

## RESEARCH ARTICLE

# The organization of the zebrafish pallium from a hodological perspective

Julián Yáñez<sup>1,2</sup>  | Mónica Folgueira<sup>1,2</sup>  | Ibán Lamas<sup>1</sup>  | Ramón Anadón<sup>3</sup> 

<sup>1</sup> Department of Biology, Faculty of Sciences, University of A Coruña, Coruña, Spain

<sup>2</sup> Centro de Investigaciones Científicas Avanzadas (CICA), University of A Coruña, Coruña, Spain

<sup>3</sup> Department of Functional Biology, Faculty of Biology, University of Santiago de Compostela, Santiago de Compostela, Spain

## Correspondence

Julián Yáñez, Department of Biology, Faculty of Sciences, University of A Coruña. Campus da Zapateira, 15008 A Coruña, Spain.  
Email: [julian.yanez@udc.es](mailto:julian.yanez@udc.es)

## Abstract

We studied the connections (connectome) of the adult zebrafish pallium using carbocyanine dye tracing and ancillary anatomical methods. The everted zebrafish pallium (dorsal telencephalic area, D) is composed of several major zones (medial, lateral, dorsal, central, anterior, and posterior) distinguishable by their topography, cytoarchitecture, immunohistochemistry, and genoarchitecture. Our comprehensive study reveals poor interconnectivity between these pallial areas, especially between medial (Dm), lateral/dorsal (DI, Dd), and posterior (Dp) regions. This suggests that the zebrafish pallium has dedicated modules for different neural processes. Pallial connections with extrapallial regions also show compartmental organization. Major extratelencephalic afferents come from preglomerular nuclei (to DI, Dd, and Dm), posterior tuberal nucleus (to Dm), and lateral recess nucleus (to DI). The subpallial (ventral, V) zones dorsal Vv, Vd, and Vs, considered homologues of the striatum, amygdala, and pallidum, are mainly afferent to DI/Dd and Dp. Regarding the efferent pathways, they also appear characteristic of each pallial region. Rostral Dm projects to the dorsal entopeduncular nucleus. Dp is interconnected with the olfactory bulbs. The central region (Dc) defined here receives mainly projections from DI–Dd and projects toward the pretectum and optic tectum, connections, which help to delimiting Dc. The connectome of the adult pallium revealed here complements extant studies on the neuroanatomical organization of the brain, and may be useful for neurogenetic studies performed during early stages of development. The connectome of the zebrafish

**Abbreviations:** *ac*, anterior commissure; *APN*, accessory pretectal nucleus; *bc*, brachium conjunctivum; *CCb*, cerebellar corpus; *ch*, habenular commissure; *CM*, mammillary nucleus; *CP*, central posterior thalamic nucleus; *CPe*, central pretectal nucleus; *cpo*, posterior commissure; *cpop*, postoptic commissure; *D*, diffuse nucleus of the hypothalamic inferior lobe; *Dc*, central zone of the dorsal telencephalic area; *DI*, lateral zone of the dorsal telencephalic area; *DId*, dorsal part of *DI*; *Dlv*, ventral part of *DI*; *Dm*, medial zone of the dorsal telencephalic area; *Dma*, anterior part of *Dm*; *Dmp*, caudal part of *Dm*; *DP*, dorsal posterior thalamic nucleus; *Dp*, posterior zone of the dorsal telencephalic area; *DT*, dorsal tegmental nucleus; *Ent*, entopeduncular nucleus; *fr*, fasciculus retroflexus; *Hb*, habenula; *hc*, horizontal commissure; *Hv*, dorsal hypothalamic nucleus; *III*, oculomotor nucleus; *IL*, lateral inferior lobe; *Ip*, interpeduncular nucleus; *lfb*, lateral forebrain bundle; *LI*, lateral lemniscus; *lot*, lateral olfactory tract; *mif*, medial longitudinal fascicle; *mot*, medial olfactory tract; *MT*, mesencephalic tegmentum; *nl*, nucleus isthmi; *nlot*, nucleus of the lateral olfactory tract; *NLR*, nucleus of the lateral recess; *NLV*, lateral valvular nucleus; *nmlf*, nucleus of the medial longitudinal fascicle; *OB*, olfactory bulb; *OT*, optic tectum; *ot*, optic tract; *P*, pineal organ; *PcE*, central pretectal nucleus; *PcO*, paracommissural pretectal nucleus; *PGa*, anterior preglomerular nucleus; *PGI*, lateral preglomerular nucleus; *PGm*, medial preglomerular nucleus; *PL*, posterior lobe; *Ppa*, anterior parvocellular preoptic nucleus; *Ppp*, posterior parvocellular preoptic nucleus; *PSp*, parvocellular superficial pretectal nucleus; *PTh*, prethalamus; *PTN*, posterior tuberal nucleus; *r*, superior raphe nucleus; *rb*, retrobulbar region; *rIII*, oculomotor tract; *RL*, rostralateral nucleus; *SCh*, suprachiasmatic nucleus; *SGVN*, secondary gustatory/visceral nucleus; *sy*, sulcus ypsiloniformis; *Tg*, dorsal tegmental nucleus; *TG*, rhombencephalic tegmentum; *TLa*, torus lateralis; *TLo*, torus longitudinalis; *TPp*, periventricular posterior tubercle nucleus; *Ts*, torus semicircularis; *V*, ventral telencephalic area (subpallium); *Vc*, central nucleus of *V*; *VCb*, cerebellar valvula; *Vd*, dorsal nucleus of *V*; *Vl*, lateral nucleus of *V*; *Vp*, posterior nucleus of *V*; *Vs*, supracommissural nucleus of *V*; *Vv*, ventral nucleus of *V*

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pallium was also compared with the pallial connections reported in other teleosts, a large group showing high pallial diversity.

#### KEYWORDS

connectome, *Danio rerio*, Dil, neural tracing, telencephalon

## 1 | INTRODUCTION

The telencephalon of vertebrates is considered the main high processing center of the brain. It consists of the olfactory bulbs and telencephalic hemispheres, the latter containing two main regions: the pallium and subpallium. In tetrapods as amphibians and mammals, the pallium is organized medio-laterally in main longitudinal zones, namely, the medial pallium or (hippocampus, i.e., dentate gyrus plus Ammon's horn in mammals), the dorsal pallium (DP) or neocortex, the lateral pallium, and the ventral pallium plus its derivative pallial amygdala (Medina & Abellán, 2009; Moreno & González, 2007; Puelles et al., 2000; Watson & Puelles, 2017). This zonal pallial configuration in evaginated telencephalons of tetrapods is apparently different from that of the everted telencephalon that is present in actinopterygians. Everted telencephalons have a single T-shaped ventricle and that extends over most of the pallium, which is reversed and whose ependymal region faces to it and is covered by an extensive choroid tela (Nieuwenhuys, 1963; Nieuwenhuys, 2009a, 2009b; Northcutt & Braford, 1980). The teleost telencephalic hemispheres also consist of dorsal (D) and ventral (V) telencephalic areas homologous to the mammalian pallium and subpallium, respectively. Further subdivisions of the pallium and subpallium in teleosts are most often named based on their topographic position within this everted telencephalon. This is because there are difficulties when establishing homologies between areas of the telencephalon of teleosts and other vertebrates. Thus, in the teleost subpallium, the following areas are generally recognized in most species: the ventral (Vv), dorsal (Vd), central (Vc), lateral (Vl), supracommissural (Vs), and postcommissural (Vp) V nuclei and the entopeduncular nucleus, considered to be field homologues of different subpallial structures of mammals (Ganz et al., 2012; Gerlach & Wullimann, 2021; Maruska et al., 2017; Northcutt & Braford, 1980; Porter & Mueller, 2020). Regarding the teleost pallium (D), several major zones have been recognized in most species, named topographically as lateral (DI), dorsal (Dd), medial (Dm), central (Dc), and posterior (Dp) zones, plus the nucleus taeniae (nucleus of the lateral olfactory tract) (Meek & Nieuwenhuys, 1998; Nieuwenhuys, 1963, 2009, 2011; Northcutt, 2006; Northcutt & Braford, 1980; Wullimann & Mueller, 2004; Yamamoto, 2009; Yamamoto & Ito, 2008; Yamamoto et al., 2007). However, cytoarchitectonical identification of pallial areas is often complex and may differ substantially between authors studying the same species (compare maps of goldfish telencephalon in Northcutt, 2006, and Yamamoto & Ito, 2008).

The eversion process of the telencephalon of actinopterygians challenges establishing homologies between regions of everted and evaginated telencephalons (Braford, 2009; Nieuwenhuys, 2009a; Yamamoto, 2009). The "simple eversion model" (Gage, 1893;

Studnička, 1896) proposes that the putative homologues of the medial-to-lateral zones of tetrapods are located in a reversed lateral-to-medial sequence in teleosts (Northcutt, 2008; Wullimann & Mueller, 2004). Based on this theory, the teleost DI would correspond to the mammalian medial pallium and Dm to the mammalian ventral and/or lateral pallium (Northcutt, 2008; Wullimann & Mueller, 2004). These correspondences received some support from functional studies in cyprinids that analyzed fish behavior after lesions into these two pallial areas (Portavella, Vargas, et al., 2002; Portavella, Torres, et al., 2004). Some aspects of "the simple eversion model" have been challenged recently by anatomical and developmental studies that drew a more complex picture of eversion (Folgueira et al., 2012; Mueller & Wullimann, 2009; Yamamoto, 2009; Yamamoto et al., 2007). In this sense, it has been shown that eversion is more complex than a simple medial to lateral out-folding of the neural tube (Folgueira et al., 2012; Furlan et al., 2017). Anyway, the organization of the pallium shows important differences between species and in advanced teleosts, such as acanthopterygians, and the pallium often exhibits much more complex cytoarchitectonical subdivisions without known mammalian correlates (Burmeister et al., 2009; Hagio et al., 2020; Kawaguchi et al., 2019; Maruska et al., 2017).

Deciphering the connectivity of the vertebrate pallium is basic for understanding the functions of its different regions, as well as their role in specific behaviors. A number of experimental studies have been performed in rat and mouse, main mammalian models in neurobiology. As a result, the connectivity of the pallium in murine species is known in great detail (Oh et al., 2014; Paxinos, 2014; Zingg et al., 2014). In teleosts, tract tracing studies have reported the connections of pallial areas in several species, mostly in goldfish (Northcutt, 2006; Yamamoto & Ito, 2005a, 2005b, 2008), electric fish (Correa & Hoffmann, 1999; Giassi et al., 2012; Giassi et al., 2012; Sas et al., 1993; von der Emde & Precthl, 1999), rainbow trout (Folgueira et al., 2004a, 2004b), and some acanthopterygians (Hagio, Sato, et al., 2018; Hagio, Kawaguchi, et al., 2021; Murakami et al., 1983). The results of these studies reveal some conserved pallial connections, among them the projections to Dp from the olfactory bulb and to DI and/or Dm from preglomerular nuclei (Folgueira, Huesa, et al., 2002; Folgueira, Anadón, et al., 2004a, 2004b, 2005; Kato et al., 2012; Northcutt, 2006; Striedter, 1991; Yamamoto & Ito, 2005a, 2008; Yang et al., 2007; Zupanc, 1997). Pallial projections to the hypothalamus, posterior tubercle, pretectum, and optic tectum were also reported (Folgueira et al., 2004a, 2004b; Yáñez et al., 2018).

The zebrafish (*Danio rerio*) is a model fish species in neurobiology. A large number of studies in embryos and larvae have revealed the great potential of zebrafish for studying the development of specific neural populations and circuits, as well as their relationship with well-characterized behaviors (Antinucci et al., 2019; Kunst et al., 2019; Lal

et al., 2018; Miyasaka et al., 2009; Turner et al., 2016; von Trotha et al., 2014). Knowledge of the zonal organization of the adult zebrafish pallium is mainly based on studies using cytoarchitecture and expression patterns of some markers and transgenes (Wullimann et al., 1996; Wullimann & Mueller, 2004; Castro et al., 2006; Mueller et al., 2011; Ganz et al., 2012, 2014; Diotel et al., 2015; Furlan et al., 2017; Lal et al., 2018; Bloch et al., 2020; Porter & Mueller, 2020). With the exception of the olfactory system (Miyasaka et al., 2009, 2014; Gayoso et al., 2011, 2012; Kermen et al., 2020), the connectivity of the adult zebrafish pallium has received scarce experimental attention. The aim of the present study is to comprehensively analyze the connections of the pallial zones of the adult zebrafish and contribute to the understanding of brain circuitry in teleosts. Our connectivity results in the adult pallium are complementary to those of single cell studies in larval zebrafish (Miyasaka et al., 2014; Kunst et al., 2019), and may facilitate comparison with larval connectivity data and also with hodological studies in adult brains of other fishes.

## 2 | MATERIAL AND METHODS

### 2.1 | Animals

Forty-four adult zebrafish (*Danio rerio*) of both sexes, ranging 28–41 mm in length, were used in this study. Fish were kept in aquaria at 28 °C under standard conditions (Aleström et al., 2019). Fish were euthanized by tricaine methanesulfonate salt overdose (MS222, Sigma-Aldrich, St. Louis, MO, USA) in freshwater, followed by exsanguination by perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PFA) through the heart. Heads were kept overnight in the same fixative at 4 °C, and then brains were dissected out and maintained in fresh fixative until tracing experiments were performed. As tracing experiments are done in fixed tissue, this minimizes animal suffering. All experiments conformed to Spanish and European Community's guidelines on animal care and experimentation.

### 2.2 | Carbocyanine dye tracing

Procedures used for carbocyanine dye tracing were similar to those applied in previous studies in zebrafish and other teleosts (Folgueira et al., 2004a, 2004b, 2006; Yáñez, Busch, et al., 2009; Yáñez, Souto, et al., 2017). Briefly, the carbocyanine dye Dil (catalog no. D282; Invitrogen, Eugene, OR, USA) was applied using a minute insect pin (000) with different strategies depending on accessibility to the targeted areas (see below and Table 1). Dil was used alone or in combination with the carbocyanine dye DiO (catalog no: D275; Invitrogen, Eugene) for double labeling (1 case).

For superficial nuclei/areas, a small crystal of Dil was directly inserted in the brain under a stereomicroscope, and then the area was sealed with melted 3% agarose in 0.1 M phosphate buffer pH 7.4 (PB). For deeper nuclei/areas, brains were embedded in 3% agarose and transversally sectioned (rostralwards or caudalwards, as appropriate) on a Vibroslice (Campden, Instruments, UK) until reaching the area of

**TABLE 1** Summary of labeling experiments conducted in this study using intact whole brain (in toto) or sectioned brain. For the procedure in sectioned brains, it is indicated if the projections were studied toward rostral or caudal in the brain. For abbreviations, see the list

Brain structure	No. of experiments	Labeling procedure
Dma	2	in toto
Dm(1/2)	2	1 sectioned brain to caudal, 1 in toto after hemilobectomy
Dmp	2	in toto
DI (rostral)	1	in toto
Dld	2	in toto
Dlv	1	in toto & double labeled in contralateral Dp
Dd	4	in toto
Dp	3	2 in toto, 1 sectioned to caudal
Dc	2	sectioned brain to caudal
PGI	3	2 in toto, 1 sectioned to rostral
Tla	3	in toto
HL (lateral)	3	in toto
HL (medial)	1	sectioned brain to rostral
PL	2	in toto
PTN	2	sectioned brain to rostral
OB	3	in toto
OT (rostral)	4	in toto
raphe	1	sectioned brain to rostral

interest. Some transverse sections were stained with 1% cresyl violet and observed under a microscope to facilitate identification of the nuclei/area of interest. Once identified, a small crystal of the tracer was applied onto the corresponding area/nucleus under a stereomicroscope and then sealed with melted 3% agarose. To assess with precision the structures accessed, photographs of brains with the crystals in injected areas were taken the day of application. For the labeled areas/nuclei accessed and the number of experiments per area/nuclei, see Table 1. Precision in Dil application procedures helped to reduce to a minimum the number of animals employed.

After Dil application, brains were incubated in PFA in the darkness at 37 °C for 5–30 days to allow diffusion along cell membranes. Shorter incubation times were used to label intratelencephalic connections, while longer ones to label extratelencephalic connections. After incubation, agarose blocks containing the labeled brains were transversally sectioned (70–80 μm in thickness) on a Vibroslice and sections were mounted in 50% (v/v) glycerine-0.2 M phosphate buffer.

Sections were analyzed and photographed under a fluorescence microscope (Eclipse 90i; Nikon, Tokyo, Japan) using appropriate fluorescence filter set for Dil and a digital camera (DP70; Olympus, Tokyo). Occasionally, images of two or three focal planes were taken using camera control software (DP Manager, Nikon) and rendered into an axial projection. For plate composition, single-channel images were inverted and converted into gray scale. Images were optimized

for contrast and brightness using CorelDraw Graphics Suite (Ottawa, Canada, RRID:SCR\_014235). For most sections, tissue autofluorescence allowed identification of various brain areas and structures without additional counterstaining methods. During composition of plates, autofluorescence was minimized to show labeled structures over little background.

With regard to the tracing method used here, it should be noted that Dil diffuses both retrogradely and anterogradely along neuronal processes. This implies that the dye uptake by an axon can lead to both retrograde labeling of the soma and retrograde/anterograde labeling of collaterals and branches (Folgueira et al., 2004a). Thus, connections need to be confirmed by application of the dye to putative targets of a given region.

### 2.3 | Complementary material

For the regional analysis of the zebrafish brain and comparison with the topography revealed in Dil experiments, various series of transverse sections stained with general methods (Nissl) or immunohistochemistry against different enzymes and peptides (GAD, ChAT, TH, neuropeptide Y, calretinin, galanin, FMRF, KLH) used in previous studies of our group (Castro et al., 2006; Gayoso et al., 2011; Turner et al., 2016) were at our disposal (for details of methods, see these publications). In addition, we used conventional HuC/D immunofluorescence for staining neurons in three additional brains. Briefly, paraformaldehyde perfused brains were cryoprotected with 30% sucrose in PB, included in Tissue-Tek (Sakura Finetek Europe, Zuid-Holland, Netherlands) frozen with liquid nitrogen and sectioned on a cryostat (14  $\mu$ m thickness). Sections were incubated sequentially in normal goat serum (G6767, Sigma-Aldrich; dilution 1:1), monoclonal mouse anti-HuC/D (IgG2b, cloneA21271, Invitrogen RRID:AB\_221448) diluted in PBS containing NGS 1:500 and 0.1% Triton X-100 for 48 h, and finally with Alexa Fluor 568-coupled goat anti-mouse (A-11031, ThermoFisher; dilution 1:50; RRID:AB\_144696), for 2 h at room temperature.

For assessing pallial regions, we consulted published photomicrographs of transverse sections showing pallial distributions of various other markers (Mueller et al., 2011; Ganz et al., 2014; Bloch et al., 2019, 2020; Porter & Mueller, 2020).

### 2.4 | Nomenclature used

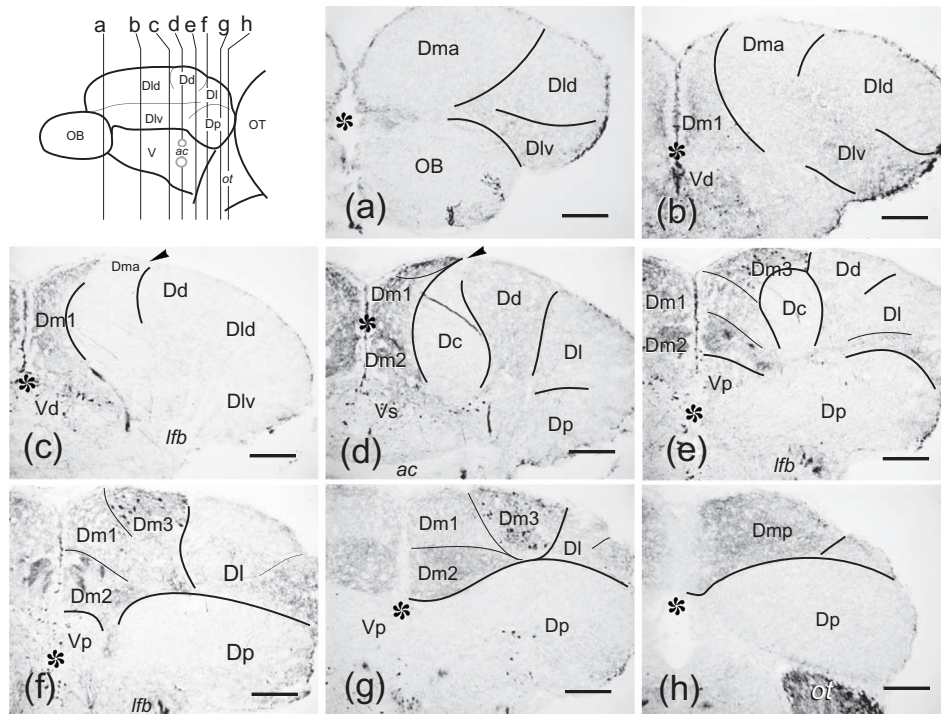
Unless otherwise stated, the general brain nomenclature used here is based on the atlas of adult zebrafish brain (Wullmann et al., 1996), excepting for the pallium. For convenience, the pallial regions studied here and their names (Dma, Dla, Dm, Dc, Dd, Dl, and Dp) are mainly based in Castro et al. (2006) and Furlan et al. (2017). The correspondence between these pallial regions with some regions proposed in alternative models of zebrafish pallial organization (Mueller et al., 2011; Ganz et al., 2014; Porter & Mueller, 2020) will be addressed appropriately in the text.

## 3 | RESULTS

### 3.1 | Regions of the zebrafish pallium approached experimentally

As mentioned previously, the zones of the zebrafish pallium experimentally accessed are mostly named on those described in Castro et al. (2006), providing also correspondences with the pallial organization proposed by other authors (Porter & Mueller, 2020). The regions of the zebrafish dorsal telencephalic area or pallium (D) were mapped with Nissl staining and calretinin immunohistochemistry (Figures 1 and 2), to assess their locations in order to apply tracers precisely. The distinguishable pallial regions in which we centered Dil application experiments were the anterior medial and lateral divisions of the anterior pallium (Dma, Dla of Castro et al., 2006), the extensive medial and lateral regions (Dm, Dl), a central region or nucleus of D (Dc; a part of the region named as DP by Porter & Mueller, 2020), a posterior pallium region (Dp; named IOP by Porter & Mueller, 2020) that is located in a caudo-lateral location, and a small dorsal region (Dd) located just lateral to the sulcus ypsiloniformis included in the Dl by Porter and Mueller (2020). Dma is a rostromedial part of the pallium just rostral to Dm from which it can be distinguished by the lack of CR-immunoreactive innervation (Castro et al., 2006; present observations). The remainder Dm can be subdivided into three to four subregions (Dm1, Dm2, Dm3, and Dmp) distinguishable using immunohistochemical methods (Castro et al., 2006; present results, Figure 2). Owing to the difficulty for distinguishing limits between these areas in fresh tissue, for tracer application to Dm, we only considered two regions, the commissural (Dm1 plus Dm2) and caudal (Dm3 plus Dmp), although some subdivisions were distinguishable by their afferents (see below). We considered Dc as the central region (nucleus) mainly recognizable by its very rich innervation by parvalbumin-immunoreactive fibers and other differential immunochemical signatures (Castro et al., 2006; von Trotha et al., 2014; Furlan et al., 2017; Porter & Mueller, 2020), as well as by differential efferents (present results). In recent proposals, a homonymous Dc is a much wider region extending till the dorsorostral pole of the pallium (DP; Mueller et al., 2011; Ganz et al., 2014; Porter & Mueller, 2020), which includes most of our Dma and Dml and will be discussed below in the light of present experimental results. Zebrafish Dp (not to be confused with that homonymous region of Porter & Mueller, 2020) is separated from Dl by a lamina of neurons, and shows different immunochemical signatures (Castro et al., 2006) and olfactory connections (Miyasaka et al., 2009; Gayoso et al., 2011, 2012; present results), as in other teleosts. Although Dp is adjacent to the nucleus of the lateral olfactory tract (nLot, nucleus taeniae) (Porter & Mueller, 2020), this nucleus was not directly accessed in our experiments because of the difficulty to apply Dil here without affecting Dp and the tract. Dd (as used by us) is a region adjacent to and hardly distinguishable from Dl with immunochemical markers but some heterogeneity in distribution of immunochemical markers was noted inside these pallial regions. Examples of the areas accessed in experiments of Dil application to various brain regions are included in the Figure 3.





**FIGURE 2** (a–h) Photomicrographs of transverse sections through the zebrafish pallium at precommissural (a–c), commissural (d), and postcommissural (e–h) levels, showing calretinin immunoreactivity. Note differences in fiber density and presence of immunoreactive cells that were used to outline zones and subregions. Asterisks: midline telencephalic ventricle. Arrowheads: sulcus ypsilonformis. For abbreviations, see the list. Scale bars: 100  $\mu\text{m}$

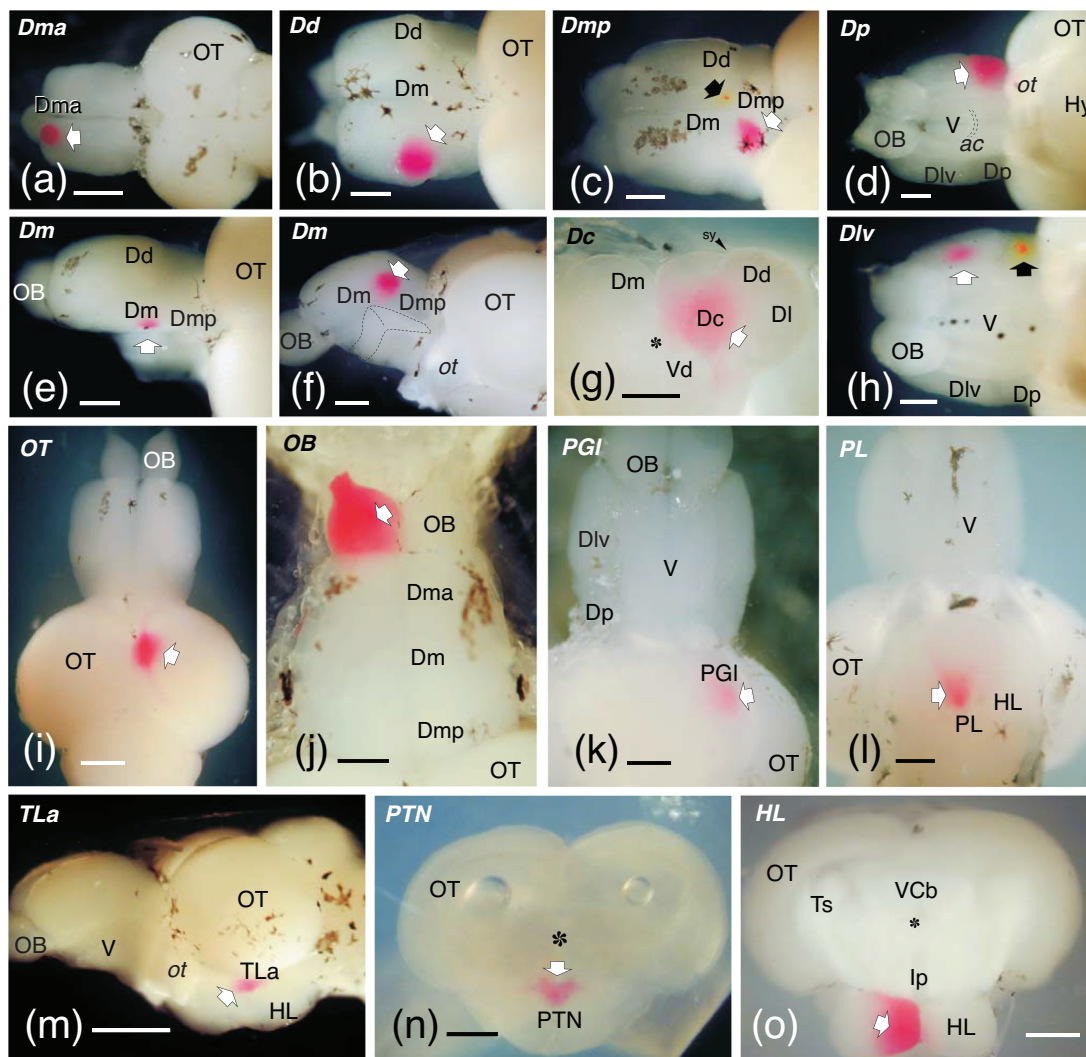
olfactory bulb (not shown) and the retrobulbar periventricular ventral telencephalic area (Figure 4a). At commissural and postcommissural levels, cell bodies and processes were labeled bilaterally in the posterior Dm (Figures 4c–d and 5b).

Numerous extratelencephalic cell bodies and fibers were labeled, mostly ipsilaterally (Figures 4e–k). Labeled cells (Dm afferents) were mostly observed in the thalamus, posterior tuberal nucleus (PTN), preglomerular complex, tegmentum, and isthmus. Some labeled fibers coursed through the rostralateral nucleus of the thalamus and the habenulae, crossing at the habenular commissure (Figures 4e and 5c) and ascending in the contralateral forebrain bundle. In the pretectal area, labeled fibers coursed ipsilaterally in the central pretectal nucleus, and, bilaterally, in the pretectal paracommissural nucleus, where occasional labeled cells were also observed (Figures 4f–g and 5d–f). More caudally, a few cells were labeled ipsilaterally in the dorsal posterior thalamic nucleus (DP) and the PTN (Figures 4g–h and 5i–k), and bilaterally in a small nucleus dorsal to the postoptic commissure (Figure 5h) that may be the suprachiasmatic nucleus. Labeled fibers were observed coursing in the lateral and medial forebrain bundle (Figures 4g). A number of cell bodies were labeled in the lateral and anterior preglomerular nuclei (Figures 4g and 5g), but only a few cell bodies were labeled more caudally in the medial preglomerular nucleus (Figures 4h and 5l). Labeled fibers from the lateral forebrain bundle also coursed in the dorsal and medial region of the ipsilateral hypothalamic inferior and posterior lobes (PLs) (Figures 4i–j and 5m). In the mesencephalon, some cells and fibers were labeled in the ipsilateral tegmentum, and some fibers reached

the superior raphe at isthmic levels (Figures 4i and 5m–n). A few cells were also labeled in the rostral part of the secondary gustatory/visceral nucleus (Figures 4j and 5o).

### 3.3 | Tracer application to the medial anterior region of D (Dma)

Medial (Dma) and lateral (Dla) anterior regions were distinguished by Castro et al. (2006) in the rostral pallium with immunohistochemical methods. Here, we describe the connections of Dma (Figure 6). We observed that connections of Dma with other pallial and subpallial regions were scarce. After applying a minute Dil crystal to the very rostral pole of Dma, we observed densely labeled structures in Dma but not in Dla (Figure 6a), showing a sharp limit between these two areas. This supports the distinction between these two regions by Castro et al. (2006). Dm1 was labeled as a caudal continuation of the Dma neuropil till the level of the rostralmost portion of the sulcus ypsilonformis, but no labeling was observed in Dm2, Dm3, or posterior Dm (Figures 6b–e). From the densely labeled Dma, bundles of labeled fibers coursed ventrolaterally and caudally, entered the lateral forebrain bundle and were followed to the (dorsal) entopeduncular nucleus, where a few cell bodies were labeled retrogradely. This projection has been previously reported based on NPY expression in zebrafish (see Turner et al., 2016). Some labeled fibers crossed the midline in the anterior commissure, but no cell bodies were labeled in the contralateral side (Figures 6d–f). No labeled fibers or cell bodies



**FIGURE 3** Photographs of brains showing examples of the areas labeled with Dil (outlined arrows) and DiO (black arrows) in various types of experiments. Most applications were done in toto. (a)–(c), and (j) are dorsal views. (d), (h), (k), and (l) are ventral views and (m) is a lateral view. (e) and (f) are dorsal and medial views of the same brain in which the left hemisphere was removed. Examples also include applications to transverse-sectioned en-block brains (g, n, o). In (a–f), (h), and (m), rostral is to the left; in (i–l), rostral is to the top. In (g), (n), and (o), dorsal is to the top. Scale bars: 500  $\mu\text{m}$  (a, i, m); 250  $\mu\text{m}$  (b–h, j–l, n–o)

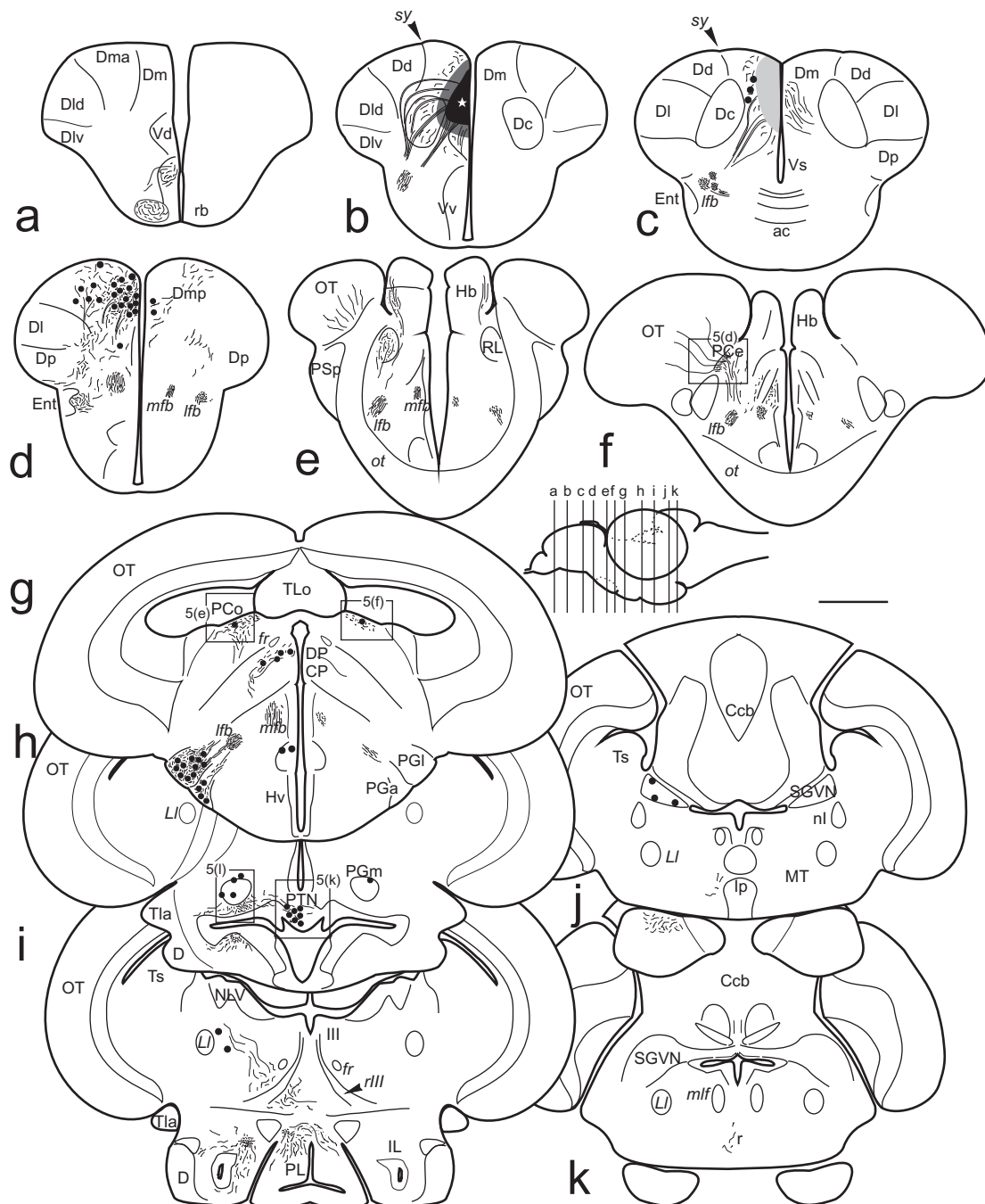
were observed caudal to the dorsal entopeduncular nucleus in these experiments.

### 3.4 | Tracer application to the caudal region of Dm (Dm3/Dmp)

In the pallium, Dil application to the caudal region of Dm (Figure 7) led to labeling cell bodies and fibers in Dm2, but not in Dma or other pallial regions (Figures 7a–d). Some labeled cells in Dm2 showed a complex morphology, with thorny dendrites and beaded fibers that might form terminal fields within Dm2 (Figure 7d). In the subpallium, some labeled cell bodies were observed in Vd/Vs and also in the entopeduncular nucleus at the level of the anterior commissure (Figures 7c

and e–f). Labeled fiber bundles coursed ventrolaterally, most of them across Dc but some across Dp, to join the lateral forebrain bundle (Figure 7g). Caudal to the telencephalon, occasional cell bodies were labeled in the ipsilateral ventral hypothalamus and the nucleus of the hypothalamic PL (not shown). A few cell bodies and dendritic processes were also labeled bilaterally in the medial preglomerular nucleus (Figure 7l).

In one experiment, in which there was some diffusion from Dm3 into the most caudal Dl/Dd, labeling was more extended. In the ipsilateral telencephalic lobe, subventricular neurons were labeled in a lateral region of Dl. These cells showed pear-shaped perikarya with long dendrites (Figure 7h). In the preoptic region, some cell bodies were labeled bilaterally in the posterior periventricular preoptic nucleus and in the suprachiasmatic nucleus (Figures 7i–j). A few cell bodies



**FIGURE 4** (a–k) Schematic drawings of transverse sections of the zebrafish brain showing structures labeled after application of Dil to the medial zone of the pallium, including Dm1 and 2. Sections are arranged from rostral to caudal and the section levels are indicated in the figurine of the lateral view of the brain. Black circles: retrogradely labeled cells. Small dots and lines: labeled fibers. Shaded areas in (b–c) represent the diffusion area of the tracer from the application point (white star). Scale bar for sections: 500  $\mu$ m

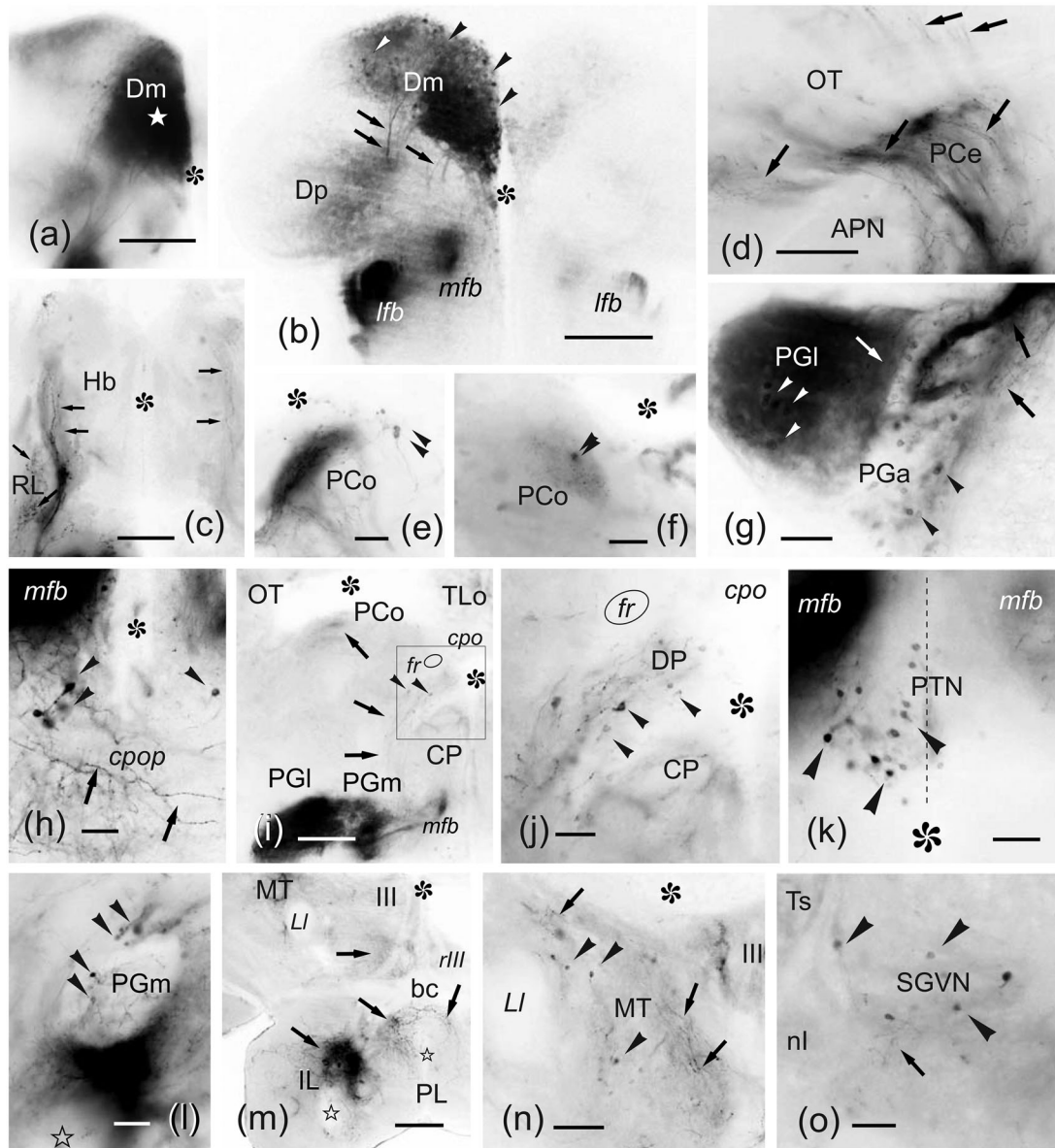
were also labeled in the ventral hypothalamus (Hv), in the medial preglomerular nucleus (PGm) (Figures 7k–l), and occasionally in the hypothalamic PL (not shown). Labeled fibers coursing in the medial forebrain bundle reached the periventricular nucleus of the posterior tubercle bilaterally, but no labeled cell bodies or terminal fields were observed in the PTN (not shown). Caudally, labeled fibers were followed in the ipsilateral ventral mesencephalic tegmentum toward the caudal region of the lateral nucleus of the valvula (NLV)/secondary

visceral nucleus, where a few labeled cells were also observed (not shown).

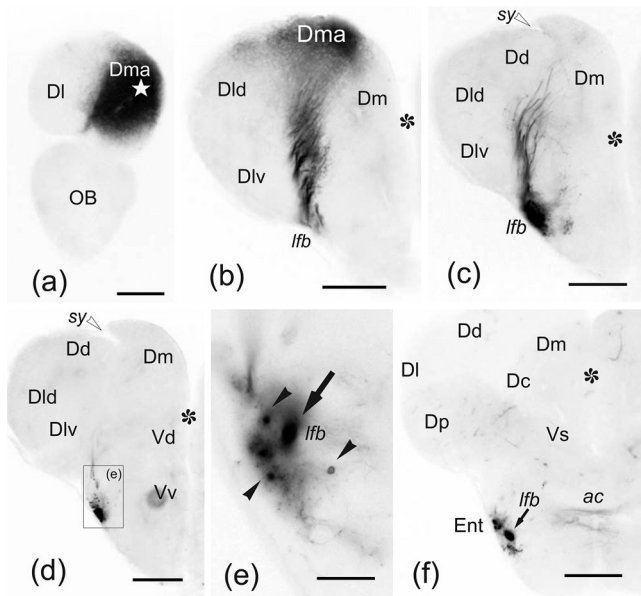
### 3.5 | Tracer application to the lateral zone of the pallium (DI)

The lateral zone of the zebrafish pallium (DI) is composed of dorsal (Dld) and ventral (Dlv) regions that can be distinguished using





**FIGURE 5** (a–o) Photomicrographs of transverse sections of the zebrafish brain showing labeled structures after application of Dil to the medial zone of the pallium (Dm1–2). The correspondence with the levels of Figure 4 is indicated. (a) Section close to the application point (white star) (see Figure 4b). (b) General view of the posterior telencephalic lobe showing numerous labeled cells in caudal Dm (see Figure 4d). (c) Detail of the habenular region showing labeled varicose fibers in the ipsilateral rostromedial nucleus and in a lateral plexus in both habenulae (see Figure 4e). (d) Labeled varicose fibers coursing through the ipsilateral central pretectal nucleus (see Figure 4d) and entering the optic tectum. (e–f) Detail of labeled cell bodies and fibers in the ipsi- (e) and contralateral (f) paracommissural pretectal nucleus (see Figure 4g). (g) Section through the anterior and lateral preglomerular nuclei, showing numerous labeled cells and fibers (see Figure 4g). (h) Detail of a small group of retrogradely labeled cell bodies close to the medial forebrain bundle and dorsal to the postoptic commissure. (i–j) General view (i) and detail (j) of the ipsilateral dorsal posterior thalamic nucleus showing a few cell bodies and fibers labeled. Note also the dense neuronal labeling in the medial and lateral preglomerular nuclei. (k) Detail showing retrogradely labeled cell bodies in the ipsilateral side of the posterior tubular nucleus (the broken line indicates the midline) (see Figure 4h). (l) Detail showing a few cell bodies and fibers labeled in the medial preglomerular nucleus (see Figure 4h). (m) Panoramic view showing numerous labeled fibers in the ventral mesencephalic tegmentum around the third nerve root, and in the hypothalamic inferior and posterior lobes (see Figure 4i). (n) Detail of labeled cell bodies and fibers between the lateral lemniscus and the oculomotor nucleus in the medial mesencephalic tegmentum. (o) Retrogradely labeled cell bodies in the rostral part of the secondary gustatory-visceral nucleus (see Figure 4j). Arrowheads point to cell bodies. Arrows point to fibers and fiber tracts. For abbreviations, see the list. Asterisk: ventricle. Outlined star: recesses of the inferior lobes. Medial is to the right. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (a–b, i, m); 100  $\mu\text{m}$  (c–d, n); 50  $\mu\text{m}$  (e–h, j–l, o)



**FIGURE 6** (a–f) Photomicrographs of transverse sections through the telencephalic lobes of zebrafish, from rostral to caudal, showing labeled cell bodies (black arrowheads) and fibers (arrows) after application of Dil to the rostralmost medial zone of the pallium (Dma). (a) Labeling of Dma around the accessed region (white star). (b–c) Sections of more caudal regions showing scant Dma connections with adjacent pallial regions (b), and small fiber bundles coursing toward the (lfb) through deeper pallial regions. (d–f) A terminal field labeled in the entopeduncular nucleus. In (e), note afferent neurons to Dma (arrowheads). Asterisk: telencephalic ventricle. Midline is to the right. Ipsilateral side is to the left. (e) is a detail of the squared area in (d). For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. White arrowheads: sulcus ypsilonformis. Scale bars: 200  $\mu\text{m}$  (a–d, f); 50  $\mu\text{m}$  (e)

immunochemical markers (Castro et al., 2006). As Dil was applied to unstained brains, we could not distinguish clearly between both regions, so they will be considered together as DI in this section (Figure 8).

Application of Dil crystals to DI at intermediate (precommissural) levels led to intense labeling of DI ipsilaterally at most levels, including the rostralmost DI or DIa, as well as weaker labeling in contralateral DI. We could not distinguish individual cell bodies or fibers in the ipsilateral DI because it was very intensely labeled, but we could appreciate cells in the contralateral DI (Figures 8a–e). In addition, some fibers were labeled bilaterally in Dc and in part of the ipsilateral Dd. In the olfactory bulbs, some mitral cells with their characteristic glomerular dendrites were labeled bilaterally (Figures 8a–b). Moreover, labeled fibers, but no cell bodies, were observed in the ipsilateral Dp and bilaterally in the lateral forebrain bundle (Figures 8c–f and 9g, i–l). In the subpallium, abundant cell bodies were labeled in dorsal Vv (Vv-d) and also in the retrobulbar region, mostly ipsilaterally (Figures 8a–c and 9a–f). Other labeled cell bodies were observed in caudal Vv, Vd, and in Vs at the level of the anterior commissure (Figures 8d–e and 9g–i). Some cell bodies were also labeled close to the ipsilateral forebrain bundle, likely corresponding to the entopeduncular nucleus (Figures 8d, f and 9j).

In extratelencephalic regions, a number of cell bodies were labeled in the lateral and anterior pregglomerular nuclei and the lateral recess nucleus (Figures 8h–i and 10c–e), whereas some cell bodies were labeled in the dorsal posterior thalamic nucleus. Occasional small cell bodies, as well as fibers and terminals, were labeled in the habenulae (Figures 8g and 10a). In the rostral rhombencephalon, cell bodies were observed in the raphe caudal to the interpeduncular nucleus (Figures 8k and 10f).

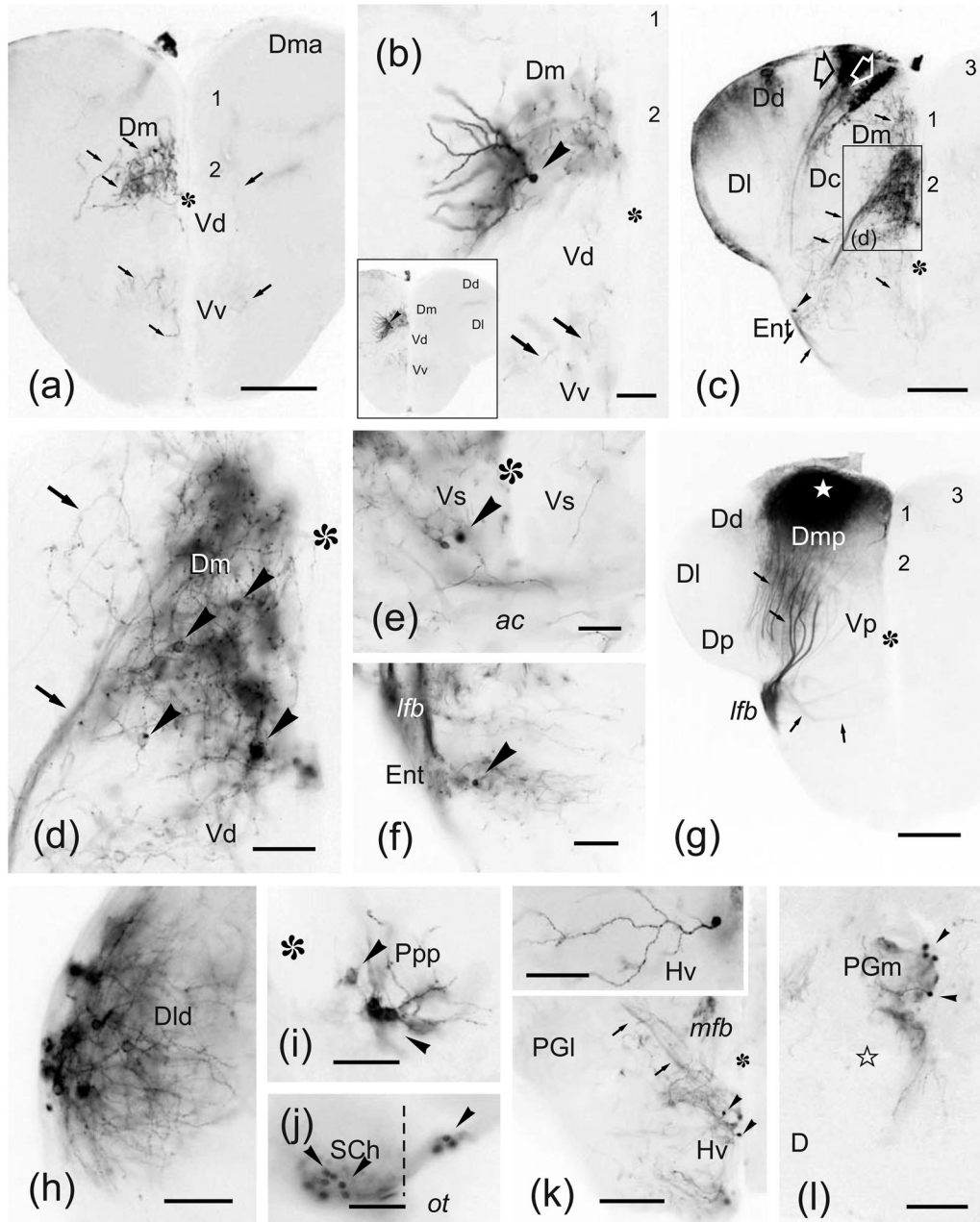
### 3.6 | Tracer application to the dorsal/dorsolateral zone of the pallium (Dd/Dld)

As the dorsal and lateral regions of the pallium are quite similar and have no clear-cut boundary, our cases of Dil application to Dd at precommissural levels may have affected Dld, so we will refer to this zone as Dd/Dld in this section. These experiments led to strong labeling of ipsilateral DI and Dd close to the application point, as well as to weaker labeling in the ipsilateral Dma and DIa (Figures 11a–e and 12a). The intense labeling precluded distinguishing individual cell bodies or fibers in these areas. In addition, dense areas of labeled fibers were observed bilaterally in Dc, whereas Dlv and Dp were mostly free of labeling, with the exception of being traversed by labeled fiber bundles (Figures 11e–f and 12c–f). Some cell bodies were labeled in the contralateral Dd/Dld and in the ipsilateral pallium, mostly in Dd, but also occasionally in DI and posterior Dm. Some labeled fibers crossed in the dorsal bundles of the anterior commissure to reach the contralateral Dma, Dd, Dc, and Dld. In the ipsilateral subpallium, numerous cell bodies were labeled in Vd and dorsal Vv, and a few cells in Vs and the ventral entopeduncular nucleus (Figures 11a–f and 12b–g). Most labeled Vd neurons were observed at the same level of the Dil application point. Fiber bundles labeled from Dd/Dld coursed ventrally and joined the lateral forebrain bundle. In the preoptic area, a few labeled cell bodies were observed in the anterior parvocellular preoptic nucleus (Figure 11f).

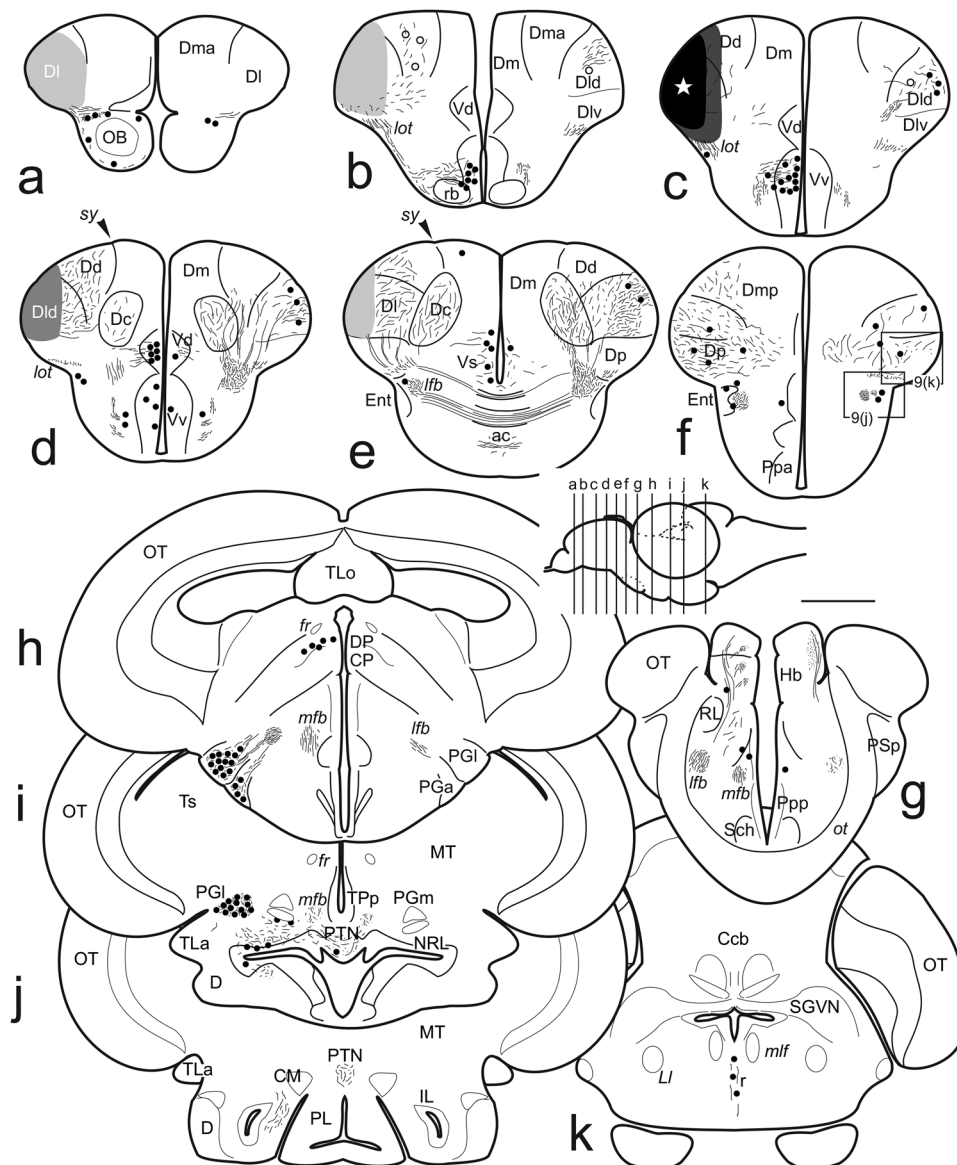
In extratelencephalic regions, a few cells were observed in the prethalamus and a few fibers traveling in the lateral forebrain bundle reached the rostralateral nucleus and the ipsilateral habenula (Figure 11g). More caudally, on the ipsilateral side, we observed a number of intensely labeled cell bodies in the lateral pregglomerular nucleus, some cell bodies labeled dorsal to the medial pregglomerular nucleus, and a few occasionally labeled ones in the periventricular tuberal nucleus and the medial part of the lateral hypothalamic lobe (Figures 11h–j and 12h–i). In addition, we observed labeled fibers ipsilaterally in the hypothalamic inferior and PLs (Figure 12j).

### 3.7 | Tracer application to the posterior zone of the pallium (Dp)

All applications of Dil to Dp were performed in toto, targeting the ventrolateral portion in order to avoid the lateral olfactory tract and nucleus. These experiments led to very intense labeling of mitral cells



**FIGURE 7** (a–l) Photomicrographs of transverse sections through the brains of two zebrafish showing labeled cells (arrowheads) and fibers (arrows) after application of Dil to the caudal dorsomedial zone of the pallium (Dmp/Dm3). (a–g) Panoramic views (a, c, g) and details (b, d, e, f) of transverse sections through the telencephalic lobe in an experiment with application of a very small Dil crystal to the area marked with a white star (in g). Note that labeled pallial neurons and fibers in levels away the area of application are mostly restricted to ipsilateral Dm2 (a–d), and a few cells in the subpallium: Vs (e) and entopeduncular nucleus (f). In (c), a few ependymal cells and processes were also labeled (outlined arrows). In (g), too, labeled small fiber bundles extend between Dm and the lateral forebrain bundle through the central region of the pallium. Photographs are ordered from rostral to caudal: (a–d) precommissural, (e–f) commissural, and (g) postcommissural levels. Numbers 1–3 indicate Dm subdivisions. (h–l) Sections of a brain where the area of application of Dil to caudal Dm was more extended showing some ipsilateral neurons labeled in Dld (h), the posterior periventricular preoptic nucleus (i), suprachiasmatic nucleus (j), ventral hypothalamus (k), and medial preglomerular nucleus (l). Ipsilateral side is to the left and the midline is indicated by broken lines or defined by the ventricle (asterisk). Outlined star: lateral recess. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (a, c, g, k); 100  $\mu\text{m}$  (b, e–f–j, l); 50  $\mu\text{m}$  (d, h–i)

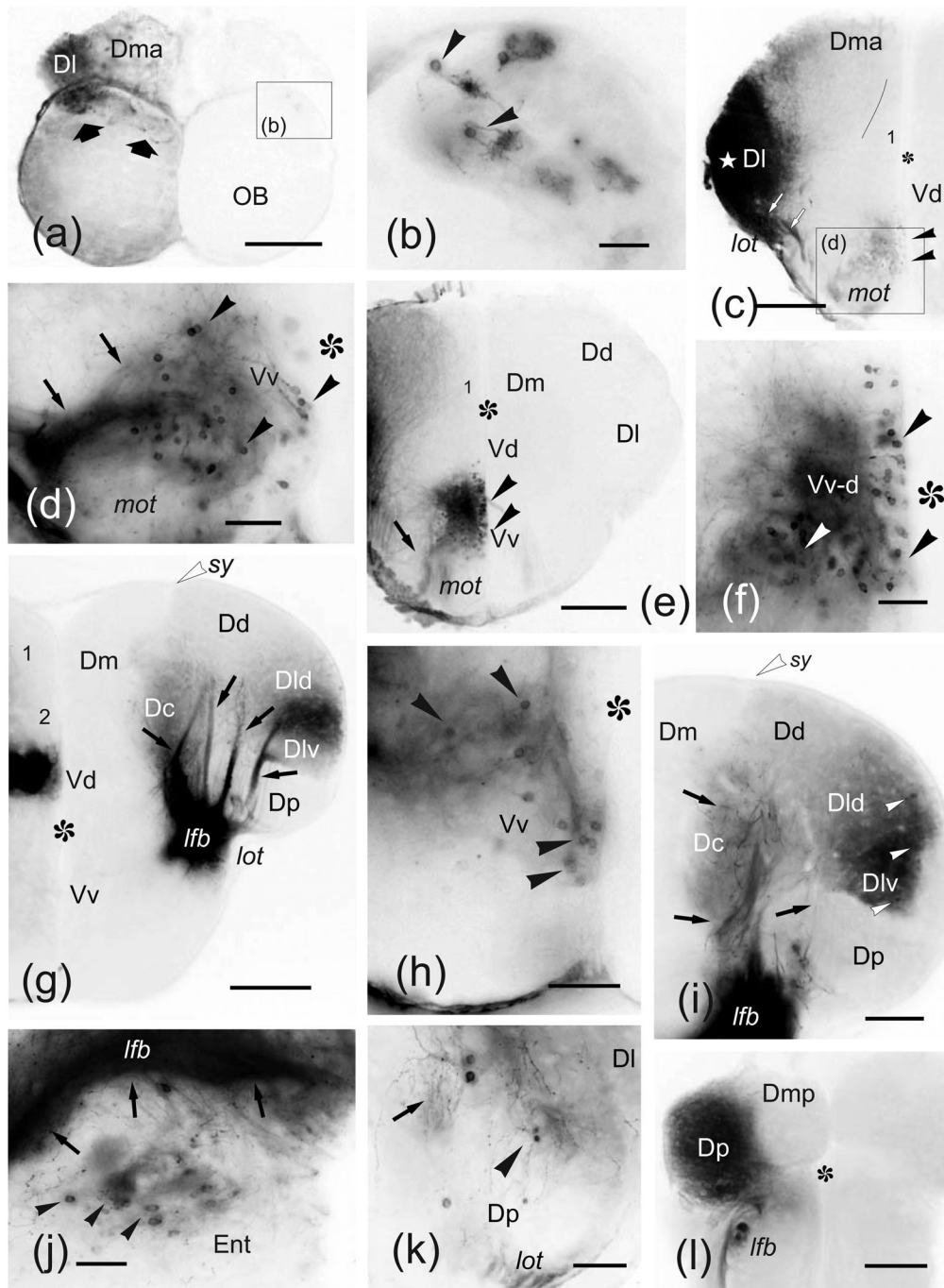


**FIGURE 8** (a–k) Schematic drawings of transverse sections of the zebrafish brain showing the distribution of cells and fibers labeled after application of Dil to the lateral zone of the pallium (DI). The level of sections from rostral to caudal is indicated in a lateral view of the brain. Black circles, retrogradely labeled cells. Small dots and lines, labeled fibers and tracts. Shaded areas in (a–e) represent the areas with dense labeling by the tracer from the point of application of the small Dil crystal (white star in c). Ipsilateral is at the left. Scale bar for sections: 500  $\mu$ m

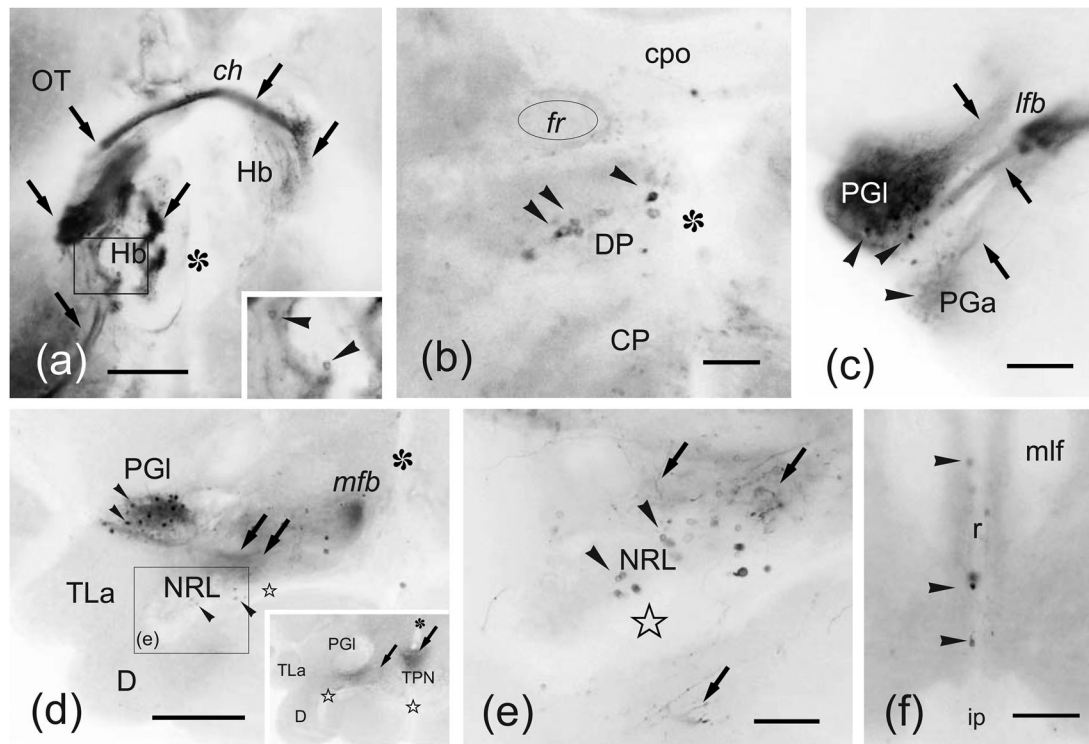
and, less intensely, fibers in the inner granular layer of the ipsilateral olfactory bulb. A few labeled mitral cells, with their characteristic dendrites branching in the olfactory glomeruli, were also observed in the contralateral olfactory bulb. These observations, with labeling on both sides, demonstrate the bilateral olfactory bulb projection to Dp (Figures 13a and 14a). In the pallium, some cell bodies and fibers were labeled in Dm (mainly ipsilaterally) and in Dp (Figures 13b–e and 14c–d). Numerous cell bodies were also labeled in the ipsilateral subpallium, mainly in the dorsal region of Vv (Vv-d, a most dorsal periventricular group), Vs and Vp (Figures 13b–e and 14b–c, f). Labeled fibers were observed bilaterally in the medial and lateral olfactory tracts, as well as crossing caudally in the anterior commissure (Figures 13a–d and 14a–b). Small cell bodies were also labeled in the entopeduncular nucleus, close to the lateral olfactory tract and fore-

brain bundles, and occasionally in the anterior parvocellular preoptic nucleus (Figures 13d–e and 14c–e).

Most of the extratelencephalic structures labeled after Dil application to Dp were fibers. Rostrally, some of these fibers were observed in the ipsilateral habenula, crossing to the contralateral side through the habenular commissure (Figures 13f and 14g). Some labeled fibers exited the lateral forebrain bundle (lfb) toward the dorsal posterior thalamic nucleus (DP), where occasional periventricular cell bodies were observed, and the lateral preglomerular nucleus (PGI) (Figures 13g and 14h–j). Numerous labeled fibers from the medial forebrain bundle coursed throughout the lateral hypothalamus to the torus lateralis (TLa), diffuse nucleus of the hypothalamic inferior lobe, the region of the tertiary gustatory nucleus, the nucleus of the lateral recess, and a few entered the hypothalamic PL (Figures 13h–i and 14j–k, m).



**FIGURE 9** (a–l) Photomicrographs of transverse sections through the telencephalic lobes of zebrafish, from rostral to caudal, showing the labeling of cell bodies (arrowheads) and fibers (arrows) after application of Dil to precommissural (DI). Ipsilateral is to the left. (a) Sections through the olfactory bulb showing labeling in dorsal glomeruli (arrows). (b) Detail of labeled bodies (arrowheads) and glomerular dendrites of mitral cell in the dorsolateral glomerular field of the contralateral olfactory bulb. (c) Section of ipsilateral telencephalic lobe at retrobulbar level showing the extension of dense labeling around the application area (star), labeled fibers in the lateral olfactory tract (arrows) and retrogradely labeled cell bodies (arrowheads) in the subpallium. (d) Detail of the labeled cell bodies in the retrobulbar region dorsal to the medial olfactory tract. (e–f) Panoramic view (e) and detail (f) showing numerous retrogradely labeled cell bodies (arrowheads) in the ipsilateral dorsal Vv (Vv-d). (g) Panoramic view of the contralateral telencephalic lobe at a precommissural level showing pallial projections to DI from the lateral forebrain bundle (lfb). Note also a compact group of labeled cells in the ipsilateral Vd. (h) Detail of labeled cells in the ipsilateral Vv. (i) Detail of the contralateral pallium at commissural level showing labeled cell bodies (arrowheads) and areas with labeled fibers (in DI and Dc). Note the clear limit of labeling of Dlv with Dp. (j) Photomicrograph showing labeled fibers in the contralateral lateral forebrain bundle (arrows) and some labeled cell bodies close to the dorsal entopeduncular nucleus (arrowheads) at commissural level. (k) Detail of a labeled cell at the contralateral DI at postcommissural level. (l) Section through of the caudal telencephalon showing labeling in the ipsilateral posterior pallium. Asterisk: telencephalic ventricle. Numbers indicate Dm subregions. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (a, c, e, g, l); 100  $\mu\text{m}$  (d, i, k); 50  $\mu\text{m}$  (b, f, h, j)



**FIGURE 10** (a–f) Photomicrographs of transverse sections through the zebrafish brain showing labeling in extratelencephalic regions after application of Dil to precommissural DI. Arrowheads: cell bodies. Arrows: fibers and tracts. (a) Transverse section through the epithalamus showing occasional labeled cells (inset) and fibers crossing through the habenular commissure to reach the contralateral habenula. (b) Section showing labeled cells in the dorsal posterior nucleus of the thalamus. (c–e) Photomicrographs and details of sections of the hypothalamus-posterior tubercle, showing labeled cells and fibers from the ipsilateral DI in the lateral preglomerular nucleus (c–d) and nucleus of the lateral recess (e). (f) Section of the isthmus showing labeled cells in the superior raphe dorsally to the interpeduncular nucleus. Arrowheads point to labeled cells, arrows to fibers. Asterisk: third ventricle (a, b) and hypothalamic lateral recess (d–e). Outlined stars: inferior and posterior lobe recesses. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (d); 100  $\mu\text{m}$  (a, c, f); 50  $\mu\text{m}$  (b, e)

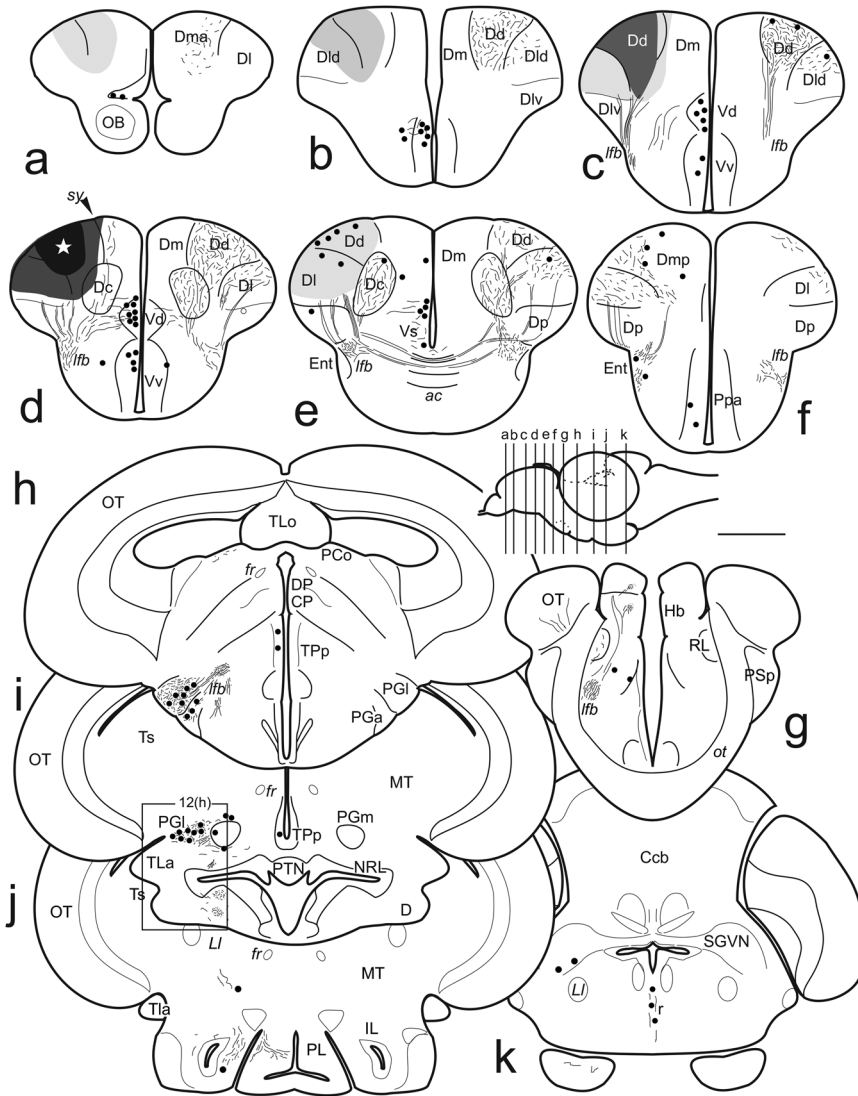
Labeled fibers coursing in the medial forebrain bundle reached the ventral periventricular hypothalamus (Hv) and the PTN, which showed a densely labeled terminal field and occasional labeled perikarya (Figures 13g–i and 14k). Fibers coursing in the lateral forebrain bundle coursed in the preglomerular region, where a number of retrogradely labeled cell bodies were observed in the anterior preglomerular nucleus (PGa) but not in the lateral preglomerular nucleus (PGL). A few lightly labeled cell bodies were observed lateral to the medial preglomerular nucleus (Figures 13g–h and 14i–j). Occasionally, an ipsilateral labeled fiber bundle was followed from the lateral hypothalamic region directly to the midbrain dorsal tegmental nucleus, which is located rostrally to the lateral NLV (Figures 14k–l). In the NLV, a few faintly labeled fibers and terminals were observed (Figure 14l). In the rostral rhombencephalon, a few cell bodies were labeled bilaterally in the rostral and ventral part of the secondary gustatory/visceral nucleus (Figures 13j and 14n), which likely corresponds to the visceral part of this complex (see Yáñez et al., 2017).

### 3.8 | Tracer application to the central zone of the pallium (Dc)

In this hodological study, we considered Dc (of Castro et al., 2006; named as Dp in Porter & Mueller, 2020) as an almond-shaped central

region located deep in the pallium at commissural levels surrounded by other pallial regions (Dm, Dd, Dl, and Dp). Owing to its location, application of Dil to Dc was made in sectioned brains approached from rostral, so these direct experiments only revealed connections with areas caudally to the application point. As Dc is also traversed by a number of fiber bundles originating or ending in other pallial areas (see present results, e.g., Dm1/2, Dm3, Dd, etc.), results obtained after direct Dil application to Dc must be contrasted with data from experiments of Dil application to other pallial zones (previous sections of results) and of extrapallial regions (see Section 3.9).

Application of Dil in the center of Dc led to strong labeling of surrounding pallial regions (Dm, Dl, Dp), mostly ipsilaterally (Figure 15a), as well as fibers and cell bodies in the supracommissural subpallium (not shown). Some labeled fibers coursed in the lateral and medial forebrain bundles and a few of them decussate in the anterior commissure. Caudally, labeled fibers could be followed to the pretectum, where abundant fibers and terminals were observed in the central (Figure 15b) and paracommissural pretectal nuclei, and in the dorsal posterior thalamic nucleus (DP), mainly ipsilaterally (not shown). Some labeled fibers also entered the rostral optic tectum (Figure 15c). Somas were labeled bilaterally in the preglomerular complex (PGL and PGM) and ipsilaterally in the posterior (PTN) and periventricular (TPp) tuberal nuclei. Labeled fibers were also observed in the



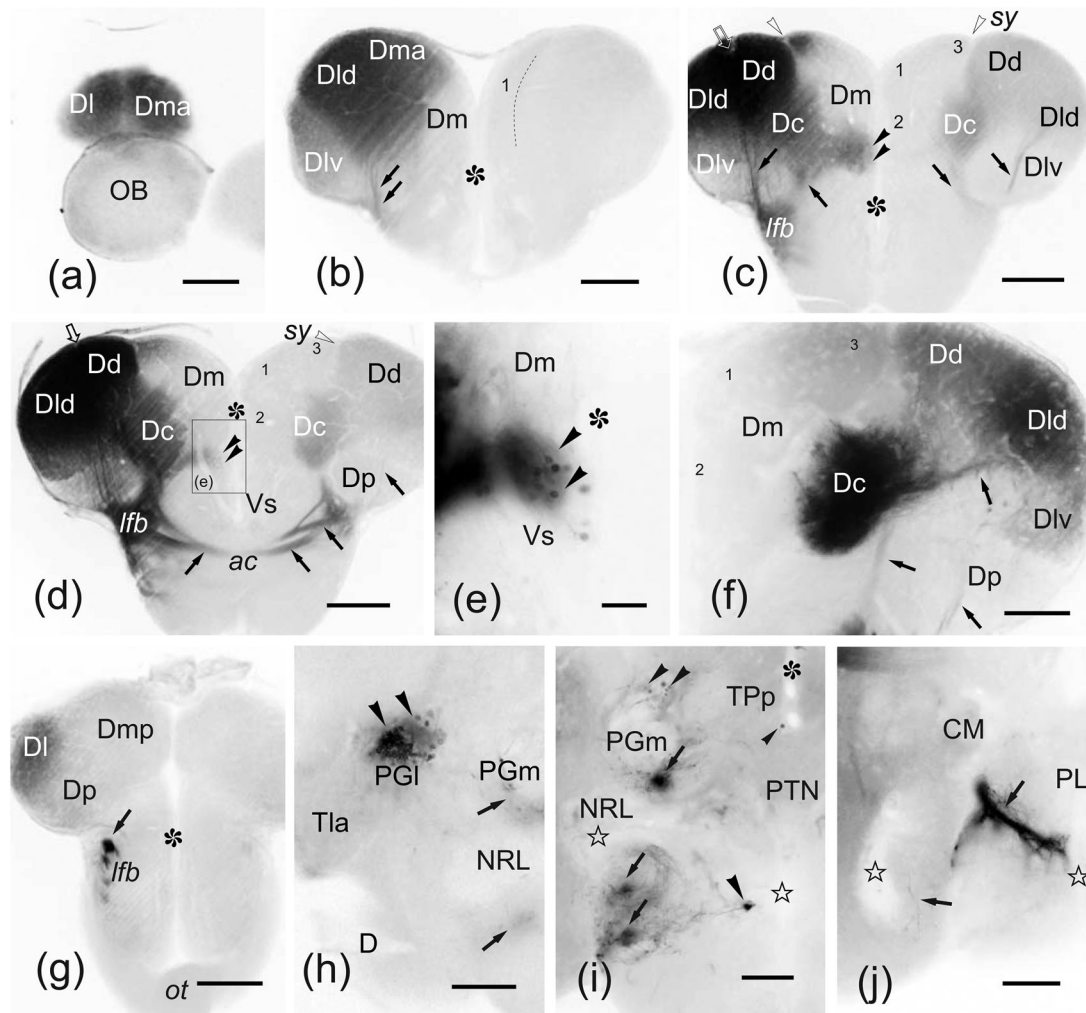
**FIGURE 11** (a–k) Schematic drawings of transverse sections of the zebrafish brain showing structures labeled after application of a Dil crystals centered in the dorsal zone of pallium (Dd) at precommissural levels. In (a–f), note that tracer diffusion (gray areas) also extends to neighboring ipsilateral pallial regions (DI and Dc), and also to the contralateral lobe. Note also ipsilateral labeling in Vd. In (g–k), labeled extratelenchephalic structures, mostly ipsilateral, are represented. Sections are arranged from rostral to caudal and the levels of the sections are indicated in the figurine of the lateral view of the brain. Black circles, retrogradely labeled cells. Small dots and lines, labeled fibers. Shaded areas in (a–e) represent the high- or less-dense labeled regions from the application point (white star in d). Scale bar for sections: 500  $\mu$ m

caudomedial region of the ipsilateral dorsal periventricular hypothalamic nucleus and bilaterally in the dorsal region of the hypothalamic PL (Figures 15d–f). Some cell bodies and many fibers were also labeled bilaterally in the medial mesencephalic tegmentum, where some fibers decussate at the level of the oculomotor nucleus, and in the lateral valvular nucleus, the superior raphe and the secondary gustatory/visceral nucleus (Figures 15g–i). Finally, a small nucleus was labeled in the dorsomedial rhombencephalic tegmentum at the level of entrance of the trigeminal nerve (Figure 15j): The nucleus appears to be the dorsal portion of superior raphe nucleus.

### 3.9 | Pallial connections revealed by retrograde tracing from extrapallial areas

Direct application of Dil to the various pallial regions, as reported above, revealed the presence of afferent perikarya in other pallial and extrapallial regions, as well as labeled fibers. As most of the pal-

lium regions of zebrafish were accessed directly from the ventricular surface, which is away the main telencephalic tracts, the labeled perikarya represent bona fide afferent neurons to these regions. However, the fibers labeled in other brain regions might not be originated in neurons of the approached pallial region, but from neurons in other centers that send collaterals both to the problem pallium region and to the putative target. This is possible because the free diffusion of Dil along cell membranes (indistinctly in retrograde and anterograde directions) characteristic of this small lipophilic molecule. A way to demonstrate that fibers are actually efferent of the problem center is to apply Dil to the putative target in order to reveal the presence of labeled perikarya in that pallial center. Here, we report the pallial neuronal populations and fibers labeled after Dil application to various extratelenchephalic regions that showed labeled neurons or fibers after Dil application to pallial zones. These experiments included application of Dil to the anterior and lateral preglomerular nuclei, the TLa, the lateral and medial regions of the hypothalamic inferior lobes, the PTN, the optic tectum, and the olfactory bulb (Figures 16–19). In the case of the olfactory bulb, the goal of these injections was helping to



**FIGURE 12** (a–j) Photomicrographs of transverse sections of zebrafish brain showing intra- (a–g) and extratelencephalic (h–j) structures labeled in an experiment of application of Dil to Dd (open arrow in c–e). Correspondences with schemes of Figure 11 are indicated. Black arrowheads: cell bodies. Arrowheads: fibers and fiber bundles. (a–g) Sections through rostral (a–b), precommissural (c), commissural (d–f), and postcommissural (g) telencephalic levels, showing ipsilateral (at left) and contralateral (at right) labeling of pallial areas well delimited mainly in Dd, Dld, Dc, and Dma, and also in the subpallial Vd. Dm and Dp are mostly free of labeling. In e, note a detail of Vd showing retrogradely labeled cells (black arrowheads). (h–j) Sections through the ipsilateral hypothalamus showing dense labeling of the lateral preglomerular nucleus (h) and bundles of labeled fibers (black arrows) coursing from the lateral forebrain bundle (see g) and reaching caudally the hypothalamic posterior lobe (j), as well as occasional labeled cells (black arrowheads) dorsal to the PGm, Hv, and periventricular TPp (i) (correspondence of (h–j) with levels depicted in Figure 11i–j is partial). Numbers 1–3 indicate Dm subdivisions. Asterisks: ventricle. Stars: inferior lobe recess. All photomicrographs are negative images of fluorescent data. For abbreviations, see the list. Scale bars: 200  $\mu\text{m}$  (b–d, g); 100  $\mu\text{m}$  (a, f, h–j); 50  $\mu\text{m}$  (e)

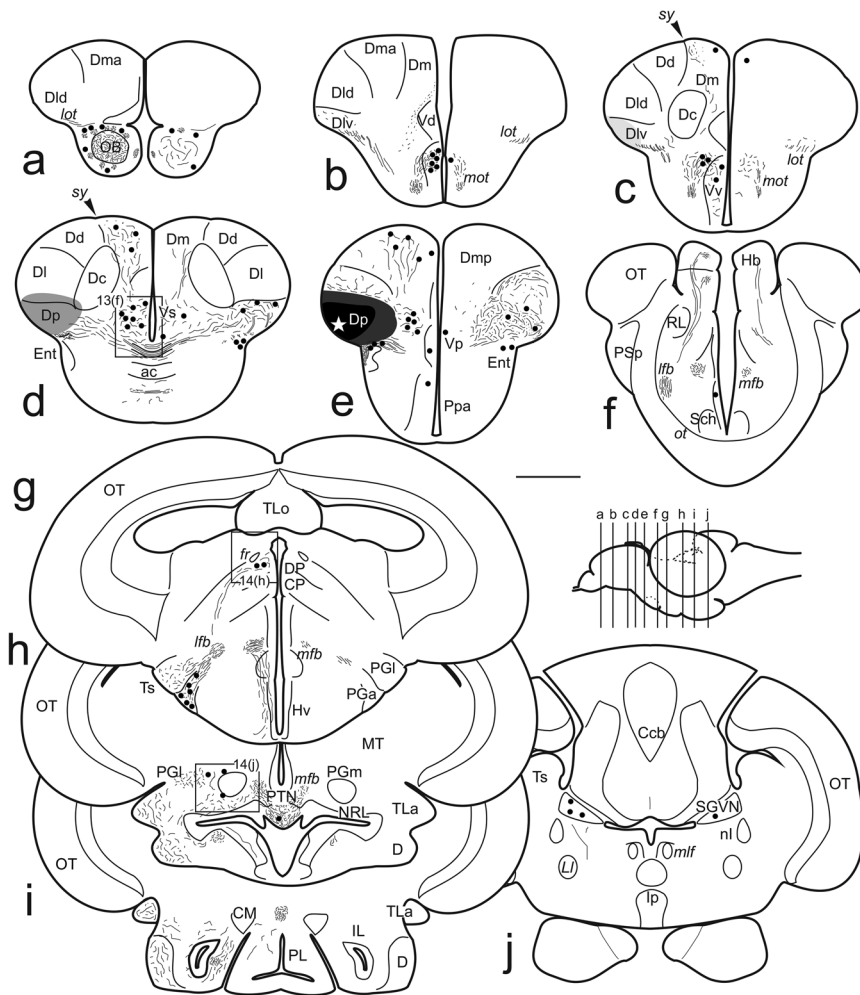
delimit Dp anatomically, facilitating thus the approach to this region. Other zebrafish extratelencephalic brain regions analyzed by us with tracing experiments that did not show connections with the pallium were not considered (see Yáñez et al., 2009; Yáñez et al., 2017; Yáñez et al., 2018).

### 3.9.1 | Preglomerular complex

Application of Dil to the lateral preglomerular nucleus (PGI), also affecting the attached anterior preglomerular nucleus (PGa), led to labeling abundant fibers in the ipsilateral pallium, mainly at precom-

missural and commissural levels of Dl, Dd, Dm, and Dc (Figure 16). Retrogradely labeled cells were also noted in the pallium, and these cells were mostly observed in a deep (away the ventricle) ventral zone of Dm at commissural and postcommissural levels (Figures 16d–g and 19b–c). A few cell bodies were also labeled in Dd (not shown), where also a number of varicose fibers could be observed coming from the lateral forebrain bundle. In addition, we observed a few faintly labeled cell bodies in dorsal Dp (Figures 16i–j), which may represent cells that project to the TLa and/or the diffuse nucleus and whose axons could get labeled as they traversed the region of the lateral preglomerular nucleus (see the next paragraph).





**FIGURE 13** (a–j) Schematic drawings of transverse sections of the zebrafish brain showing structures labeled after application of Dil to the posterior zone of the pallium (Dp) using a ventrolateral approach. The level of sections is indicated in the lateral view of the brain. Black circles, retrogradely labeled cells. Small dots and lines, labeled fibers. Shaded areas in (c–e) represent the diffusion area of the tracer from the application point (white star in e). Scale bar for sections: 500  $\mu$ m

### 3.9.2 | Torus lateralis

Application of Dil to the TLa led to ipsilateral labeling of cell bodies in dorsal Dp forming a horizontal band at postcommissural levels, and occasionally cells in Dc and Dm (Figures 16k–o and 19c). Some labeled fibers were also seen ascending through the lateral forebrain bundle and reaching Dd (Figures 16k–l). In the subpallium, we observed retrogradely labeled cell bodies in Vv-d and labeled axons coursing in the medial forebrain bundle. No cells were labeled in the other pallial areas.

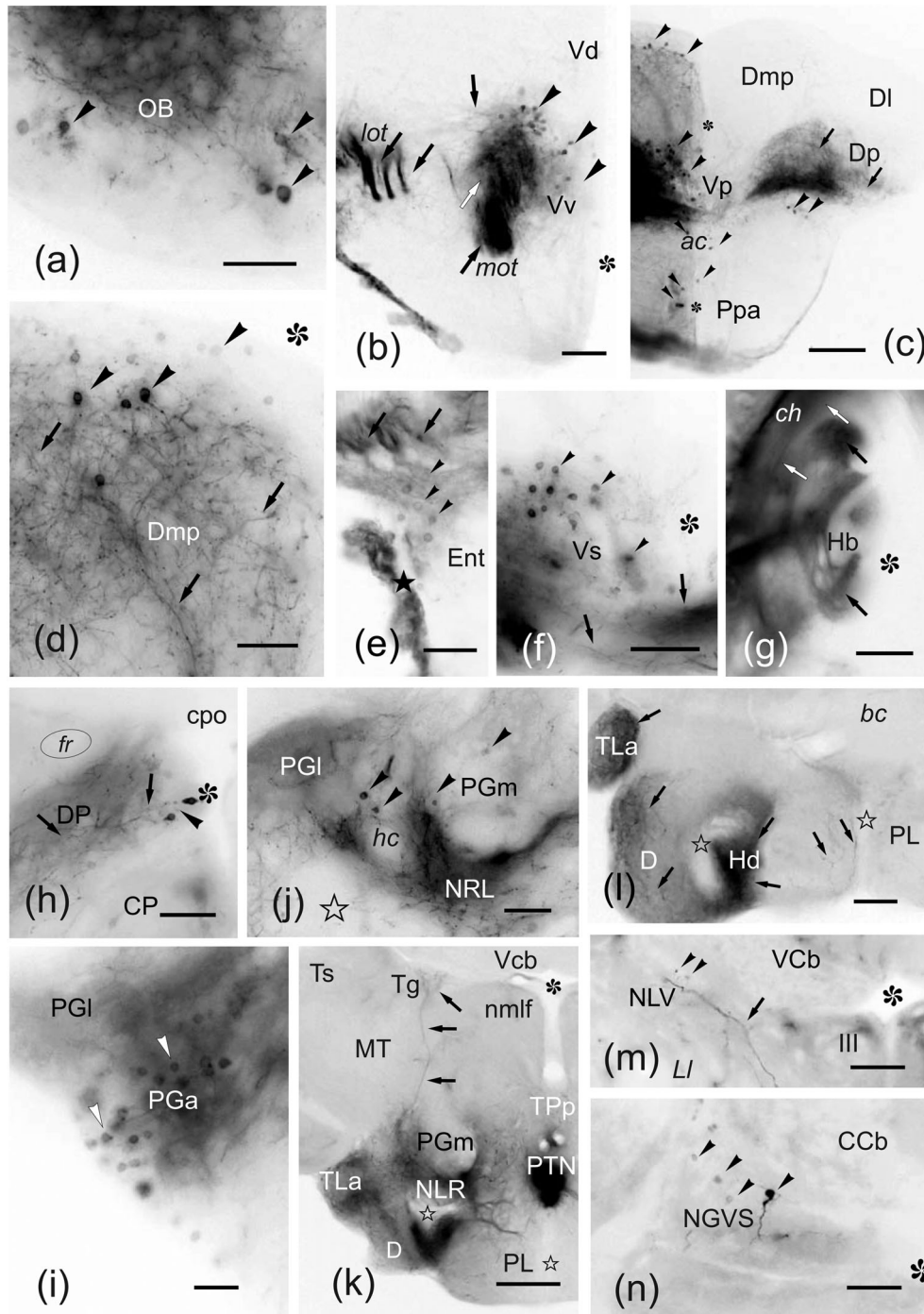
### 3.9.3 | Lateral hypothalamic lobes

Application of Dil to the lateral region of the hypothalamic inferior lobe led to weak labeling of a terminal field in precommissural Dd, and to labeling of cell bodies in the ventromedial and dorsolateral regions of Dm at commissural levels (Dm1, Dm2, Dm3) (Figures 16p–u and 19b). Occasional faintly labeled cell bodies were observed in Vs. At postcommissural levels, however, a number of retrogradely labeled cells were observed in Dp and a few cells in the posterior region of Dm (Dmp) and the ventral entopeduncular nucleus (Figure 16u). No cells were labeled in the other pallial areas.

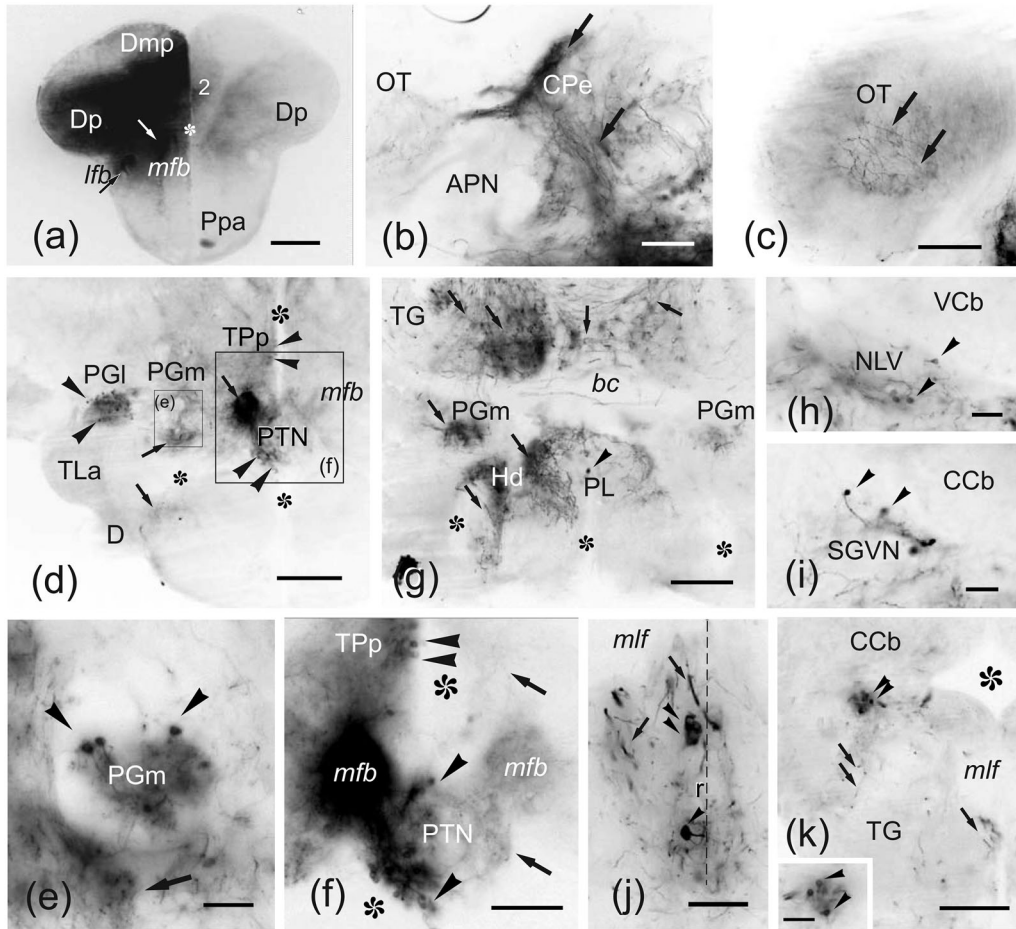
Dil application to the medial region of the hypothalamic inferior lobe led to labeling of two groups of cells in Dm with different rostro-caudal distribution: one group was located in the border with Dd from pre-commissural to commissural levels, and the other group was located in ventromedial Dm from commissural to caudal levels (Figures 16v–z and 19a–c). These two Dm cell groups join caudally in the ventromedial region of Dmp (Figure 16zz). No cells were labeled in Dp or other pallial areas in these experiments.

### 3.9.4 | Posterior hypothalamic lobe and posterior tubercle

Application of Dil to the ventral region of the hypothalamic PL led to labeling of numerous cell bodies in the subpallium (Vd and Vv-v), and a few cell bodies and some fibers in regions of Dm (Figures 17a–g and 19a–c). In the precommissural pallium, a few multipolar cells and some fibers were labeled in periventricular Dm (Dm1) (Figures 17a–b). More caudally, only a few cells were labeled in Dm (Dm1) at commissural levels (Figures 17e–f) and some cells at the ventral border of medial Dp (Figure 17g). These latter cells belong to Dp that was not assessed.



**FIGURE 14** (a–n) Photomicrographs of transverse sections of the zebrafish brain showing intra- (a–f) and extratelencephalic (g–n) labeled cells (arrowheads) and fibers (arrows) after application of Dil to ventral Dp. (a) Section through the ipsilateral olfactory bulb showing mitral cell perikarya and numerous labeled afferent fibers in the granular layer. (b) Detail of the precommissural subpallium showing numerous labeled neurons in dorsal Vv as well as fibers in the medial and lateral olfactory tracts. (c) Panoramic view at the level of the application point showing bilateral labeling in Dp and ipsilateral Dmp. (d) Detail of labeled cells and fibers in the ipsilateral Dmp. (e) Detail of labeled cells in the ipsilateral entopenduncular nucleus. Black star, meningeal tissue. (f) Detail of labeled neurons in Vs. (g) Detail of the ipsilateral habenula showing labeled fibers in the habenular neuropil (black arrows) and the tracts of the habenular commissure (white arrows). (h) Detail of labeled periventricular cells and fibers in the dorsal posterior thalamic nucleus (DP). (i) Detail of labeled cells in the ipsilateral anterior preglomerular nucleus. (j) Section through the ipsilateral preglomerular region showing some labeled cells between the lateral and medial preglomerular nuclei, and numerous fibers in the lateral recess nucleus (NLR). (k–l) Sections through rostral (k) and intermediate (l) levels of the hypothalamic inferior lobes showing a number of labeled fibers in the dorsal tegmental nucleus (Tg), the lateral hypothalamus (TLa, D, NLR, Hd), and posterior tuberal nucleus (PTN). (m) Section through the mesencephalic tegmentum showing fibers in the lateral valvular nucleus (TLa, D, NLR, Hd), and posterior tuberal nucleus (PTN). (n) Section through the rostral isthmus showing labeled cells in the secondary gustatory/visceral nucleus. Asterisk: ventricle. Outlined stars: inferior lobe recess. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (c, l, k); 100  $\mu\text{m}$  (b, g, j, m); 50  $\mu\text{m}$  (a, d–f, h–i, n)



**FIGURE 15** (a–k) Photomicrographs of transverse sections of the zebrafish brain ordered from rostral to caudal showing cells (arrowheads) and fibers (arrows) labeled after application of Dil to Dc in a brain en-block sectioned at commissural level. (a) Panoramic view of a section of the telencephalic lobes caudal to the application point. Note that Dp and Dm2 were heavily labeled, probably en-passant, through the various fibers bundles traversing Dc between major telencephalic tracts and more superficial pallial areas. Ipsilateral is to the left. (b) Section through the ipsilateral pretectum showing dense innervation of the central pretectal nucleus (CPe) by labeled fibers. (c) Section through the rostral optic tectum showing labeled varicose fibers. (d–f) Section through the hypothalamus-posterior tuberal area (d) and details (e–f) showing labeled cells and fibers in the ipsilateral PGI, PGm, and PTN. (g) Sections through the caudal hypothalamus and midbrain tegmentum showing bilateral labeled projections to the PGm, the hypothalamic posterior lobe and the tegmentum, and ipsilateral projection to Hv. (h–j) Details showing labeled cells in the lateral valvular nucleus (h), the secondary gustatory/visceral nucleus (i), and the superior raphe nucleus (j). (k) Section at the level of the trigeminal nerve exit showing a group of labeled cells in the ipsilateral dorsal rhombencephalic tegmentum and fibers in the reticular area. Asterisk, ventricle. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (a, c–d, g, k); 100  $\mu\text{m}$  (b, f); 50  $\mu\text{m}$  (e, h–j, inset in k)

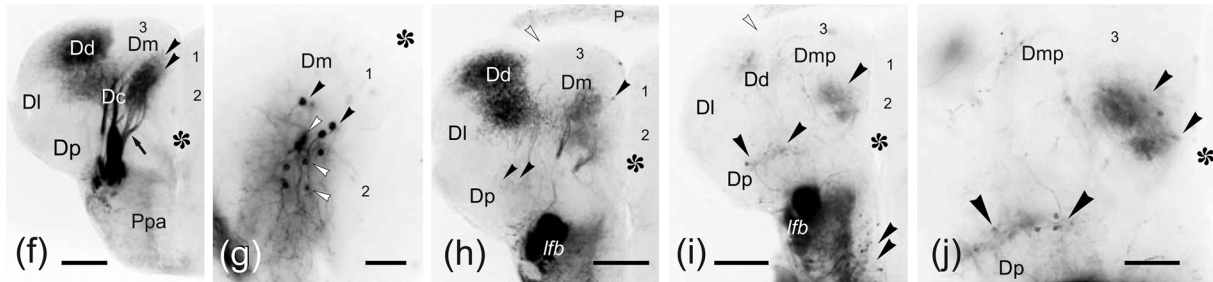
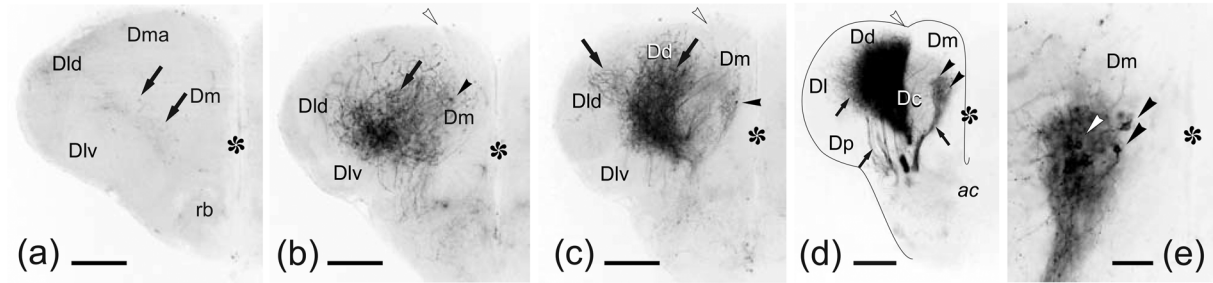
Application of Dil to the PTN led to bilateral labeling of cell bodies in the subpallium (Vv-d and Vs) from retrobulbar to commissural levels (Figures 17h–o and 19a–c). In the pallium, only a faintly labeled cell was observed in Dp. Labeled fibers were observed in the periventricular region of ventral Dm at commissural level (Dm2), in the border between Dd and Dm, and in ventral Dmp.

### 3.9.5 | Olfactory bulbs

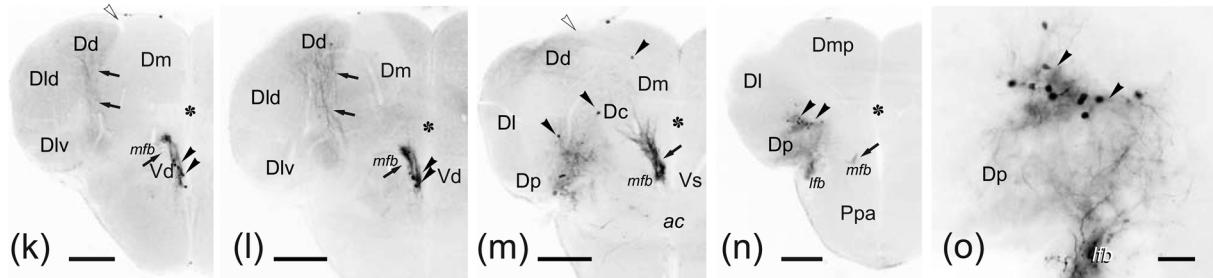
Application of Dil to an olfactory bulb led to strong labeling of the medial (mot) and lateral (lot) olfactory tracts extending up to Dp (Figures 18a–c and 19b–c). At precommissural levels, although only the

ipsilateral lot was intensely labeled, varicose fibers were also observed bilaterally in a restricted area close to the lot that could correspond in part with the nucleus of the lot (nlot) of Porter and Mueller (2020). At commissural level, ipsilateral-labeled fibers crossed the anterior commissure. Some of them turn rostrally coursing in the contralateral mot. At commissural and postcommissural levels, a large number of labeled fibers innervated the ipsilateral Dp and also crossed in the anterior commissure toward the contralateral Dp. A few labeled fibers from the mot were also seen into the Dm, mostly at commissural level (Figures 18a–c). Labeled cell bodies were observed in Vd and Dp, the latter being obscured by the high number of labeled fibers. For further details of the anatomy of bulbo-pallial connections, see the studies by Gayoso et al. (2011, 2012).

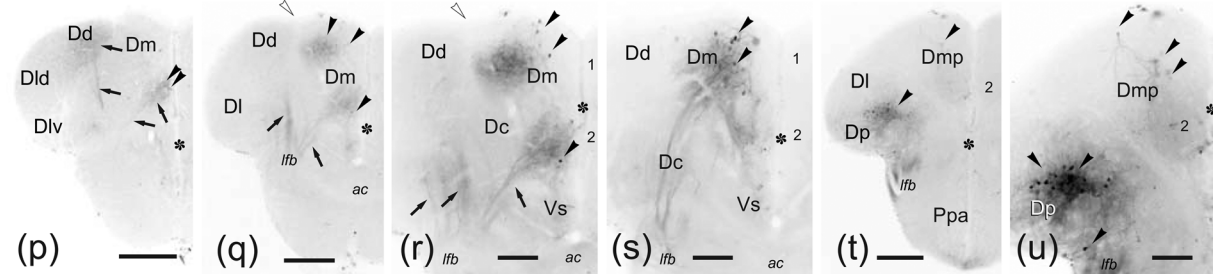
from PGI-PGa



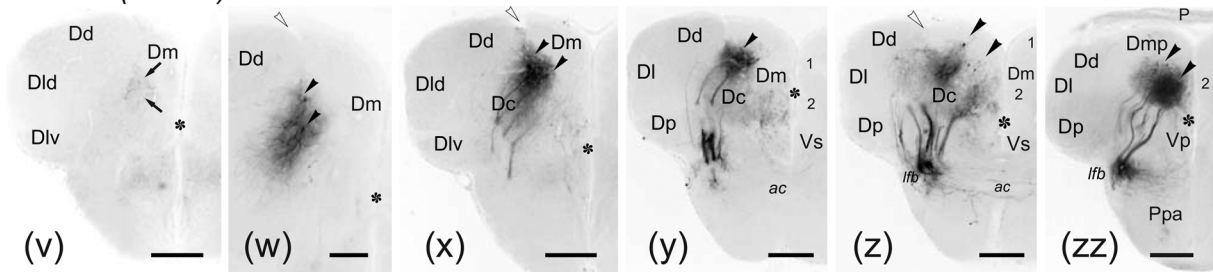
from TL<sub>a</sub>



from HL(lateral)

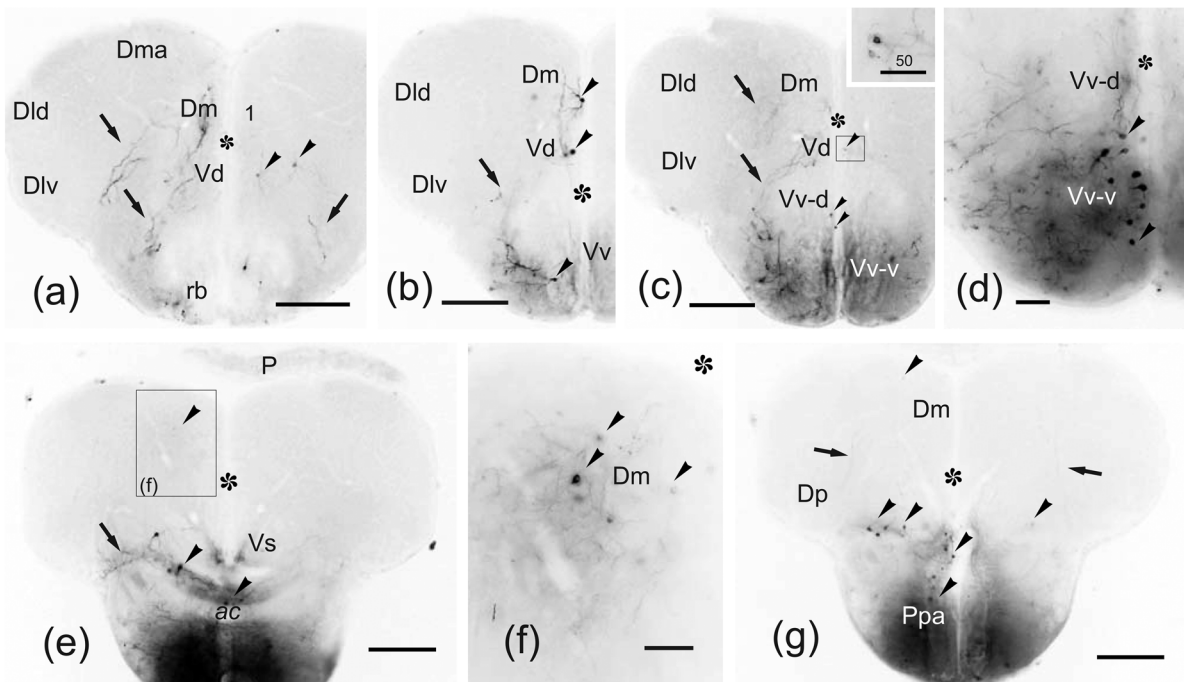


from HL(medial)

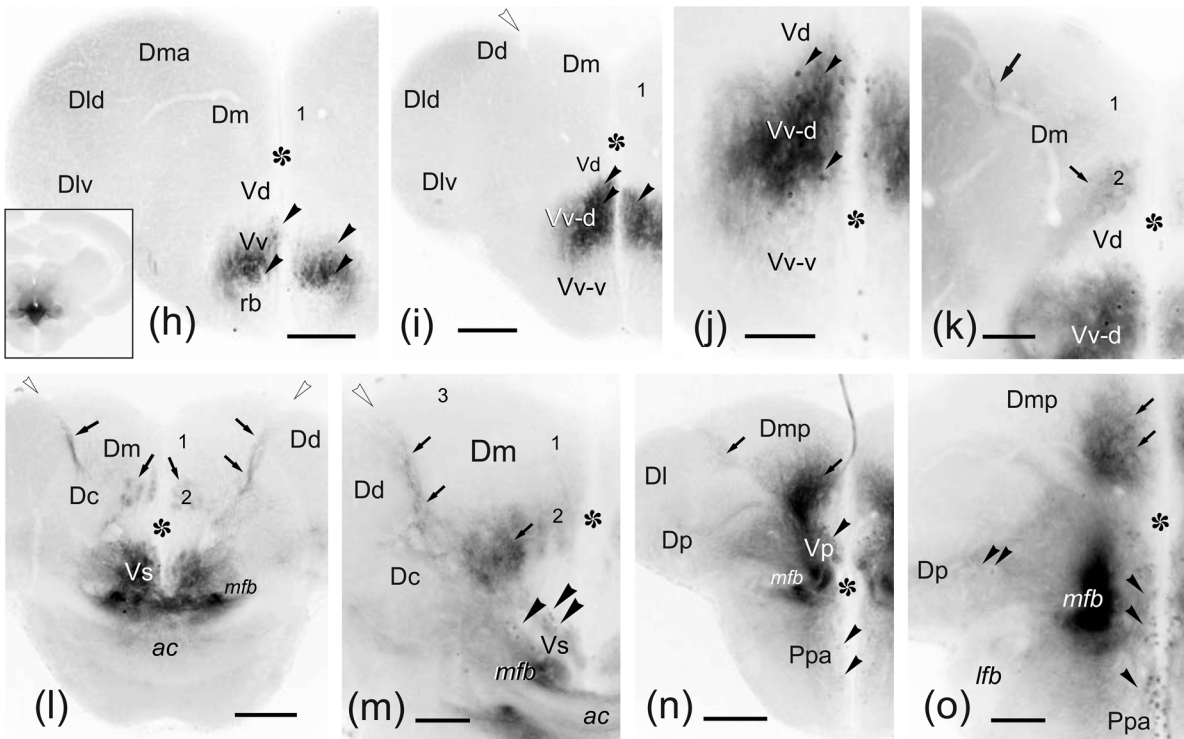


**FIGURE 16** (a–zz) Photomicrographs of transverse sections through the telencephalic lobes showing labeled cells (black arrowheads) and fibers (arrows) after application of Dil to the extratelencephalic areas indicated at top left of each picture group. (a–j,) Labeling from the lateral preglomerular nucleus, (k–o) from the torus lateralis, and (p–u) from the lateral part of the hypothalamic lobes; (v–zz) from the medial part of the hypothalamic lobes. Ipsilateral is to the left. Numbers 1–3 indicate the Dm subdivisions. Open arrowheads point to the sulcus ypsilonformis. Asterisk, midline ventricle. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu$ m (a–d, f, h–i, k–n, p–q, t, v, x–zz); 100  $\mu$ m (j, r–s, u, w); 50  $\mu$ m (e, g, o)

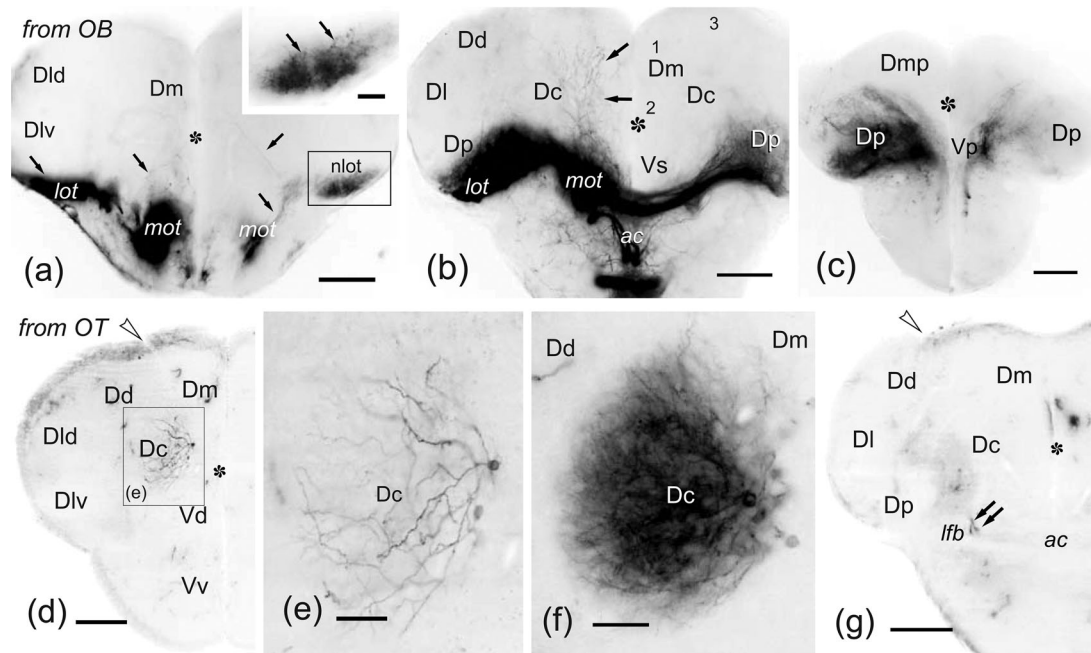
from PL (ventral)



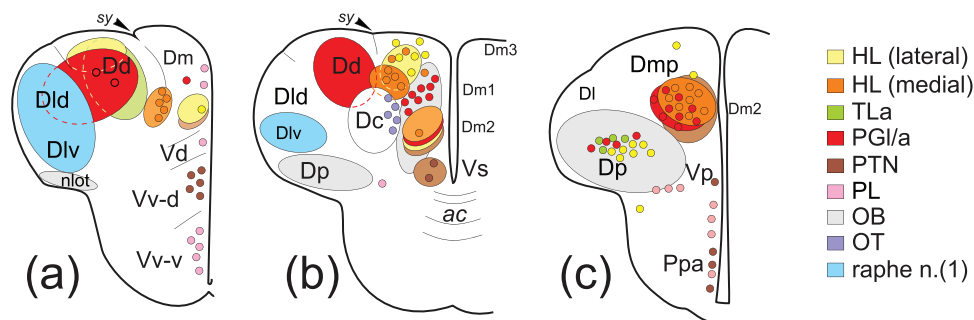
from PTN



**FIGURE 17** (a–o) Photomicrographs of transverse sections through the telencephalic lobes showing labeled cells (black arrowheads) and fibers (arrows) after application of Dil to the ventral part of the hypothalamic posterior lobe (a–g) and to the posterior tuberal nucleus (PTN) (h–o). Open arrowheads point to the sulcus ypsilonformis. Numbers 1–3 indicate Dm subdivisions. Asterisk, midline ventricle. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (a–c, e, g, h–i, l, n); 100  $\mu\text{m}$  (j–k); 50  $\mu\text{m}$  (d, f)



**FIGURE 18** (a–g) Photomicrographs of transverse sections at precommissural (a, d–e) commissural (b, g) and postcommissural (c) levels through telencephalic lobes showing labeled cells (arrowheads) and fibers (arrows) after Dil application to the olfactory bulb (a–c) and rostral optic tectum (d–g). Note the heavy bilateral olfactory projections on the nlot (detailed in a) and Dp in a–c, as well as fibers in Dm at commissural level (b). Note also projections on the lateral region of Vs and Vv (for details of projections, see Gayoso et al., 2011, 2012). (d–g) Note the intense labeling of Dc neurons extending dendritic trees to the central neuropil (d–f) at precommissural levels and sending axons to the optic tectum via the lateral forebrain bundle (g). No labeled cells were observed at commissural telencephalic levels (g). Ipsilateral is to the left. Numbers 1–3 indicate Dm subdivisions. Asterisk, ventricle. For abbreviations, see the list. All photomicrographs are negative images of fluorescent data. Scale bars: 200  $\mu\text{m}$  (a–b, d, g); 100  $\mu\text{m}$  (b); 50  $\mu\text{m}$  (e, inset in a); 25  $\mu\text{m}$  (f)



**FIGURE 19** (a–c) Schematic drawings of transverse sections through the zebrafish telencephalon at precommissural (a), commissural (b), and postcommissural (c) levels summarizing the main fields receiving olfactory and caudal extratelencephalic afferents (depicted as colored regions) and pallial and subpallial efferent cells projecting to these regions (color-coded dots). For abbreviations, see the list. (1) Raphe-telencephalic connections are based in Lillesaar et al. (2009) and in present results

### 3.9.6 | Optic tectum

Application of Dil to the rostral optic tectum led to strong labeling of large cell bodies located in the precommissural region of Dc (Figures 18d–g and 19b). These cells have pear-shaped perikarya and extend lateral dendrites that branch profusely in the neuropil at the center of Dc. These cell bodies and neuropil form together an almond-shaped central region of the pallium that extends for about 250  $\mu\text{m}$

rostrally to the anterior commissure. This labeled region was located deep in the pallium and has no contact with either the rostral or dorsal ventricular zone from which it is separated by other pallial areas. This Dc neuropil was also recognizable in other experiments as a target of numerous fibers labeled from DI and Dd neurons (see Sections 3.5 and 3.6). These results allow to characterize the zebrafish Dc as a deep nucleus in the central region of the pallium (for alternative definitions of zebrafish Dc, see discussion).

## 4 | DISCUSSION

### 4.1 | Anatomical regions of the zebrafish pallium

Recent molecular studies of the zebrafish telencephalon are introducing new views regarding the regional organization of the zebrafish pallium, especially attending the characterization of rostral Dm, Dc, Dd, and Dl. Establishment of connections of neuronal groups is the result of the expression of a large number of regulatory factors, proteins, signals, and receptors directing the birth and progressive specification of neuron types, guiding the axon growth, selective search for one or several types of target neurons, and establishment of functional synapses (Kwan et al., 2012). Thus, the patterns of connections reflect the differential characteristics of various neuronal populations, and reveal the areal/topographical organization of neural centers, even though they appear rather homogeneous. Following this idea, connectional results presented here may reveal hidden properties of pallial territories and thus be useful for assessing the organization of pallium in zebrafish.

As suggested previously using immunohistochemical methods (Castro et al., 2006), present results support the notion that major pallium regions (Dm, Dl) are not homogeneous rostrocaudally, because rostral parts are distinguishable from more caudal portions by connections. Castro et al. (2006) showed that the lateral part of the rostral pallium (Dla) can be distinguished from more caudal Dl and Dc based on the distribution of different markers, among others the calcium-binding protein calretinin. Parvalbumin (PV) expression also allows distinguishing in Dl a rostral region that lacks PV from more caudal regions with intense PV-positive cells (figure S4-A1-2 in Aoki et al., 2013, Supplemental information). Rostrocaudal differences in these main areas have been reported in the trout (Castro et al., 2003; Folgueira et al., 2004b) and goldfish (Northcutt, 2006). Recent studies named a wide region that comprises to the entire rostral pole of the zebrafish pallium as “Dc” (Mueller et al., 2011; Porter & Mueller, 2020), which includes our Dma and Dla and more caudal territories. The use of the name Dc in this sense causes homonymy confusion with the traditional definition of Dc in studies of different teleosts as a large-celled central region (Nieuwenhuys, 1963), as used here. Moreover, the anterior pallium differs from the rest of the pallium, and may be divided into medial and lateral divisions (Dma and Dla) (Castro et al., 2006). Additional support for this distinction comes from present experiments of Dil application to Dma and Dla, or more caudal regions of Dm and Dl. Thus, the anterior pallium of zebrafish appears immunohistochemically and hodologically different from more caudal regions, including Dc used in the traditional meaning. We have not applied Dil to more caudal parts of Wullimann et al.’s Dc, and its possible rostrocaudal diversity was not determined.

Based on the distribution of parvalbumin positive fibers in zebrafish pallium, Mueller et al. (2011) delimit a “new Dc (DP)” that includes territories such as our Dma, Dla, and Dd. Ganz et al. (2014) interpreted their genoarchitectonic results in the zebrafish pallium according to this model, although their Figures 1–3 show inconsistent expression of *eomesa*, *emx3* and *Prox1*, from rostral to caudal in the regions labeled as Dc and Dld. The results of connections of these pallium regions

(present results) do not support well the united “new Dc” and are better interpreted in terms done by Castro et al. (2006).

New neurogenetic data indicate the progressive generation of the zebrafish pallium in a temporal and medio-lateral zonal sequence (Dirian et al., 2014; Furlan et al., 2017). Results of these studies suggest that the deepest pallial neurons (possibly our Dc neurons among others) are generated early during development from the pallial ventricular zone, forming the core of the pallium, and then the ventricular zone gives rise to neurons (Dma, Dm, Dla, Dl, Dd, and Dp?) that surround the Dc core, as proposed by early models (see Nieuwenhuys & Meek, 1990). If the new neurogenetic ideas on the zebrafish pallium organization (Dirian et al., 2014; Furlan et al., 2017) also apply to advanced teleosts that exhibit much more complex pallia need to be investigated. For further discussion on the organization and connections of Dc, see Section 4.5.

Results in zebrafish and other species indicate a high complexity in the pallial subdivisions of teleosts. For instance, the existence of neurochemical differences between rostral Dm and more caudal parts of the pallium has also been reported in trout (Castro et al., 2003) and goldfish (Northcutt, 2006). In goldfish, Northcutt’s (2006) cytoarchitectonic study proposed a more complex subdivision of main pallial areas, which is in contrast with the subdivision of the pallium in the same species done by Yamamoto and Ito (2008), more similar to the zebrafish “new model” of Mueller et al. (2011). Complex rostral to caudal subdivision of the main pallial areas is outstanding in some acanthopterygians, both in medial and lateral regions (see Burmeister et al., 2009; Dewan & Tricas, 2014; Maruska et al., 2017; Hagio et al., 2020).

### 4.2 | The medial zone of the zebrafish pallium (Dm) is organized in territories with differential connections

The zebrafish Dm is considered to be topologically similar to the cortical amygdala of mammals (Porter & Mueller, 2020), despite its topographically medial location, which results from the eversion process. Our results indicate the existence of regional differences within Dm with regard to its connections, despite that all individual zones could not be accessed separately by technical reasons. In general, Dm exhibits abundant internal connections. Dm1 shows reciprocal connections with Dma, and projects to Dm3 and probably Dm2. Thus, Dm1/2 and Dm3 appear connected, whereas no direct connection between Dma and Dm3 was observed. However, connections of zebrafish Dm subregions with other pallial regions are rather limited. Only the applications of Dil to Dm3 labeled some neurons in a lateral region of Dl. Thus, zebrafish Dm, excepting its caudal part, appears largely independent from other pallial regions.

From a comparative point of view, the connections of zebrafish Dm with other pallial zones resemble those reported in goldfish (Northcutt, 2006) and *Gymnotus carapo* (Giassi et al., 2012), but not those reported in the rainbow trout (Folgueira et al., 2004b) and *Sebasticus marmoratus* (Murakami et al., 1983). Although similar, Dl-caudal Dm connections are much more limited in zebrafish than in

goldfish (Northcutt, 2006). In the rainbow trout, most intrapallial connections of Dm were restricted to other Dm subregions (Folgueira et al., 2004b), and no connections with DI were observed. The intrapallial pattern of connections of zebrafish Dm also differs from the abundant interconnections between Dm and Dc reported in *S. marmoratus* (Murakami et al., 1983). Finally, in the mormyrid *Gnathonemus petersii*, tracer application in the auditory and lateral line related areas of Dm led to labeling pallial neurons only in Dm (von der Emde & Prechtel, 1999).

With regard to their connections with extrapallial territories, the zebrafish Dma, Dm1/2 and caudal Dm3/Dmp, show remarkable differences between them. In general, Dma connections with extrapallial nuclei are scant, showing mainly projections to the (dorsal) entopeduncular nucleus, from which it also appears to receive fibers (Turner et al., 2016). Zebrafish Dma shows more restricted connections than those reported for rostral Dm (Dmr) in goldfish (Northcutt, 2006), suggesting that the zebrafish Dma approached by us may not correspond in extension to goldfish Dmr. In goldfish and carp, Yamamoto and Ito (2008) reported afferents and neurons labeled from the preglomerular complex to Dm mostly at its commissural levels, but not rostrally. Compared to Dma, intermediate (Dm1/2) and caudal (Dm3) regions of Dm show more broad connections. Present results show that Dm1/2 receives abundant innervation from the lateral and anterior preglomerular nuclei and the PTN, and projects fibers to the thalamus, pretectum, and the hypothalamic lobes. Among afferent to the precommissural and commissural region of Dm are notable the fibers from the preglomerular complex that form a terminal field in parallel to the ependymal surface. As shown in toto in the brain of a transgenic zebrafish line, this terminal field originating from a preglomerular population expressing GFP does not extend along all Dm, and fibers probably are collaterals of the projection of these neurons on DI (see below) (Bloch et al., 2020). A study in transgenic lines of zebrafish has identified a neuronal population in Dm (120A-Dm neurons) that projects to the hypothalamus and is essential for fear conditioning (Lal et al., 2018). This 120A-Dm population resembles those cells observed here after Dil application in the hypothalamus. Lal et al. (2018) also show projections from this Dm population to Vs and the entopeduncular nucleus, in line with present results. With regard to Dm3/Dmp, our experiments reveal afferents from neurons of the dorsal entopeduncular nucleus, the preoptic/suprachiasmatic region, ventral hypothalamus, and PTN. These results indicate that afferents to Dm3/Dmp mostly differ from those to Dm1/2.

From a comparative point of view, the pattern of Dm efferents observed in zebrafish seems more restricted than that reported for the caudal Dm of goldfish (Northcutt, 2006). This suggests that the region approached by us in zebrafish is only equivalent to a part of Northcutt's caudal Dm in goldfish. Comparison with other teleosts indicates that Dm connections with preglomerular nuclei and the tertiary gustatory nucleus are conserved. That is the case for the rainbow trout, for instance, in which Dm is reciprocally connected with the preglomerular nuclei and receives afferents from the tertiary gustatory nucleus (Folgueira et al., 2003, 2005). In *S. marmoratus* (Murakami et al., 1983), there are connections between Dm and the

acoustic lateral preglomerular nuclei, as well as direct projections from the Dm and Dd to the acoustic region of the torus semicircularis. In goldfish, tracer injections in the tertiary gustatory nucleus labeled fibers and many neurons in dorsal Dm (Kato et al., 2012). Reciprocal connections between the preglomerular complex and division 2 of Dm, but not from rostral Dm, have been reported in *Gymnotus* (Corrêa & Hoffmann, 1999; Giassi et al., 2012; Giassi et al., 2012), and between dorsal Dm and the auditory preglomerular nucleus in the carp and goldfish (Yamamoto & Ito, 2005a, 2005b, 2008). In *Gnathonemus*, the auditory/lateral line ventral preglomerular nucleus projects on Dm (von der Emde & Prechtel, 1999). In addition, a few afferent fibers to Dm originate from the commissural nucleus of Cajal in carp (Uezono et al., 2015), but not in tilapia or zebrafish (Yoshimoto & Yamamoto, 2010; Yáñez et al., 2017; present results). Together, these observations reveal both shared and specialized traits in connections of Dm in different teleost species.

Results of Lal et al. (2018) on the relation of Dm with fear conditioning, finding several transgenic lines affecting the 120A-Dm population and showing reduced performance in avoidance responses, suggest that zebrafish Dm is functionally equivalent to the mammalian pallial amygdala, which is in line with similar proposals on the base of neurochemical results (Porter & Mueller, 2020).

### 4.3 | The lateral zone of the zebrafish pallium (DI)

In zebrafish, the lateral zone of the pallium (DI) comprises a wide region dorsal and rostral to Dp, lateral to Dc and the sulcus ypsilonformis (Figure 1). This region is not homogeneous from rostral to caudal, as noted by the differential innervation by NPY-immunoreactive fibers (very dense in the dorsal part of precommissural DI; Castro et al., 2006), the differential expression of the cannabinoid receptor 1 (cb1) in rostral DI (Lam et al., 2006) and the central-caudal distribution of parvalbumin-immunoreactive neurons in DI (see supplemental figure S4 in Aoki et al., 2013). These data allow distinguishing roughly anterior, middle, and posterior regions in DI, with the middle one consisting of a dorsal and a ventral part (Castro et al., 2006). Moreover, dorsal DI appears to be continuous with Dd in zebrafish, which makes it difficult to distinguish between these two regions.

Regarding its intrapallial connections, Dil application to DI led to widespread labeling in the pallium, including Dd, Dc, and Dp. This may indicate the existence of numerous interconnections between DI and other pallial areas. However, as there is no clear anatomical boundary between dorsal DI and Dd, this labeling could be the result of Dil unspecific diffusion, invading areas other than DI. In zebrafish, Dil application to DI results in strong labeling in the ipsilateral DI, precluding observation of labeled cell bodies, and in weaker labeling in the contralateral DI, where labeled cells could be observed, with fibers passing through the anterior commissure. Likewise, massive ipsilateral labeling was observed in DI of *Gymnotus* after iontophoretic injections of BDA (Giassi et al., 2012), which produced more localized application than our method employed here with Dil. In *Gymnotus* experiments, pallial labeling from DI does not surpass the limits of DI



except for its projections to Dc, showing clear zonal restriction. Intense interconnectivity between DI and Dd subregions occurs in a mormyrid (*Apteronotus leptorhynchus*; Elliot et al., 2017), as in zebrafish. These profuse interconnections between DI and other pallial regions may explain why parvalbumin immunoreactive fibers that originate in neurons located in the middle region of zebrafish DI appear evenly distributed throughout all parts of DI, including DIa, Dc, and Dd (Mueller et al., 2011).

With regard its intratelencephalic connections, zebrafish DI receives many projections from the contralateral DI, and also from neurons in the subpallium (Vv, Vd, Vs), unlike Dm. Of these subpallial regions afferent to DI, Vd showed the largest population of labeled cells. Bulk application of tracers to the zebrafish subpallium (centered in Vv and Vd) also revealed retrogradely labeled neurons and fibers in pallial regions (DI, Dd, Dm, Dc, Dp) (Rink & Wullimann, 2004), which suggests that connections may be reciprocal. In goldfish and carp, DI receives afferents from Vd and Vs (Northcutt, 2006; Yamamoto & Ito, 2008), and in trout, Vd, Vv, and Vs are also afferent to pallial areas (Folgueira et al., 2004a; 2004b). These subpallial regions (Vv, Vd, Vs) may correspond to the mammalian pallidum, striatum, and subpallial amygdala, as proposed on the base of a variety of molecular markers (Ganz et al., 2012; Porter & Mueller, 2020). However, discussion about possible homologies in detail with mammals is out of our focus and the reader is referred to these molecular studies. The interhemispheric interconnections of DI via the anterior commissure may be topographically organized, as observed in trout (Folgueira et al., 2004b), leading probably to topographical contralateral regulation of activity as reported for contralateral projections of the olfactory bulbs (Kermen et al., 2020). Moreover, the connectivity observed between DI and subpallial centers may be related with the roles of DI in learning and spatial memory reported in goldfish (Portavella et al., 2002; Ocaña et al., 2017; Rodríguez-Expósito et al., 2017). Experimental evidence after ablation of DIv in goldfish indicates that this zone of the pallium is necessary for development of allocentric, relational spatial learning and memory, but not for egocentric, nonrelational strategies for orientation (Rodríguez et al., 2002; Rodríguez-Expósito et al., 2017). Based on these results, it has been proposed that the goldfish DIv is functionally comparable to the hippocampus of mammals. Whether this is the case for zebrafish, who is phylogenetically close to goldfish, needs to be further investigated.

With regard to the extratelencephalic afferents to zebrafish DI, the most important comes from the lateral and anterior preglomerular nuclei. This DI afferent was very clearly demonstrated using a transgenic zebrafish line in which the lateral preglomerular neurons express GFP (Bloch et al., 2020); interestingly, this projection only covers the middle region of DI, and simultaneously projecting on Dm, as indicated above, providing main inputs to two well-separated pallial regions. This input was proposed to be part of an ascending visual pathway retina-tectum-preglomerular complex-pallium (Bloch et al., 2020). A recent neurogenetic study using the same transgenic line suggested that many preglomerular neurons originate in the embryonic mesencephalon near the midbrain-hindbrain boundary (Bloch et al., 2019). This teleost visual circuit appears to be very unlike the visual circuits

of amniote vertebrates, indicating convergent evolution. How this DI circuitry compares with that of the mammalian hippocampus is unclear.

#### 4.4 | Considerations about the dorsal zone of the pallium (Dd)

As indicated above, the dorsal zone of the zebrafish pallium (Dd) extends laterally to Dm, between the sulcus ypsilonformis and dorsal DI (Figure 1). The Dd and dorsal DI zones are so poorly differentiated cytoarchitectonically in the adult zebrafish that can be considered as a single area judging from our tracing results. In electric fishes as *Gymnotus* and *Apteronotus*, immunohistochemical markers such as the kinase CaMKIIa clearly distinguish between both pallial zones (Giassi et al., 2012). In zebrafish, despite the difficulty to differentiate between Dd and DI, this domain is far from being homogeneous rostrocaudally with regard to the expression of gene markers or fiber innervation. For instance, numerous Dd+DI neurons express eomesa rostral to the anterior commissure but not caudal to it (Ganz et al., 2014). In middle and caudal levels, Dd+DI shows numerous parvalbumin positive perikarya, but not in rostral levels (DIa). A small zone of Dd at the level of the anterior commissure shows calretinin-immunoreactive cells, which are lacking in rostral and caudal levels of Dd or in DI (Castro et al., 2006). Likewise, notable regional density differences in innervation by NPY-positive fibers were reported among rostral and caudal DI and Dc (Castro et al., 2006). Thus, these differences in expression patterns and innervation reveal a more complex area than suggested by cytoarchitectonic analysis. While with respect to cytoarchitectonical aspects, the Dd and DI areas may be largely viewed as a continuum, functional differentiation would be based on the information processed in their different parts.

#### 4.5 | The central zone of the zebrafish pallium (Dc)

Although the central zone of the teleost pallium (Dc) is considered a heterogeneous region in most teleost species, it was generally identified cytoarchitectonically by the presence of large neurons in low to moderate density (Northcutt & Braford, 1980; Nieuwenhuys, 1998). DI tracing results from the optic tectum in adult zebrafish allowed us characterizing Dc as an almond-shaped central pallial area with a medial shell of cell bodies with profuse dendritic arbors distributed throughout its central neuropil. Moreover, this Dc neuropil also receives massive afferents from dorsal DI/Dd. The extension of this neuropil corresponds to a similar region that is conspicuously innervated by PV-immunoreactive fibers (see Dc in figure 5E of Mueller et al., 2011; figure 2F of Porter & Mueller, 2020, labeled as Dp) and Prox1-immunoreactive fibers (see figure 5B in Ganz et al., 2014); these fibers appear to originate from PV-positive neurons of DI, which is in agreement with our tracing results, revealing neurochemical signatures of this afferent system to our Dc. In contrast with neighbor regions, this almond-shaped Dc neuropil is poorly innervated by GAD-immunoreactive fibers (Castro et al., 2006). Together, these results

in zebrafish indicate that Dc is distinguishable from the other pallial regions pallial by its specific neuronal population, connections, and immunohistochemistry. We conclude that this zebrafish central nucleus is the pallial area giving rise to a pallial dorsal efferent pathway in zebrafish, relaying information mainly from dorsal DI-Dd and projecting toward the pretectum (Yáñez et al., 2018) and rostral optic tectum (present results). In electric fish, similar Dc neurons were also characterized neurochemically as glutamatergic cells that receive diffuse projections from DI and project to the tectum; they have been compared with layer 5 and layer 6 neurons of the mammalian pallium that project to the superior colliculus, the optic tectum homologue (Harvey-Girard et al., 2012).

Hodological data show that the zebrafish Dc (as characterized here) is clearly distinct by its pattern of connections from the other pallial areas, including those rostradorsal pallial regions included in "Dc" by Mueller et al. (2011). A cytoarchitecturally distinct Dc central zone well separated from DI and rostral pallium was recognized in the telencephalon of various teleosts (Murakami et al., 1983; Giassi et al., 2012; Harvey-Girard et al., 2012; Elliott et al., 2017). Hodologically, Dc neurons project to the optic tectum in goldfish (Grober & Sharma, 1981) and the goby *Acanthogobius flavimanus* (Hagio et al., 2018; Hagio et al., 2021), which is similar to that shown in zebrafish. However, in the goby, tectal afferent neurons are located in a central part (Dcm) adjacent to Dm within a large Dc. In trout, a small but well-defined Dc, similar to that shown in zebrafish, was demonstrated by retrograde labeling from the paracommissural pretectum (Folgueira et al., 2004b). In the carp, large Dc neurons and occasional small neurons in Dm were also labeled from the ipsilateral auditory torus semicircularis (Echteler, 1984). A central region (Dc) receiving rich innervation from DI was reported in goldfish (Northcutt, 2006). In the electric fish *Gymnotus*, a conspicuous Dc is richly innervated with fibers from DI (Giassi et al., 2012; Giassi et al., 2012), as noted in zebrafish. In *Sebastiscus*, Dc is an anatomically very well-defined central area that maintains connections with other pallial areas and gives rise to extensive projections to various brain regions (Murakami et al., 1983).

#### 4.6 | The posterior zone of the zebrafish pallium (Dp)

The posterior zone of the pallium (Dp) of teleosts and other ray-finned fishes is the main target of mitral cells of the olfactory bulbs (Nikonov et al., 2005; Huesa et al., 2006). Our results expand our knowledge regarding the connectivity of this telencephalic area in zebrafish, showing its extratelencephalic connections, mainly with preglomerular nuclei and hypothalamus. Present results confirm projections of mitral cells to Dp in zebrafish shown by Dil labeling (Gayoso et al., 2011, 2012) and single-cell labeling (Miyasaka et al., 2009, 2014). Single-cell labeling shows that most mitral cells project their axons to Dp bilaterally (Miyasaka et al., 2014), and the contralateral projections are specifically organized (Kermen et al., 2020). Tract-tracing experiments further reveal that the olfactory bulb-Dp connection is reciprocal, as indicated by the numerous neurons labeled in Dp after Dil applica-

tion into the olfactory bulb (Gayoso et al., 2011, 2012) and the abundance of fiber labeled from Dp in the inner granular layer of the bulb (present results). Similar results obtained with neural tracing methods were reported in different teleosts, as *S. marmoratus*, rainbow trout, and goldfish (Murakami et al., 1983; Folgueira et al., 2004a; Northcutt, 2006) and in a chondrosteian (Huesa et al., 2000, 2006). In the goldfish, a ventral region of DI (DI-v), rostral to Dp, also maintains reciprocal connections with the olfactory bulb (Northcutt, 2006). This DI-v olfactory region was also observed in zebrafish, appearing as a rostral extension of Dp. Recent molecular studies in adult zebrafish identify DI-v with a rostral part of the nucleus of the lateral olfactory tract (nLot; Porter & Mueller, 2020). For details of differential connections in zebrafish of the different olfactory bulb glomerular fields and the subpallium, the reader is directed to studies of Gayoso et al. (2011, 2012).

With regard to intrapallial connections of Dp, only the application of Dil to DI led to labeling of some fibers and cell bodies in Dp. These results, showing connections between DI and Dp, differ from those reported in other species, such as the rainbow trout (Folgueira et al., 2004b), *S. marmoratus* (Murakami et al., 1983), or *Gymnotus* (Giassi et al., 2012). In the rainbow trout, Dp was interconnected with Dm but not with DI-Dd (Folgueira et al., 2004b). Similarly, in *S. marmoratus*, numerous Dp neurons project to ventral Dm but not to DI, whereas dorsal Dm projects to Dp (Murakami et al., 1983). In *Gymnotus*, DI and Dp are not interconnected and their limit is very sharp after tracing experiments from DI (Giassi et al., 2012). In goldfish, application of HRP to the caudal Dm, DI, or DI-v produced labeling of neurons and fibers in Dp (Northcutt, 2006). This pattern observed in goldfish seems less specialized than that in zebrafish, also a cyprinid, and in other teleosts studied.

With regard to the subpallial connections of Dp, our experiments in zebrafish labeled numerous cell bodies ipsilaterally in the dorsal region of Vv (Vv-d) and, bilaterally, in the supra commissural nucleus (Vs), with fibers crossing in the anterior commissure. The Vs and Vv neurons, as for most subpallial nuclei, are GABAergic (Mueller & Guo, 2009). Accordingly, these nuclei may contribute to the abundance of GAD-immunopositive (inhibitory) fibers observed in Dp (Castro et al., 2006). In the rainbow trout, Dp receives projections from Vv and projects to the Vd (Folgueira et al., 2004a, 2004b). Moreover, interconnections between Dp and Vv were reported in *S. marmoratus* (Murakami et al., 1983).

Regarding extratelencephalic connections of zebrafish Dp, one of the most consistent extratelencephalic pathway labeled after Dil application to Dp was that to the PTN. As most mitral cell axons project to both Dp and the posterior tubercle in zebrafish (Miyasaka et al., 2014), the fibers in the posterior tubercle we observed after Dil application to Dp probably represent, in fact, mitral cell axons labeled retrograde-antegradely from Dp because Dil diffuses freely along cell membranes. Results of Dil application to the posterior tubercle ruled out direct projections from Dp to the PTN, because only an occasional neuron was labeled in Dp. Likewise, labeled fibers observed in the habenulae after Dil application to Dp also appear to be en-passant labeled mitral cell fibers (Miyasaka et al., 2014), as Dil tracing from the habenula did not reveal labeled cell bodies in Dp (Turner et al., 2016).

However, Dp neurons were retrogradely labeled from the TLa and the lateral region of the hypothalamic lobes, which also appear to be targets of ascending gustatory and visceral pathways (Yáñez et al., 2017), indicating convergence of chemical information from olfactory and taste pathways on these areas. On the other hand, extratelencephalic afferents to Dp (from the anterior and medial preglomerular nuclei, PTN, and secondary gustatory/visceral nucleus) appear very scant in zebrafish by comparison with the numerous afferent sources to Dp reported in the rainbow trout (Folgueira et al., 2004b). In this latter species, two subregions (dorsal and ventral) can be identified within Dp based on connection patterns (Folgueira et al., 2002; Folgueira et al., 2004a, 2004b). Thus, it seems that there are important differences between this two species regarding Dp organization and circuitry.

#### 4.7 | Other pallial afferents

The pallium of teleosts receives diffuse projections from various regulatory systems, as revealed by the presence of fibers immunoreactive to various peptides and neurotransmitters using immunohistochemical methods (Batten et al., 1990; Kaslin & Panula, 2001). The cells of origin of these fibers are difficult to label with Dil because the extensive axonal branching characteristic of these cells limits the amount of tracer diffusing to cell perikarya, which at most appear faintly or very faintly labeled. In any case, the Dil-labeled cells from the pallium observed in the zebrafish raphe (labeled from DI and Dd) may be part of the serotonergic system, and those labeled in the hypothalamic lateral or posterior recess region (from DI) may be part of the histaminergic system (Kaslin & Panula, 2001; Xavier et al., 2017), which send numerous fibers to rostral pallial regions. Further analysis and discussion of these and other regulatory systems are away from our present purposes.

## 5 | CONCLUSIONS

We present the results of the first comprehensive study of connections of the adult zebrafish pallium with tracing methods. This study reveals a wide intrazonal connectivity in pallial regions that is in contrast with the poor interzonal connections observed, especially between medial (Dm), dorsolateral (DI, Dd), and posterior (Dp) regions. This suggests that the zebrafish pallium has a modular organization (Figure 19), with dedicated modules for different neural processes. Pallial afferents also show compartmental organization. Most extratelencephalic afferent fibers come from preglomerular nuclei, whereas subpallial afferents to DI and Dp originate from Vd, Vs, or dorsal Vv, regions that may be comparable with the mammalian striatum, amygdala, or pallidum (Wullimann & Mueller, 2004; Porter & Mueller, 2020). Moreover, output pathways of the pallium appear characteristic of different regions. Interestingly, zebrafish Dc neurons characterized here originate the main pallial output toward the pretectum and optic tectum. In electric fish, these neurons were also characterized neurochemically as glutamatergic cells that project to the tectum and have been compared with

layer 5 and 6 neurons of the mammalian pallium that project to the superior colliculus (Harvey-Girard et al., 2012). Finally, we hope that the connections of the adult zebrafish pallium revealed in our experiments may be useful for neurogenetic studies of individual pallial neurons or the roles of different pallial zones in the zebrafish behavior.

#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. All authors had access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

#### DATA SHARING AND AVAILABILITY STATEMENT

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

#### AUTHOR CONTRIBUTIONS

Study concept and design: JY and RA. Acquisition of data: JY, IL. Analysis and interpretation of data: JY, MF, and RA. Drafting of the manuscript: JY, MF, and RA.

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#### ORCID

Julián Yáñez  <https://orcid.org/0000-0002-4559-8398>

Mónica Folgueira  <https://orcid.org/0000-0003-2927-7516>

Ibán Lamas  <https://orcid.org/0000-0002-6526-0079>

Ramón Anadón  <https://orcid.org/0000-0003-3260-1209>

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