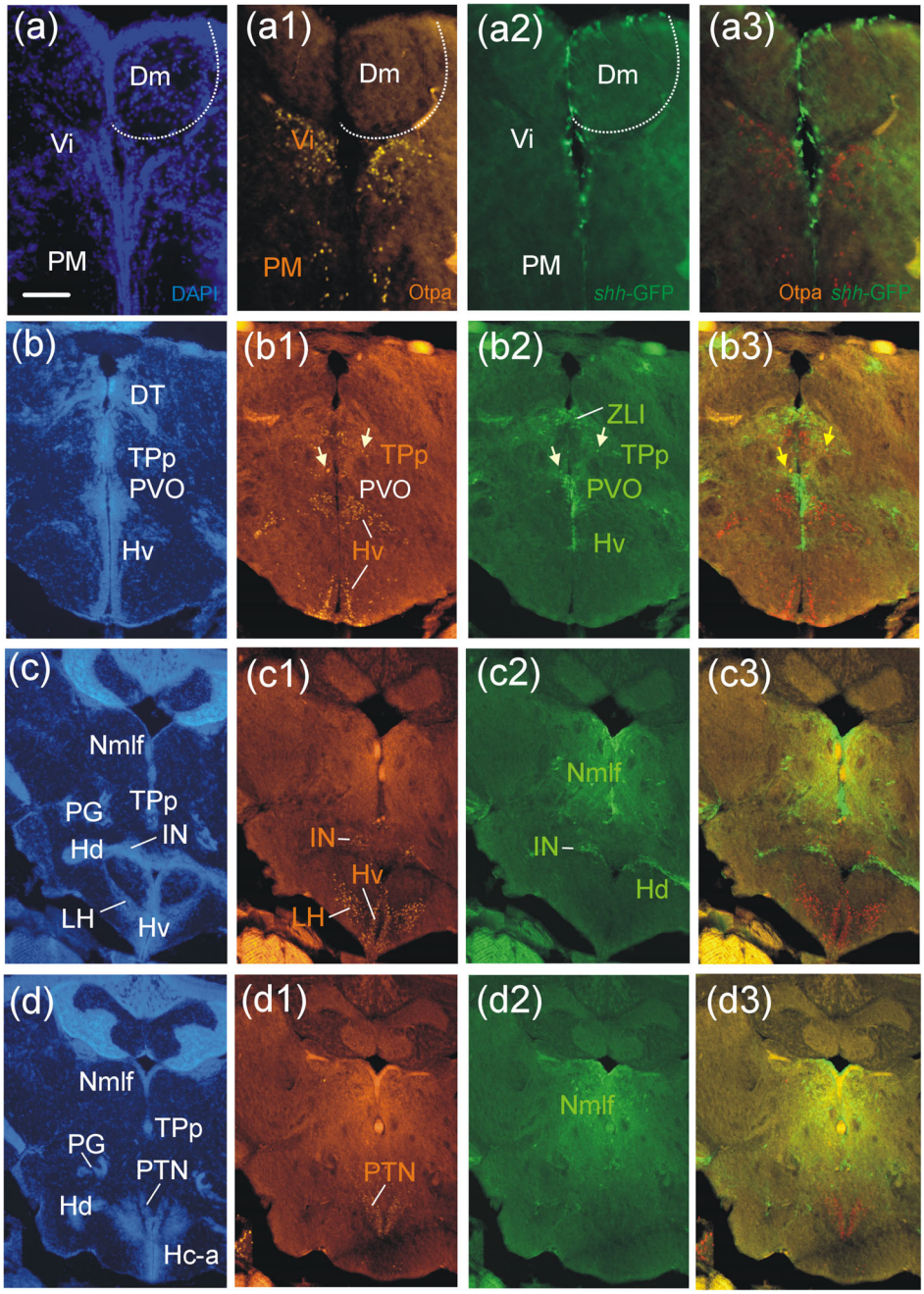

FIGURE 9 Distribution of Otpa-positive cells compared to *shh*-GFP cells in the adult zebrafish brain. Four transverse section levels in DAPI stain (a-d), Otpa immunostain (a1-d1), GFP immunostain (a2-d2) and merged pictures (a3-d3). (a-a3): telencephalon and preoptic region. (b-b3 to d-d3): three levels of posterior tuberculum and hypothalamus. White arrows in (b1-b2) point to the only *shh*-GFP cells double-labeled with Otpa. See text for more details. Scale bar = 250 µm. Abbreviations: Dm, medial zone of dorsal telencephalon; Hc-a, caudal zone of periventricular hypothalamus in front of posterior recess; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; LH, lateral hypothalamic nucleus; Nmlf, nucleus of medial longitudinal fascicle; PG, preglomerular complex; PM, magnocellular preoptic nucleus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; TPp, periventricular nucleus of posterior tuberculum; Vi, intermediate nucleus of ventral telencephalon; ZLI, zona limitans intrathalamica

the intermediate hypothalamic nucleus (IN), contains *shh*-GFP-positive cells (Figure 9c2). The IN also shows Otpa-positive cells, but these two markers do not colocalize in the same cells, as the Otpa cells are more remote from the lateral recess ventricular surface than the *shh*-GFP cells (Figure 9c1-c3). The prerecess part of the caudal periventricular hypothalamic zone (Hc-a) contains no *shh*-GFP cells (Figure 9d-d3), but the (Otpa-free) Hc-b part does so in the larva (see Section 4).

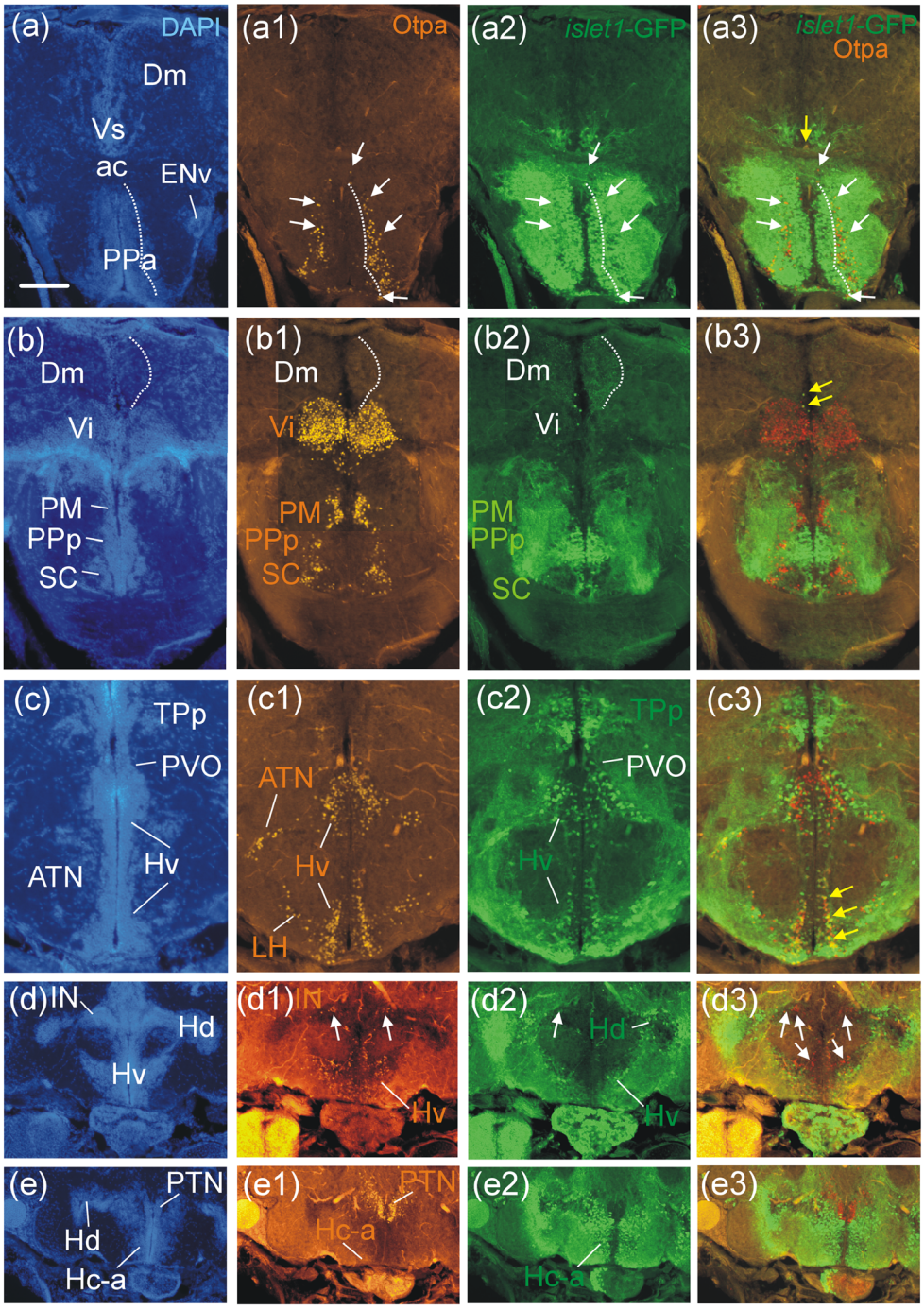

FIGURE 10 Distribution of Otpa-positive cells compared to *islet1*-GFP cells in the adult zebrafish brain. Five transverse sections levels in DAPI stain (a-e), Otpa immunostain (a1-e1), GFP immunostain (a2-e2) and merged pictures (a3-e3). (a/b-a3/b3): telencephalon and preoptic region. Note one double-labeled cell in the supraoptic ventral telencephalic nucleus (Vs), yellow arrow in (a3) and two further ventricularly located double-labeled cells in (b3) (yellow arrows; see text). (c/d/e) to (c3/d3/e3): three levels of posterior tuberculum and hypothalamus. Several double-labeled cells sit in the ventral periventricular hypothalamic zone (Hv), yellow arrows in (c3). Note single labeled Otpa and *islet1*-GFP cells in the intermediate hypothalamic nucleus (IN), white arrows pointing dorsally in (d1-d3), and single labeled *islet1*-GFP cells in the ventral periventricular hypothalamic zone (ventrally pointing white arrows in d3). See text for more details. Scale bar = 250 µm. Abbreviations: Ac, anterior commissure; ATN, anterior tuberal nucleus; Dm, medial zone of dorsal telencephalon; ENv, ventral entopeduncular nucleus; Ha, habenula; Hc-a, caudal zone of periventricular hypothalamus in front of posterior recess; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; PM, magnocellular preoptic nucleus; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SC, suprachiasmatic nucleus; TeO, optic tectum; TPp, periventricular nucleus of posterior tuberculum; Vi/Vs, intermediate/supracommissural nucleus of ventral telencephalon

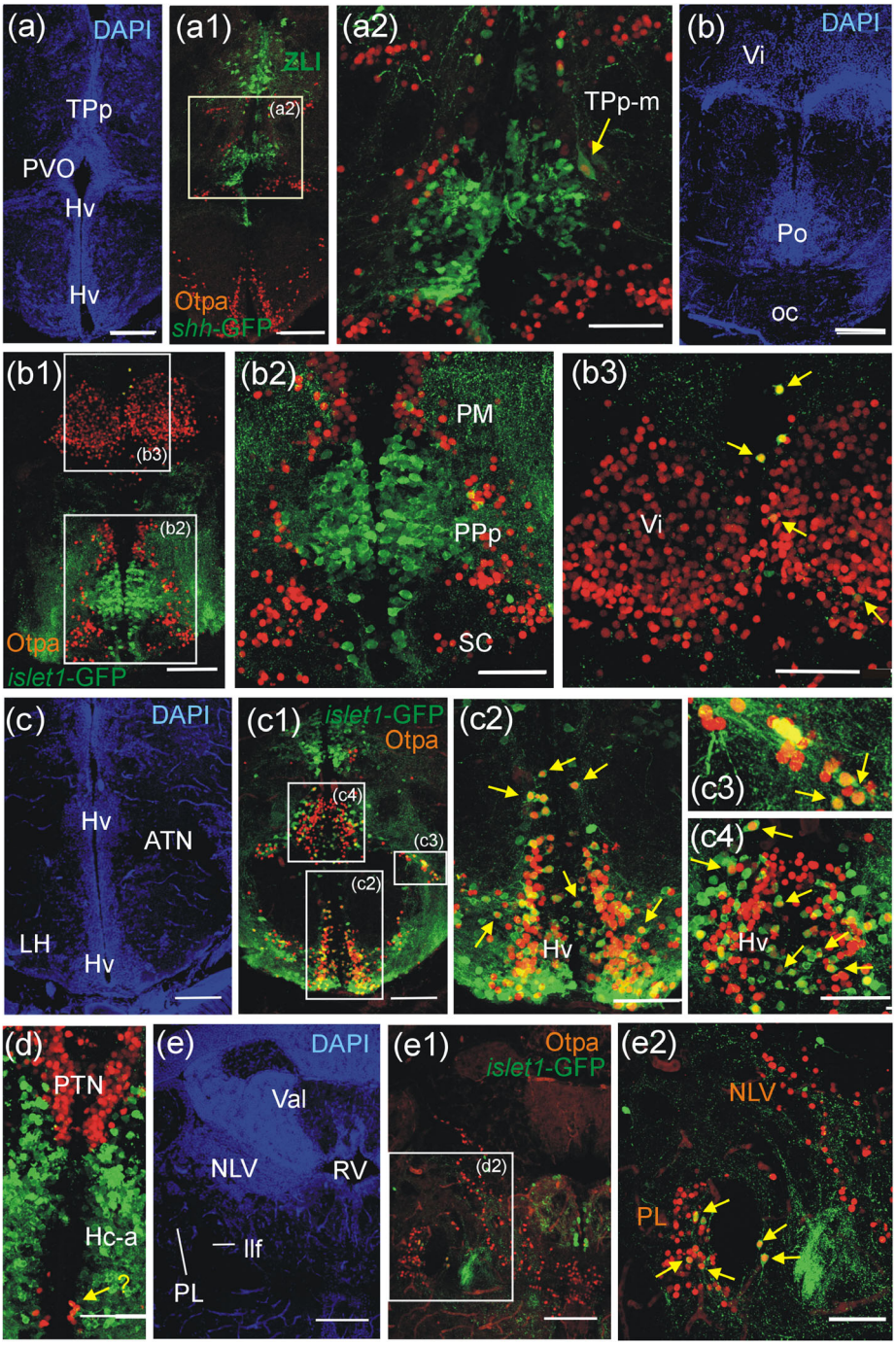

FIGURE 11 Confocal microscopic analysis of Otpa-positive cells in adult zebrafish brains of *shh*-GFP or *islet1*-GFP transgenic zebrafish line brains shows cellular co-localization of Otpa and *shh*-GFP in TPp-m cells (a–a2) and of Otpa and *islet1*-GFP in few midline pallial cells and within Vi (b-b3), massive such co-localization in Hv and some in ATN (c-c4) and Hc-a (d), as well as in PL (e-e2). Abbreviations. ATN, anterior tuberal nucleus; Hc-a, caudal zone of periventricular hypothalamus in front of posterior recess; Hv, ventral zone of periventricular hypothalamus; LH, lateral hypothalamic nucleus; llf, lateral longitudinal fascicle; NLV, nucleus lateralis valvulae; oc, optic chiasma; PL, perilemniscal nucleus; PM, magnocellular preoptic nucleus; Po, preoptic region; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; RV, rhombencephalic ventricle; SC, suprachiasmatic nucleus; TPp, periventricular nucleus of posterior tuberculum; TPp-m, magnocellular cells of the periventricular posterior tubercular nucleus; Val, valvula cerebelli; Vi, intermediate nucleus of ventral telencephalon

3.4.2 | Otpa and *islet1*-GFP

Telencephalon. In contrast to the zebrafish dorsal telencephalic zones (Dm/Dl/Dp/Dd/Dc; see Figure 2), some ventral telencephalic nuclei (Vv/Vd/Vs; see Figure 2) contain many *islet1*-GFP cells (shown here only for Vs in Figure 10a2, but see Baeuml et al., 2019 for Vv/Vd). Very few *islet1*-GFP cells extend into the ventricular lining of the intermediate nucleus of the ventral telencephalon (Figure 10b2). These few cells and one Vs cell colocalize with Otpa (yellow arrows in Figure 10a3/b3; 11b1/b3). Also, very few of the densely packed Otpa-positive cells, which define the intermediate nucleus of the ventral telencephalon (Vi; Figure 10b1) exhibit *islet1*-GFP (Figure 11b-b3; confocal picture). Furthermore, a distinct *islet1*-GFP positive terminal field is seen in the posteromedial part of the medial zone of the dorsal telencephalon (Dm; Figure 10b2, white dotted line), which likely originates from axons of *islet1*-GFP positive cells in the preoptic region, or of ventral telencephalic nuclei. This terminal field over Dm was misidentified by Baeuml et al. (2019) as covering the intermediate nucleus of the ventral telencephalon (Vi), which is clearly not the case as can be seen in Figure 10b-b3.

Preoptic region. The zebrafish preoptic region (PPa, PPp, PM, SC; Figure 10a-b) contains many *islet1*-GFP cells. In the anterior parvocellular preoptic nucleus (PPa), cells at the periphery of the dense *islet1*-GFP expression domain are not cellularly double-labeled with Otpa (most dorsal and most ventral white arrows on right brain side in Figure 10a1-a3). Also in the core of the *islet1*-GFP domain, it appears that Otpa cell clusters fill gaps of *islet1*-GFP negativity (two white arrows on each brain side in Figure 10a1-a3). Overall, Otpa-positive cells in PPa are generally at the periphery of this nucleus (Figure 10; dotted line separates medial PPa cells without interspersed Otpa-positive cells from peripheral cells, where Otpa positivity does occur). Similarly, in the posterior parvocellular preoptic nucleus (PPp) and suprachiasmatic (SC) nucleus, Otpa-positive cells lie peripherally to *islet1*-GFP cells, whereas Otpa-positive cells lie very medial in the magnocellular preoptic nucleus (PM,; Figure 10b). There is no cellular overlap of *islet1*-GFP and Otpa in PPp, PM and SC (Figure 11b2; confocal picture).

Posterior tuberculum. The zebrafish periventricular nucleus of the posterior tuberculum (TPp) contains many *islet1*-GFP cells (Figure 10c2) which include small TH-positive cells (TPp-s), but not magnocellular cells (TPp-m) (see Baeuml et al., 2019). We saw no overlap of *islet1*-GFP with Otpa in the TPp. The PVO (Figure 10c) contains neither Otpa (this study, see Figure 6) nor *islet1*-GFP cells (Baeuml et al., 2019). The PTN contains many Otpa-positive cells, which are never coincident with *islet1*-GFP cells (Figures 10e; 11).

Hypothalamus. The ventral (Hv), dorsal (Hd), and preposterior recess part of the zebrafish caudal periventricular hypothalamic zone (Hc-a) contain many *islet1*-GFP cells (Figures 10c-e; 11c-d). Within Hd, the intermediate hypothalamic nucleus (IN) also contains Otpa-positive cells, but they are not *islet1*-GFP-positive (Figure 10d1-d3, dorsally directed white arrows). The Hv exhibits both Otpa-positive and *islet1*-GFP positive cells. The two markers appear to be colocalized in the ventral aspect of Hv (Figure 10c3, yellow arrows). This was confirmed in confocal analysis, which further also showed such colocalization in the dorsal part of Hv and in some cells of the anterior tuberal nucleus (ATN) (Figure 11c-c4). Furthermore, in the prerecess caudal periventricular hypothalamic zone (i.e., Hc-a), Otpa may be colocalized in some *islet1*-GFP cells (Figure 11d). The confocal analysis, furthermore, revealed that the hindbrain perilemniscal nucleus contains cells with colocalization of *islet1*-GFP and Otpa (Figure 11e-e2). Overall, however, cellular colocalization of Otpa and *islet1*-GFP in the adult zebrafish brain is rare in most locations with the exception of the hypothalamus.

4 | DISCUSSION

One fundamental realization of the present study is that, overall, there is continued expression of Otpa in the same general adult zebrafish regions as in larval brains (see below), an observation similarly made for *islet1* and *shh* previously (Baeuml et al., 2019; Wullimann & Umeasalugo, 2020). This suggests that we recognize here the adult derivatives of Otpa-expressing larval regions.

4.1 | Orthopedia and catecholaminergic cells

The distribution of the transcription factor Orthopedia a (Otpa) and the rate-limiting enzyme for the biosynthesis of catecholamines, tyrosine hydroxylase (TH), was immunohistochemically analyzed here in the adult zebrafish brain (summarized in Table 2 and Figure 12a/b).

Telencephalon. The only *otp* expression domain in the tetrapod telencephalon is in the medial amygdala (Bardet et al., 2008; Bupesh et al., 2011; Medina et al., 2011 Abellán et al., 2013). The intermediate ventral telencephalic nucleus (Vi; nucleus described by Levine & Dethier, 1985) has been identified as the teleostean homolog of the tetrapod medial amygdala, based on various lines of evidence (including Otpa positivity, kin odor-related olfactory input and ERK signaling, efferent projections to the tuberal hypothalamus) (Biechl et al., 2017; Gerlach & Wullimann, 2021). However, the Vi does not contain catecholaminergic cells (Figure 12a,b; Table 2).

Preoptic region. Although all adult zebrafish preoptic nuclei contain dopaminergic (Yamamoto et al., 2011) and Otpa-positive cells (this study), these cells never colocalize cellularly, but form distinct nonoverlapping clusters (Figure 12a,b; Table 2). Otpa does colocalize with various neuropeptides in the zebrafish preoptic supraopto-paraventricular region (SPV) (Del Giacco et al., 2006; Eaton & Glasgow, 2007; Fernandes et al., 2013; Herget et al., 2014; Affaticati et al., 2015). Larval expression studies of *otpa/b*, neuropeptides, as well as of various additional regulatory markers (*arx*, *dlx5a*, *islet1*, *ngn1*, *sim1a*, *foxg1*) (Herget et al., 2014; Affaticati et al., 2015) yielded a molecular neuroanatomical definition of the neurosecretory preoptic domain within the larger zebrafish preoptic region. Additional data in adult zebrafish showed that the oxytocin and vasopressin containing magnocellular preoptic

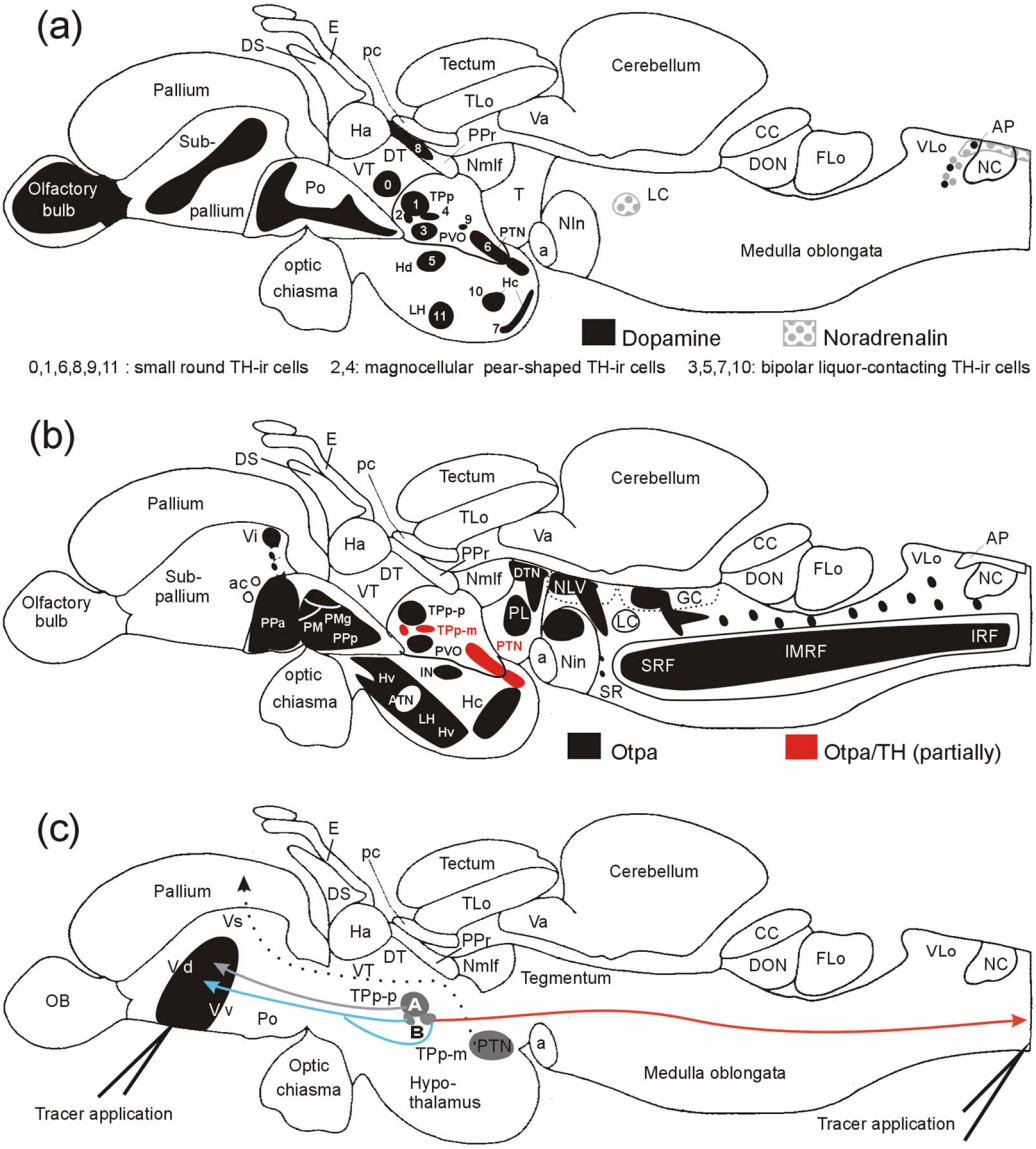

FIGURE 12 Schematic lateral view of adult zebrafish brain shows summary of (a) TH-positive brain nuclei (modified from Rink & Wullimann, 2002a; Wullimann & Rink, 2002), and (b) Otpa domains. (c) Schematic lateral view of adult zebrafish brain shows telencephalic and spinal connections of posterior tubercular nuclei. In (a), Arabic numbers, which were initially introduced in our developmental studies (Rink & Wullimann, 2002a; Wullimann & Rink, 2002), are also indicated for diencephalic and hypothalamic dopaminergic cell groups. Note that descending spinal projections of dopaminergic parvocellular periventricular posterior tubercular cells (TPp-p; Becker et al., 1997), and ascending projections of dopaminergic positive TPp-p and magnocellular pear-shaped periventricular posterior tubercular cells (TPp-m; Rink and Wullimann, 2001) have been previously established in the adult zebrafish brain (in larvae: Tay et al., 2011). Furthermore, TH-negative posterior tuberal (PTN) cells project to the zebrafish pallium (Rink & Wullimann, 2001, 2004; confirmed in goldfish by Northcutt, 2006). We propose the hypothesis that there are two populations of TPp-m cells, one with exclusive ascending projections to the striatum, and one with additional spinal projections, plus additional TPp-p projections to the striatum as well as ascending projections to the pallium from the posterior tuberal nucleus. (c) is modified from Rink and Wullimann (2002b). Abbreviations: A, ansulate commissure, ac anterior commissure; AP, area postrema; ATN, anterior tuberal nucleus; CC, crista cerebellaris; DON, descending octaval nucleus; DS, saccus dorsalis; DT, dorsal thalamus; DTN, dorsal tegmental nucleus; E, epiphysis; Flo, facial (sensory) lobe, GC, central gray; Ha, habenula; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; LC, locus coeruleus; LH, lateral hypothalamic nucleus; LR lateral hypothalamic recess; NC, commissural nucleus of Cajal; NLV, nucleus lateralis valvulae; Nmlf, nucleus of medial longitudinal fascicle; NIn, interpeduncular nucleus; OB, olfactory bulb; oc, optic chiasma; pc, posterior commissure; PL, perilemniscal nucleus; PM, magnocellular parvocellular preoptic nucleus; PMg, gigantocellular part of PM; Po, preoptic region; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; PM, magnocellular preoptic nucleus; PPr, periventricular pretectum; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SR, superior raphe; SRF, superior reticular formation; T, midbrain tegmentum; TLo, torus longitudinalis; TPp-m, magnocellular cells of the periventricular posterior tubercular nucleus; TPp-p, parvocellular cells of periventricular posterior tubercular nucleus; Va, valvula cerebelli; VLo, vagal (sensory) lobe; Vd/Vi//Vs/Vv, dorsal/intermediate/supracommissural/ventral nucleus of ventral telencephalon; VT, ventral thalamus

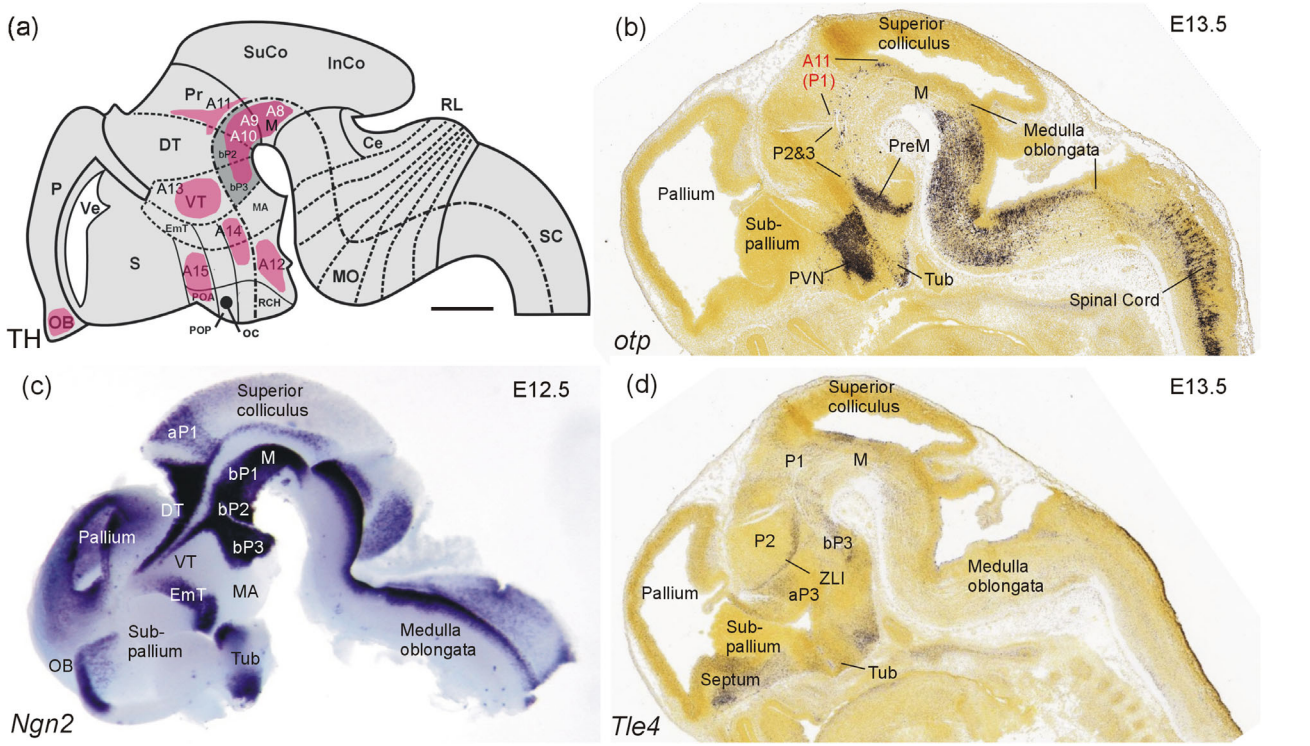

FIGURE 13 The embryonic mouse basal diencephalon. (a) Schematic sagittal view of the embryonic mouse brain (modified from Vernier & Wullimann, 2009, based on data by Smeets & González 2000; Marín et al., 2005; Björklund & Dunnett, 2007; Vitalis et al., 2000) shows embryonic mammalian dopamine systems. Note position of A11 in the alar plate of prosomere 1 (P1). (b) Embryonic mouse brain sagittal section shows all major *otp* expression domains (taken from Developmental Mouse Brain Atlas, Allen Institute) except for that in the medial amygdala which is out of section plane. Note strong expression in the alar plate of P1 and sparse expression in the basal plate of P1 through P3. (c) Embryonic mouse brain sagittal section shows expression of bHLH gene *ngn2* (modified from Osório et al., 2010). Note the large domain selectively demarcating the region of interest, that is, the basal plate portions of midbrain and P1 through P3 (incl. spear-shaped expression domains flanking in addition the zona limitans intrathalamica between dorsal and ventral thalamus). (d) Embryonic mouse brain sagittal section shows expression domains of the gene*Tle4* (transducin-like enhancer of split 4; taken from Developmental Mouse Brain Atlas, Allen Institute) which has a potential role in basal diencephalic regions (P1–P3). Scale bar: 500 μm (applies to all panels). Abbreviations: A8-A14, mammalian dopaminergic cell groups (see text). aP1/2, alar plates of prosomeres 1/2; bP1-3, basal plates of prosomeres 1–3; Ce, cerebellum; DT, dorsal thalamus; EmT, eminentia thalami; InCo, inferior colliculus; M, midbrain tegmentum; MA, mammillary hypothalamus; MO, medulla oblongata; OB, olfactory bulb; P, pallium; P1-P3, prosomeres 1–3; POA, anterior preoptic area; Pr, pretectum; PreM, premammillary region; PVN, paraventricular nucleus; RCH, retrochiasmatic area; RL, rhombic lip; S, subpallium; SC, spinal cord; SuCo, superior colliculus; Tub, tuberal hypothalamus; Ve (telencephalic), ventricle; VT, ventral thalamus; ZLI, zona limitans intrathalamica

(PM) and posterior parvocellular preoptic (PPp) nuclei are homologs of the tetrapod paraventricular and supraoptic nuclei (Herget et al., 2014). Together, this allowed to recognize the preoptic neurosecretory domain as the teleostean homolog of the tetrapod SPV (Osório et al., 2010; Moreno et al., 2012; Domínguez et al., 2013, 2015). Thus, the developmental role of *otp* in the telencephalic and preoptic (SPV) domains has been relatively well clarified.

Remaining hypothalamus and posterior tuberculum. In mammals (e.g., mouse; Morales-Delgado et al., 2011; Puelles et al., 2012) and other amniotes (Bardet et al., 2008), there are two *otp* domains within the basal plate hypothalamus (Figure 13b), one in the tuberal hypothalamus and one in the preretromammillary/premammillary hypothalamus (Figure 13b). The amniote tuberal *otp* domain compares well with our adult zebrafish Otpa-positive domain in the ventral periventricular hypothalamic zone (Hv). The *otp* domain in the mammillary hypothalamus compares partly to our Otpa-positive domain in the prerecess caudal periventricular hypothalamic zone (Hc-a). Teleosts, however, have two *otp* paralogues, *otpa* and *otpb*, and they are both expressed in the hypothalamus (Fernandes et al., 2013; Wolf & Ryu, 2013; Herget et al., 2014). While the *otpb* domain is included in the *otpa* domain in certain regions (like the SPV) (Herget et al., 2014) and overlaps also largely in Hv, *otpb* has a much more caudal extent in the basal plate hypothalamus (Fernandes et al., 2013; Wolf & Ryu, 2013; Herget et al., 2014), extending clearly into the caudal periventricular hypothalamic zone surrounding the posterior recess (Hc-b) where *otpa* is absent (this study). Thus, when comparing the mouse mammillary *otp*-expressing domain to the zebrafish one, *otpb*-expressing cells in the latter are to be included (see below).

Zebrafish orthologs of diagnostic genes expressed in rodent premammillary (*lef1*), tuberomammillary (*lhx6*), supramammillary (*irx5a*), and mammillary nucleus (*foxb1.2*) were studied in the zebrafish embryonic brain to define the homologs of the mammillary hypothalamus

as opposed to the tuberal one (Wolf & Ryu, 2013). Clearly, the bilateral *lef1* expression defines the part of Hc surrounding the posterior recess, that is, the Hc-b described in the adult brain here. Furthermore, rostral to the *lef1* expression domain, a midline expression domain of *foxb1.2* has been interpreted as mammillary body or nucleus (Wolf & Ryu, 2013). Whether or not this area develops into the adult zebrafish corpus mamillare (CM; see Figures 1; 3; 4) needs further investigation. In any case, the respective connectivities of these similarly named structures in mammals and teleosts are different (Northcutt & Wullimann, 1988). The embryonic zebrafish *lhx6* domain lies ventral to the *foxb1.2* domain and is hard to compare to a distinct adult hypothalamic structure. The *irx5a* domain lies more dorsocaudally to the *foxb1.2* domain and extends considerably rostrally in the midline, resembling our adult PTN there. Lateral to the midline *foxb1.2* domain, a distinct *otpa*/*otpb* domain is present on each brain side, which is caudally contiguous with the bilateral *lef1* domain (i.e., the Hc-b), while only *otpb* is also expressed in Hc-b (Wolf & Ryu, 2013). This embryonic *otpa* domain, thus, resembles our Otpa-positive domain in Hc-a (Figures 1, 3, 7, 8). Although it is difficult to relate this embryonic situation to the adult stage, one could reasonably conclude that the teleostean mammillary hypothalamus includes the *foxb1.2*, *lhx6*, and *lef1* embryonic expression domains and maybe (at least caudally) the *irx5a* domain, and that these domains develop into the adult CM (*foxb1.2*) and its surrounding regions (*lhx6*), the prerecess (*otpa*) and recess-surrounding part (*lef1*) of the Hc (i.e., Hc-a and Hc-b) and, hypothetically, part of the PTN (*irx5a*).

A different concept suggested that the zebrafish embryonic or larval mammillary hypothalamus is more restricted (Schredelseker & Driever, 2020). Accordingly, it would include only the *otpa* domain (their perimammillary and periretromammillary regions) and the *foxb1a* (revised name for *foxb1.2;* see ZFIN database) domain (their mammillary and adjacent *shh*-expressing retromammillary regions; Schredelseker & Driever, 2020). However, their mammillary hypothalamus would explicitly not include the *lxh6* and *lef1* domains, which were attributed to the tuberal hypothalamus (Schredelseker & Driever, 2020). Thus, the adult (prerecess) Otpa-positive Hc-a and the Otpa-free CM and its surroundings would derive from the embryonic mammillary hypothalamus. However, *otpb* clearly extends into the *lef1* domain and, thus, a comparison to mammals (Morales-Delgado et al., 2011; Puelles et al., 2012)—which have only one *otp* gene—suggests that Hc-b (the *lef1* domain) should be included into the perimammillary (and therefore mammillary hypothalamus) region. Again, the difficulties of relating embryonic or larval neuroanatomy to the differentiated adult stage call for more developmental investigation.

We conclude from our analysis of Otpa expression in basal plate hypothalamic areas in the adult zebrafish that the ventral periventricular hypothalamic zone (Hv) with its migrated anterior tuberal (ATN) and lateral hypothalamic (LH) nuclei correspond to the *otp*-expressing basal hypothalamic tuberal field seen in the mouse (Figures 12b; 13b), because these zebrafish structures express, at least partly, Otpa. Detailed connectional and neurochemical studies confirmed a homology of part of Hv (Forlano & Cone, 2007) with the mammalian arcuate nucleus in the lateral tuberal hypothalamus (Forlano & Cone, 2007). The dorsal periventricular hypothalamic zone (Hd) surrounding the lateral recess contains Otpa-positive cells in its intermediate hypothalamic nucleus, supporting the inclusion of Hd into the tuberal hypothalamus.

In addition, the observed extensive Otpa expression in the prerecess caudal periventricular hypothalamic zone (Hc-a) may correspond to part of the mammalian *otp*-positive preretro- and premammillary fields (e.g., mouse; Figure 13b). This is in accordance with bilateral embryonic or larval *otpa* expression in the zebrafish mammillary hypothalamus (Wolf & Ryu, 2013). As mentioned above, in the recess-surrounding part of Hc (Hc-b) there is no Otpa, but (larval) *otpb* expression exists and, thus, Hc-b qualifies for inclusion into the mammillary hypothalamus.

Finally, we will relate adult zebrafish hypothalamic and posterior tubercular Otpa expression to dopaminergic systems. In addition to zebrafish larval dopamine systems, there have been various complete descriptions of adult zebrafish dopaminergic systems using markers beyond TH (Kaslin & Panula, 2001; Rink & Wullimann, 2001; Yamamoto et al., 2010; 2011) and we will relate to these studies in the analysis of TH/Otpa-positive cells below.

The adult zebrafish ventral periventricular hypothalamic zone (Hv) and the anterior tuberal nucleus (ATN) contain Otpa-positive cells, but do not exhibit catecholaminergic cells (Kaslin & Panula, 2001; Rink & Wullimann, 2001; Yamamoto et al., 2010, 2011; this study). However, the lateral hypothalamic nucleus (LH), the dorsal periventricular hypothalamic zone (Hd), including its intermediate hypothalamic nucleus (IN), as well as both parts of the caudal periventricular hypothalamic zone (Hc-a/Hc-b) do exhibit dopaminergic cells which contain TH2 instead of (IN) or in addition (LH/PVO/Hd/Hc) to TH1 (Candy & Collet, 2005; Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010; 2011; Semenova et al., 2014). TH2 is usually not visualized with conventional TH antibodies (Yamamoto et al., 2010; 2011; Xavier et al., 2017). These studies also clarified that these "phantom" TH2-related dopaminergic cell populations contain the molecular machinery of functional dopamine cells. The teleostean TH2-containing cells in PVO, Hd, and Hc are bipolar liquor-contacting cells, and are at the same time serotoninergic (see Discussion in Rosner et al., 2020). They are lost in mammals in correlation with the loss of the *th2* gene (Yamamoto et al., 2010; Xavier et al., 2017). In two early studies, some TH-positive cells in LH (small round cell type), as well as in Hd and Hc-a (bipolar liquor-contacting cell type) were described (Rink & Wullimann, 2001), likely because the antibody unexpectedly detected some TH2 (or TH1) enzyme, and the same applies in addition for TH positivity in IN (Kaslin & Panula, 2001). Thus, Otpa potentially colocalizes with TH2 in these dopamine cells and would have evaded detection in our present analysis. Furthermore, *otpb* expression is more extended in these regions, and may be expressed in catecholaminergic cells. Regarding the posterior tuberculum, we see Otpa-positive cells in the PVO, TPp, and PTN, and in the latter two, Otpa and TH colocalize cellularly (Table 2; see also Section 4.2).

Interestingly, experimental studies in zebrafish larvae show that the main effect of reducing numbers of *th1*-expressing cells in *otpa*$^{-/-}$ zebrafish mutants is on many—but not all—magnocellular TPp-m cells (larval groups 2/4) and PTN (group 6) cells, and not on parvocellular

TPp-p (group 1) and PVO cells (group 3), which appear weakly TH-immunolabeled also in wild-type brains (Fernandes et al., 2013; their Figure 3). Furthermore, larval groups 1 (our adult 0 and 1; see first paragraph of Section 4.1.) and 3 (our PVO) are not expected to be affected, since they do not express *otpa* in zebrafish larvae (Ryu et al., 2007) and in adult brains (this study). The *otpa*$^{-/-}$/*otpb*$^{-/-}$ double mutants have an additional reducing effect on cell numbers of these dopamine cells (Fernandes et al., 2013). However, Hd (group 5) and in particular Hc (group 7) dopamine cells (which partly express *th1*, but more often *th2*; Yamamoto et al., 2011) only develop in older 5 dpf larva (Rink & Wullimann, 2002a) and were, therefore, not covered in the analysis of 3 dpf larva of Fernandes and colleagues (2013). Thus, these relatively later developing liquor-contacting dopaminergic cell populations (5,7,10) may express *otpb* and be affected in particular in *otpb* mutants. Also interesting is that hindbrain *th1*-expressing cells in *otp* double-mutants are not affected, which is consistent with our finding that they do not express Otpa.

Overall, our analysis of Otpa expression in the adult zebrafish forebrain highlights detailed correspondences between mouse and zebrafish preoptic and remaining (basal plate) hypothalamus. However, it can be further concluded that the inferior lobe (diffuse and central nuclei), a large part of the adult teleostean hypothalamus, does not relate closely to mammalian tuberal and retromammillary or mammillary hypothalamic divisions, but rather represents a teleost-specific hypothalamic hypertrophy. Recently, substantial numbers of midbrain cells (embryonic optic tectum) have been shown to enter the diffuse nucleus of the inferior lobe (Bloch et al., 2019). These cells clearly enter this hypothalamic nucleus via tangential migration, while autochthonic hypothalamic cells contribute to the diffuse nucleus of the inferior lobe via radial migration from the periventricular hypothalamic zone which identifies this nucleus as hypothalamic (Wullimann, 2020).

Hindbrain. Regarding zebrafish hindbrain Otpa expression domains, the mammalian literature agrees with *otp* expression being a basal plate marker (Simeone et al., 1994; Ju et al., 2004; Aroca et al., 2006; Figure 13b). Adult zebrafish anterior rhombencephalic structures were typically only partly Otpa-positive. These hindbrain nuclei include the dorsal tegmental and perilemniscal nuclei and nucleus lateralis valvulae, three cerebellar projecting nuclei. Also, the superior reticular formation, central gray, and interpeduncular nucleus (in particular its dorsal part) are Otpa-positive. More posterior Otpa-positive cells are seen in the intermediate and inferior reticular formation, the vagal motor nucleus, and AP. Some scattered Otpa-positive cells in the zebrafish hindbrain have no clear association with any particular described brain nucleus. In any case, none of the hindbrain Otpa-positive cells show cellular colocalization with TH (Table 2; Figure 12b).

4.2 | The vertebrate mesodiencephalic complex of dopamine cells

While there is consensus on the factual developmental and adult configuration of zebrafish dopamine systems, there are two interpretations in the literature on the comparative identity of periventricular posterior tubercular dopamine cells. This issue has previously been discussed extensively (Rink & Wullimann, 2001; Rink & Wullimann, 2002b; Ryu et al., 2007; Vernier & Wullimann, 2009; Tay et al., 2011; Wullimann, 2011; Filippi et al., 2014; Wullimann, 2014; Wullimann & Umeasalugo, 2020) and we will only briefly summarize the main arguments here.

A core issue is that the basal midbrain in teleosts lacks dopamine cells, which would qualify as the homolog of the midbrain substantia nigra/ventral tegmental area (SN/VT) of tetrapods and cartilaginous fish (review: Wullimann, 2014). The only dopamine cells with axonal projections to the teleostean subpallium (striatum) were identified in both cell types of the zebrafish periventricular posterior tuberculum (TPp-p/TPp-m; Rink & Wullimann, 2001) and were interpreted as homologous to the diencephalic part of the tetrapod SN/VT. During brain development, all vertebrates show a continuity of dopamine cells extending from the midbrain floor into all basal divisions of the diencephalon (P1 through P3; bP1 through bP3 in mouse in Figure 13a; see citations in figure legend and Discussion in Wullimann & Umeasalugo, 2020), which is now considered the mesodiencephalic dopaminergic complex (mammalian A8-A10; Fougère et al., 2021). This basal midbrain and adjacent multiprosomeric diencephalic areas are also nicely outlined *by Ngn2* expression, a marker for glutamatergic neurons (Osório et al., 2010; Figure 13c). Further support comes from studies involving *shh*. In addition to the usual transcellular signaling nature of this gene's product, basal plate *shh*-expressing cells themselves develop into the mammalian SN/VT (Joksimovic et al., 2009; Blaess et al., 2011; Hayes et al., 2011). This is also the case for dopaminergic TPp cells in zebrafish (and those of PVO/PTN) (Wullimann & Umeasalugo, 2020). These comparative facts argue in favor of identifying the teleostean TPp as the diencephalic division of the vertebrate mesodiencephalic dopamine complex.

As mentioned, the *shh*-expressing TPp cells (as also the PVO and PTN) coexpress TH (Wullimann & Umeasalugo, 2020), and dopaminergic TPp-m and PTN cells further exclusively coexpress Otpa in addition (this study). In mice, the only dopaminergic cells coexpressing *otp* are located in the alar-basal boundary pretectal region (mammalian A11; Ryu et al., 2007; Figure 12a; note that this mammalian dopamine group formerly has been considered hypothalamic, see Smeets & Reiner, 1994a). This fact together with the commonality of spinal plus striatal projections of mammalian A11 and zebrafish dopaminergic TPp cells led to the alternative interpretation that the teleostean TPp is the homolog of A11 (Ryu et al., 2007; Tay et al., 2011; Filippi et al., 2014).

Indeed, inspection of the embryonic mouse brain shows only scattered *otp*-expressing cells in bP1 through bP3, but many more in the alar-basal boundary region of P1 (A11; Figure 13a,b). In teleostean pretectal dopaminergic cells (group 8 in Figure 13a, close to the posterior commissure; corresponds to the adult PPr; see Mueller et al., 2004; Yáñez et al., 2018), Otpa and *shh* are definitely not expressed (this study; Wullimann & Umeasalugo, 2020) and these cells have different efferent connections than A11, for example, to the optic tectum (Wullimann, 1998; Kress & Wullimann, 2012). A comparable pretectal population closely associated with the posterior commissure

occurs in all other tetrapods except mammals (Smeets & Reiner, 1994a, b), speaking for A11 being something else. Thus, if A11 of mammals were homologous to the teleostean TPp, ancestral basal plate *shh/otp/th*-expressing cells must have tangentially migrated into the alar-basal boundary region of the pretectum in mammals. The sporadic *otp*-expressing cells in the embryonic mouse bP1-bP3 might then be remnants of such cells. Also, the numerous dopamine cells in bP1-bP3 would have downregulated *otp* already. Supporting this is the fact that *otp* is expressed in the nigrostriatal bundle in the embryonic mouse (Wang & Lufkin, 2000). Alternatively, A11 is a novel mammalian formation. This clearly needs further comparative investigation, for example using genes with a preponderant expression in bP1-bP3 such as *Tle4* (Figure 13d).

In the light of our present finding that dopaminergic TPp projection cells with and without Otpa expression exist, connectional studies with combined expression studies in adult zebrafish brains are called for to show whether there is correlation with different subpopulations of ascending or descending axonal projections of TPp cells (Figure 12c). It is clear, for example, that TPp-p dopamine cells do not coexpress Otpa and are GABAergic, as opposed to glutamatergic TPp-m cells (Filippi et al., 2014; review in Wullimann, 2014). Furthermore, in addition to combined ascending and descending TPp projection cells, there may be cells with only one connection, as noted for adult striatal (Rink & Wullimann, 2001) and spinal TPp projection cells (Becker et al., 1997). It is also interesting that the PTN has ascending projection cells (to pallium; see Northcutt, 2006) which are not dopaminergic (Rink & Wullimann, 2001).

4.3 | Distribution of Otpa-positive cells and *shh*-GFP and *islet1*-GFP cells

The Otpa-stained brain sections of adult *shh*-GFP transgenic zebrafish demonstrate nicely that, within Otpa-positive cells, Shh is only expressed in dopaminergic cells of the periventricular posterior tuberculum (TPp-m; see Section 3.4.1 and Figures 9; 11a). However, because Shh is a signaling factor, it may act in other regions on the development of Otpa-positive cells in the vicinity, as is true for *islet1*-expressing cells in the vertebrate brain (see Discussion in Wullimann & Umeasalugo, 2020). Therefore, we checked in adult *islet1*-GFP transgenic zebrafish brain sections for cellular colocalization of *islet1*-GFP and Otpa (Figure 10). In most locations, cells with one of those two markers are remote from or contiguous with each other (see Section 3.4.2). For example, in the anterior parvocellular preoptic nucleus (PPa), the large *islet1*-GFP domain embraces the Otpa domain. However, *islet1*-GFP cells at the border of this expression domain show no colocalization with Otpa, and additional Otpa-positive cell clusters lie in *islet1*-GFP-free holes within the PPa (see Section 3.4.2), similar to the situation in the larva (Herget et al., 2014). The fact that *islet1*-GFP cells in the adult PPa colocalize cellularly with TH (as do *islet1*-GFP cells in PPp, VT, TPp-p, see Baeuml et al., 2019), but Otpa does not (present analysis), also renders it unlikely that *islet1*-GFP and Otpa-positive cells are identical in the PPa. Furthermore, Otpa-positive cells in the adult magnocellular (PM), posterior parvocellular preoptic (PPp), and suprachiasmatic (SC) nucleus are clearly different from *islet1*-GFP cells. Previously, Filippi et al. (2012) reported in the larval zebrafish brain that all larval TH-positive cells in the preoptic region lack *islet1* expression. The latter study furthermore described larval zebrafish catecholaminergic group 1 as the only TH-positive *islet1* -expressing domain (Filippi et al., 2012). In our original description of larval zebrafish brain TH-positive domains (Rink & Wullimann, 2002a), group 1 subsumed both ventral thalamic (zona incerta homolog) and parvocellular posterior tubercular dopaminergic cells (TPp-p) of the prethalamic prosomere. The ventral thalamic TH group (zona incerta homolog) was only separately designated as 0 in the adult zebrafish brain (see Figure 12a). In line with this, group 1 (Pth1 in Filippi et al., 2012) contains both prethalamic alar (ventral thalamic) and small-celled PT dopaminergic/*islet1*-expressing cells and, importantly, these were the only dopaminergic cells affected in morpholino-based knockdowns of *islet1* (similar to knockdowns of the coexpressed *arx* gene, but through a different neurogenetic pathway; Filippi et al., 2012). These larval data are furthermore in line with our description of adult *islet1*-GFP cells in the ventral thalamus and TPp-p (Baeuml et al., 2019), and the absence of adult colocalization of Otpa and *islet1*-GFP in the ventral thalamus and TPp-p in the present study.

The only *otpa/b*-expressing larval zebrafish TH-positive groups 2/4/5/6 were previously reported not to express *islet1* (Filippi et al., 2012). However, *islet1* is expressed in TH-negative cells in the vicinity of these TH-positive groups. Since these areas contain dopamine cells using TH2 (Yamamoto et al., 2011) and express Otpa (within Hd in the IN, within Hc in Hc-a; this study) and *islet1* in TH-negative cells (Filippi et al., 2012), it is possible that colocalization of Otpa and *islet1* occurs in TH2-containing dopaminergic cells. However, in the adult intermediate hypothalamic nucleus (IN), both Otpa-positive and *islet1*-GFP cells are present, but never double-labeled (this study). In both, the ventral periventricular hypothalamic zone (Hv) and prerecess caudal zone of the periventricular hypothalamus (Hv/Hc-a), colocalization of Otpa (or Otpb) and *islet1*-GFP is seen (or possible) according to our data. In line with this, partial overlap of *otpa* and *islet1* expression was described in the embryonic and larval zebrafish brain in the Hv area, but not in Hc (Schredelseker & Driever, 2020). Overall, this indicates that the overwhelming majority of larval and adult Otpa-positive cells in the zebrafish brain neither express *shh* (with the exception of the dopaminergic magnocellular TPp cells), nor do they appear to be dependent on the Shh-*islet1* neurogenetic pathway (with the exception of hypothalamic Hv and Hc-a noncatecholaminergig Otpa-positive cells).

5 | Conclusions

We report in this article:

1. the complete array of expression domains of the *orthopedia a* (*otpa*) gene immunohistochemically in the early adult zebrafish brain including newly defined Otpa-positive domains in the ventral and

prerecess caudal periventricular hypothalamic zones and in various brainstem nuclei (for example, perilemniscal nucleus and nucleus lateralis valvulae).
2. that cellular colocalization of TH is restricted to two posterior tubercular nuclei.
3. that cellular colocalization of Otpa with the signaling factor *shh* is only present in posterior tubercular dopaminergic cells with ascending projections to the subpallium, and with the transcription factor *islet1* in the ventral hypothalamic zone.

AUTHOR CONTRIBUTION

Jaime Eugenin von Bernhardi, Daniela Biechl, Laura Miek, Ulrich Herget, Soojin Ryu, and Mario F Wullimann performed experiments. All authors contributed to the analysis of data and writing of the manuscript.

ACKNOWLEDGMENTS

We thank Bea Stiening for various laboratory-related support and the Faculty of Biology of the Ludwig-Maximilians Universität München (LMU Munich) and the Graduate School for Systemic Neurosciences (GSN), as well as the Division of Neurobiology (Prof. Benedikt Grothe) at the LMU Munich for financial and infrastructural support. We also thank Prof. Reinhard Köster (Technical University Braunschweig) for providing fixed transgenic fish used in this study. Open access funding is enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

DATA AVAILABILITY STATEMENT

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

REFERENCES

Abellán, A., Desfilis, E., & Medina, L. (2013). The olfactory amygdala in amniotes: an evo-devo approach. *Anatomical Record (Hoboken, N.J. : 2007)*, *296*(9), 1317–1332.

Affaticati, P., Yamamoto, K., Rizzi, B., Bureau, C., Peyriéras, N., Pasqualini, C., Demarque, M., & Vernier, P. (2015). Identification of the optic recess region as a morphogenetic entity in the zebrafish forebrain. *Scientific Reports*, *5*, 8738.

Aroca, P., Lorente-Cánovas, B., Mateos, F. R., & Puelles, L. (2006). Locus coeruleus neurons originate in alar rhombomere 1 and migrate into the basal plate: studies in chick and mouse embryos. *Journal of Comparative Neurology*, *496*(6), 802–818.

Baeuml, S. W., Biechl, D., & Wullimann, M. F. (2019). Adult *islet1* expression outlines ventralized derivatives along zebrafish neuraxis. *Frontiers in Neuroanatomy*, *13*, 19.

Bardet, S. M., Martinez-de-la-Torre, M., Northcutt, R. G., Rubenstein, J. L., & Puelles, L. (2008). Conserved pattern of OTP-positive cells in the paraventricular nucleus and other hypothalamic sites of tetrapods. *Brain Research Bulletin*, *75*(2-4), 231–235.

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., & Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *Journal of Comparative Neurology*, *377*(4), 577–595.

Biechl, D., Tietje, K., Ryu, S., Grothe, B., Gerlach, G., & Wullimann, M. F. (2017). Identification of accessory olfactory system and medial amygdala in the zebrafish. *Scientific Reports*, *7*, 44295.

Bloch, S., Thomas, M., Colin, I., Galant, S., Machado, E., Affaticati, P., Jenett, A., & Yamamoto, K. (2019). Mesencephalic origin of the inferior lobe in zebrafish. *BMC Biology*, *17*(1), 22.

Bupesh, M., Legaz, I., Abellán, A., & Medina, L. (2011). Multiple telencephalic and extratelencephalic embryonic domains contribute neurons to the medial extended amygdala. *Journal of Comparative Neurology*, *519*(8), 1505–1525.

Björklund, A., & Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends in Neuroscience (Tins)*, *30*, 194–202.

Blaess, S., Bodea, G. O., Kabanova, A., Chanet, S., Mugniery, E., Derouiche, A., Stephen, D., & Joyner, A. L. (2011). Temporal-spatial changes in Sonic Hedgehog expression and signaling reveal different potentials of ventral mesencephalic progenitors to populate distinct ventral midbrain nuclei. *Neural Development*, *6*, 29.

Candy, J., & Collet, C. (2005). Two tyrosine hydroxylase genes in teleosts. *Biochimica Et Biophysica Acta*, *1727*(1), 35–44.

Caqueret, A., Coumailleau, P., & Michaud, J. L. (2005). Regionalization of the anterior hypothalamus in the chick embryo. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, *233*(2), 652–658.

Chen, Y. C., Priyadarshini, M., & Panula, P. (2009). Complementary developmental expression of the two tyrosine hydroxylase transcripts in zebrafish. *Histochemistry and Cell Biology*, *132*(4), 375–381.

Del Giacco, L., Sordino, P., Pistocchi, A., Andreakis, N., Tarallo, R., Di Benedetto, B., & Cotelli, F. (2006). Differential regulation of the zebrafish orthopedia 1 gene during fate determination of diencephalic neurons. *BMC Developmental Biology*, *6*, 50.

Del Giacco, L., Pistocchi, A., Cotelli, F., Fortunato, A. E., & Sordino, P. (2008). A peek inside the neurosecretory brain through Orthopedia lenses. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, *237*(9), 2295–2303.

Domínguez, L., Morona, R., González, A., & Moreno, N. (2013). Characterization of the hypothalamus of *Xenopus laevis* during development. I. The alar regions. *Journal of Comparative Neurology*, *521*, 725–759.

Domínguez, L., González, A., & Moreno, N. (2015). Patterns of hypothalamic regionalization in amphibians and reptiles: common traits revealed by a genoarchitectonic approach. *Frontiers in Neuroanatomy*, *9*, 3.

Eaton, J. L., & Glasgow, E. (2007). Zebrafish orthopedia (otp) is required for isotocin cell development. *Development Genes and Evolution*, *217*(2), 149–158.

Fernandes, A. M., Beddows, E., Filippi, A., & Driever, W. (2013). Orthopedia transcription factor otpa and otpb paralogous genes function during dopaminergic and neuroendocrine cell specification in larval zebrafish. *Plos One*, *8*(9), e75002.

Filippi, A., Mahler, J., Schweitzer, J., & Driever, W. (2010). Expression of the paralogous tyrosine hydroxylase encoding genes th1 and th2 reveals the full complement of dopaminergic and noradrenergic neurons in zebrafish larval and juvenile brain. *Journal of Comparative Neurology*, *518*(4), 423–438.

Filippi, A., Jainok, C., & Driever, W. (2012). Analysis of transcriptional codes for zebrafish dopaminergic neurons reveals essential functions of Arx and Isl1 in prethalamic dopaminergic neuron development. *Developmental Biology*, *369*(1), 133–149.

Filippi, A., Mueller, T., & Driever, W. (2014). vglut2 and gad expression reveal distinct patterns of dual GABAergic versus glutamatergic cotransmitter phenotypes of dopaminergic and noradrenergic neurons in the zebrafish brain. *Journal of Comparative Neurology*, *522*(9), 2019–2037.

Forlano, P. M., & Cone, R. D. (2007). Conserved neurochemical pathways involved in hypothalamic control of energy homeostasis. *Journal of Comparative Neurology*, *505*(3), 235–248.

Fougère, M., van der Zouwen, C. I., Boutin, J., & Ryczko, D. (2021). Heterogeneous expression of dopaminergic markers and Vglut2 in mouse

mesodiencephalic dopaminergic nuclei A8-A13. *Journal of Comparative Neurology*, *529*(7), 1273–1292.

García-Moreno, F., Pedraza, M., Di Giovannantonio, L. G., Di Salvio, M., López-Mascaraque, L., Simeone, A., & De Carlos, J. A. (2010). A neuronal migratory pathway crossing from diencephalon to telencephalon populates amygdala nuclei. *Nature Neuroscience*, *13*(6), 680–689.

Gerlach, G., & Wullimann, M. F. (2021). Neural pathways of olfactory kin imprinting and kin recognition in zebrafish. *Cell and Tissue Research*, *383*(1), 273–287.

Hayes, L., Zhang, Z., Albert, P., Zervas, M., & Ahn, S. (2011). Timing of Sonic hedgehog and Gli1 expression segregates midbrain dopamine neurons. *Journal of Comparative Neurology*, *519*, 3001–3018.

Herget, U., Wolf, A., Wullimann, M. F., & Ryu, S. (2014). Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. *Journal of Comparative Neurology*, *522*, 1542–1564.

Higashijima, S., Hotta, Y., & Okamoto, H. (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *Journal of Neuroscience*, *20*, 206–218.

Joksimovic, M., Yun, B. A., Kittappa, R., Anderegg, A. M., Chang, W. W., Taketo, M. M., McKay, R. D. G., & Awatramani, R. B. (2009). Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. *Nature Neuroscience*, *12*, 125–131.

Ju, M. J., Aroca, P., Luo, J., Puelles, L., & Redies, C. (2004). Molecular profiling indicates avian branchiomotor nuclei invade the hindbrain alar plate. *Neuroscience*, *128*(4), 785–796.

Kaslin, J., & Panula, P. (2001). Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). *Journal of Comparative Neurology*, *440*(4), 342–377.

Kress, S., & Wullimann, M. F. (2012). Correlated basal expression of immediate early gene egr1 and tyrosine hydroxylase in zebrafish brain and downregulation in olfactory bulb after transitory olfactory deprivation. *Journal of Chemical Neuroanatomy*, *46*(1-2), 51–66.

Levine, R. L., & Dethier, S. (1985). The connections between the olfactory bulb and the brain in the goldfish. *Journal of Comparative Neurology*, *237*(4), 427–444.

Marín, F., Herrero, M. T., Vyas, S., & Puelles, L. (2005). Ontogeny of tyrosine hydroxylase mRNA expression in mid- and forebrain: neuromeric pattern and novel positive regions. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, *234*(3), 709–717.

Medina, L., Bupesh, M., & Abellán, A. (2011). Contribution of genoarchitecture to understanding forebrain evolution and development, with particular emphasis on the amygdala. *Brain, Behavior and Evolution*, *78*(3), 216–236.

Morales-Delgado, N., Merchan, P., Bardet, S. M., Ferrán, J. L., Puelles, L., & Díaz, C. (2011). Topography of somatostatin gene expression relative to molecular progenitor domains during ontogeny of the mouse hypothalamus. *Frontiers in Neuroanatomy*, *5*, 10.

Morales, L., Castro-Robles, B., Abellán, A., Desfilis, E., & Medina, L. (2021). A novel telencephalon-opto-hypothalamic morphogenetic domain coexpressing Foxg1 and Otp produces most of the glutamatergic neurons of the medial extended amygdala. *Journal of Comparative Neurology*, *529*(10), 2418–2449.

Moreno, N., Domínguez, L., Morona, R., & González, A. (2012). Subdivisions of the turtle Pseudemys scripta hypothalamus based on the expression of regulatory genes and neuronal markers. *Journal of Comparative Neurology*, *520*(3), 453–478.

Mueller, T., Vernier, P., & Wullimann, M. F. (2004). The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish *Danio rerio*. *Brain Research*, *1011*(2), 156–169.

Northcutt, R. G. (2006). Connections of the lateral and medial divisions of the goldfish telencephalic pallium. *Journal of Comparative Neurology*, *494*(6), 903–943.

Northcutt, R. G., & Wullimann, M. F. (1988). The visual system in teleost fishes. Morphological patterns and trends. *In* Atema, J., Fay, R. R., Popper, A. N., Tavolga, W. N. (Eds.), *Sensory biology of aquatic animals* (pp. 515–552). Springer.

Osório, J., Mueller, T., Rétaux, S., Vernier, P., & Wullimann, M. F. (2010). Phylotypic expression of the bHLH genes *Neurogenin2*, *NeuroD*, and *Mash1* in the mouse embryonic forebrain. *Journal of Comparative Neurology*, *518*, 851–871.

Puelles, L., Martínez-de-la-Torre, M., Bardet, S., & Rubenstein, J. L. R. (2012). Chapter 8: Hypothalamus. *In* C. Watson, G. Paxinos, L. Puelles (Eds.), *The mouse nervous system* (pp. 221–312). Academic Press-Elsevier.

Rink, E., & Wullimann, M. F. (2001). The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Research*, *889*, 316–330.

Rink, E., & Wullimann, M. F. (2002a). Development of the catecholaminergic system in the early zebrafish brain: an immunohistochemical study. *Developmental Brain Research*, *137*, 89–100.

Rink, E., & Wullimann, M. F. (2002b). Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (*Danio rerio*) lead to identification of an ascending dopaminergic system in a teleost. *Brain Research Bulletin*, *57*(3-4), 385–387.

Rink, E., & Wullimann, M. F. (2004). Connections of the ventral telencephalon (subpallium) in the zebrafish (Danio rerio). *Brain Research*, *1011*(2), 206–220.

Rosner, E., Chagnaud, B. P., & Wullimann, M. F. (2020). Serotonin systems in three socially communicating teleost species, the grunting toadfish (*Allenbatrachus grunniens*), a South American marine catfish (*Ariopsis seemanni*), and the upside-down catfish (*Synodontis nigriventris*). *Journal of Chemical Neuroanatomy*, *104*, 101708.

Ryu, S., Mahler, J., Acampora, D., Holzschuh, J., Erhardt, S., Omodei, D., Simeone, A., & Driever, W. (2007). Orthopedia homeodomain protein is essential for diencephalic dopaminergic neuron development. *Current Biology : CB*, *17*(10), 873–880.

Schredelseker, T., & Driever, W. (2020). Conserved genoarchitecture of the basal hypothalamus in zebrafish embryos. *Frontiers in Neuroanatomy*, *14*, 3.

Semenova, S. A., Chen, Y. C., Zhao, X., Rauvala, H., & Panula, P. (2014). The tyrosine hydroxylase 2 (TH2) system in zebrafish brain and stress activation of hypothalamic cells. *Histochemistry and Cell Biology*, *142*(6), 619–633.

Shkumatava, A., Fischer, S., Müller, F., Strähle, U., & Neumann, C. J. (2004). Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and lamination in the zebrafish retina. *Development (Cambridge, England)*, *131*, 3849–3858.

Simeone, A., D'Apice, M. R., Nigro, V., Casanova, J., Graziani, F., Acampora, D., & Avantaggiato, V. (1994). Orthopedia, a novel homeobox-containing gene expressed in the developing CNS of both mouse and Drosophila. *Neuron*, *13*(1), 83–101.

Smeets, W. J. A. J., & Reiner, A. (1994a). Catecholamines in the CNS of vertebrates: current concepts of evolution and functional significance. *In*: W. J. A. J. Smeets, & A. Reiner (Eds.), *Phylogeny and development of catecholamine systems in the CNS of vertebrates* (pp. 463–481). Cambridge University Press.

Smeets, W. J. A. J., & Reiner, A. (1994b). *Phylogeny and development of catecholamine systems in the CNS of vertebrates*. Cambridge University Press.

Smeets, W. J. A. J., & González, A. (2000). Catecholamine systems in the brain of vertebrates: new perspectives through a comparative approach. *Brain Research Reviews*, *33*, 308–379.

Tay, T. L., Ronneberger, O., Ryu, S., Nitschke, R., & Driever, W. (2011). Comprehensive catecholaminergic projectome analysis reveals

single-neuron integration of zebrafish ascending and descending dopaminergic systems. *Nature Communications*, *2*, 171.

Vernier, P., & Wullimann, M. F. (2009). Posterior tuberculum. *In* M. D. Binder, N. Hirokawa, & U. Windhorst (Eds.), *Encyclopedia of neuroscience* (pp. 1404–1413). Springer.

Vitalis, T., Cases, O., Engelkamp, D., Verney, C., & Price, D. J. (2000). Defects of tyrosine hydroxylase-immunoreactive neurons in the brains of mice lacking the transcription factor Pax6. *Journal of Neuroscience*, *20*, 6501–6516.

Wang, W., & Lufkin, T. (2000). The murine Otp homeobox gene plays an essential role in the specification of neuronal cell lineages in the developing hypothalamus. *Developmental Biology*, *227*(2), 432–449.

Westerfield, M. (2007). *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)* (5th ed.). University of Oregon Press.

Wolf, A., & Ryu, S. (2013). Specification of posterior hypothalamic neurons requires coordinated activities of Fezf2, Otp, Sim1a and Foxb1. *Development (Cambridge, England)*, *140*(8), 1762–1773.

Wullimann, M. F. (1998). The central nervous system. *In* D. H. Evans (Ed.), *Physiology of fishes* (pp. 245–282). CRC Press.

Wullimann, M. F., & Rink, E. (2002). The teleostean forebrain: a comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. *Brain Research Bulletin*, *57*(3-4), 363–370.

Wullimann, M. F. (2011). Basal ganglia: insights into origins from lamprey brains. *Current Biology: CB*, *21*(13), R497–R500.

Wullimann, M. F. (2014). Ancestry of basal ganglia circuits: new evidence in teleosts. *Journal of Comparative Neurology*, *522*, 2013–2018.

Wullimann, M. F. (2020). Neural origins of basal diencephalon in teleost fishes: radial versus tangential migration. *Journal of Morphology*, *281*(9), 1133–1141.

Wullimann, M. F., & Umeasalugo, K. E. (2020). 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. *Journal of Comparative Neurology*, *528*(8), 1321–1348.

Wullimann, M. F., Rupp, B., & Reichert, H. (1996). *Neuroanatomy of the zebrafish brain. A topological atlas*. Birkhäuser Verlag. https://doi.org/10.1007/978-3-0348-8979-7

Xavier, A. L., Fontaine, R., Bloch, S., Affaticati, P., Jenett, A., Demarque, M., Vernier, P., & Yamamoto, K. (2017). Comparative analysis of monoaminergic cerebrospinal fluid-contacting cells in Osteichthyes (bony vertebrates). *Journal of Comparative Neurology*, *525*(9), 2265–2283.

Yamamoto, K., Ruuskanen, J. O., Wullimann, M. F., & Vernier, P. (2010). Two tyrosine hydroxylase genes in vertebrates: new dopaminergic territories revealed in the zebrafish brain. *Molecular and Cellular Neuroscience*, *43*, 394–402.

Yamamoto, K., Ruuskanen, J. O., Wullimann, M. F., & Vernier, P. (2011). Differential expression of dopaminergic cell markers in the adult zebrafish forebrain. *Journal of Comparative Neurology*, *519*, 576–598.

Yáñez, J., Suárez, T., Quelle, A., Folgueira, M., & Anadón, R. (2018). Neural connections of the pretectum in zebrafish (*Danio rerio*). *Journal of Comparative Neurology*, *526*(6), 1017–1040.

How to cite this article: Eugenin von Bernhardi, J., Biechl, D., Miek, L., Herget, U., Ryu, S., & Wullimann, M. F. (2022). A versatile transcription factor: Multiple roles of *orthopedia a* (*otpa*) beyond its restricted localization in dopaminergic systems of developing and adult zebrafish (*Danio rerio*) brains. *Journal of Comparative Neurology*, *530*, 2537–2561. https://doi.org/10.1002/cne.25351