Evolutionary developmental biology of bitterling fish

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Chapter 4 Spatial and temporal expression of marker genes during early brain regionalization in the rosy bitterling (*Rhodeus ocellatus*)

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Abstract

During early brain development, the neural tube is partitioned into forebrain, midbrain and hindbrain regions and further subdivided into radial, longitudinal and transverse units. This regionalization is associated with the position-specific expression of developmental control genes. We investigated early brain regionalization in the rosy bitterling (*Rhodeus ocellatus*), a teleost, by examining the expression pattern of marker genes *dlx2a*, *fgf8a*, *msx3*, *pax6a*, *shha*, and *sox9b* using whole-mount *in situ* hybridization. The developmental stages examined were 10-somite, 30-somite, 5-ovl and the prim-3 stages. Our results indicate that the subdivision of brain regions has started at the 10-somite stage. At the 30-somite stage, differentiation on the DV axis has started and segmentation of the neuromeres along the AP axis is completed. At the 5-ovl and prim-3 stages, there are three and five streams of migratory neural crest cells, respectively. The cephalic flexure becomes apparent at the prim-3 stage. We compared the observed gene expression patterns in the bitterling with those in the zebrafish (*Danio rerio*). The comparison shows that transcriptional heterochrony is found between the brain and trunk region between the bitterling and the zebrafish.

Introduction

The adult brain has an extremely complex structure. None the less, features of this structure are conserved across the vertebrate subphylum. The brain develops from the embryonic neural tube (Lumsden and Krumlauf, 1996). An early sign of regionalization of the neural tube is the partitioning of its anterior end into three brain vesicles arranged in anteroposterior sequence: the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). Subsequently, the forebrain is subdivided into the telencephalon and diencephalon, and the hindbrain is subdivided into the metencephalon and myelencephalon (Mueller and Wullimann, 2016; Puelles and Rubenstein, 2003). Further regionalization events produce prosomeres in the prosencephalon (Murakami et al., 2001; Puelles and Rubenstein, 2003) and rhombomeres 1 to 7 in the rhombencephalon (Moens and Prince, 2002; Murakami et al., 2001; Trevarrow et al., 1990). This regionalization provides the foundation for neuronal differentiation and axonal outgrowth (Hanneman et al., 1988; Mendelson, 1986; Metcalfe et al., 1986; Moens and Prince, 2002; Wilson et al., 1990).

The assignment of regional identity in the neural tube, as outlined above, is accompanied by the position-specific expression of developmental genes in so-called 'signalling centers' (Gibbs et al., 2017; Reifers et al., 1998; Rhinn and Brand, 2001). Of these, the midbrain-hindbrain boundary (MHB) or isthmic organizer (IsO) is the most extensively-studied. The MHB is composed of the posterior membrane of the tectal ventricle and the rostral cerebellar thickening (Wullimann and Knipp, 2000). The expression of the zebrafish *fgf8a* in the MHB is associated with planar induction during mes- and rhombencephalon development (Gibbs et al., 2017; Puelles, 2019; Reifers et al., 1998).

The anterior neural ridge (ANR) is a forebrain signaling center. It lies at the junction between the anterior neural plate and anterior non-neural ectoderm. The expression of *fgf8a* in the ANR is essential for induction of the telencephalon (Shanmugalingam et al., 2000). The zona limitans intrathalamica (ZLI) is a neuroepithelial domain in the alar plate of the diencephalon which separates the prethalamus from the thalamus. Expression of *shha* in the ZLI alone is sufficient for diencephalic differentiation in zebrafish (Jeong et al., 2007; Scholpp et al., 2006; Vieira et al., 2005). In the hindbrain area, r4 is the earliest rhombomere to develop; it is also a signaling center. r4 promotes the development of adjacent rhombomeres, thus supporting the propagation of hindbrain segmental patterns (Maves et al., 2002).

Spatially-restricted gene expression related to these various signaling centers define transverse and longitudinal segmental boundaries in the neural tube (Hauptmann and Gerster, 2000; Lauter et al., 2013). For example, the subdivisions of the diencephalic region can be visualized in terms of gene expression patterns. According to the *updated prosomeric model* (Puelles, 2019; Puelles and Rubenstein, 2003; Wullimann and Rink, 2002), the alar plate of the diencephalon is divided into regions called P1 (pretectum), P2 (thalamus), and P3 (prethalamus). Stable *pax6a* expression is an early marker of the pretectum as well as a P1 marker, defining the forebrain-midbrain boundary (Krauss et al., 1991a; Murakami et al., 2001; Puschel et al., 1992). The gene *dlx2a* is a P3 marker whose expression is restricted to the prethalamus, mainly in the cells of the ventricular zone (Mueller et al., 2008). The mesencephalon is devoid of *pax6a* expression. The transcription factor *sox9b*, a molecular marker for the tegmentum (Chiang et al., 2001; Li et al., 2002), is expressed on the ventral side of the mesencephalon.

In the hindbrain, several genes show segmental expression patterns. The expression domain of *pax6a* extends from r2 to the posterior hindbrain region (Krauss et al., 1991b; Puschel et al., 1992). Cranial neural crest cells are derived from adjacent r1, 2, 4, and 6. The odd-numbered r3 and 5 do not give rise to migratory neural crest cells (Graham et al., 1993). In the zebrafish, *sox9b* is expressed in neural crest progenitors; its expression is downregulated after the neural crest cells start migrating (Li et al., 2002; Plavicki et al., 2014). *dlx2a* is expressed in migratory neural crest cells (Akimenko et al., 1994; Panganiban and Rubenstein, 2002; Piotrowski and Nüsslein-Volhard, 2000). It's expression marks out distinct segmented streams of neural crest cells migrating towards the primordia of all seven pharyngeal arches (Rocha et al., 2020).

Highly conserved gene expression patterns are the foundation of the emergent concept of a *neural genoarchitecture*. This concept describes the neuroanatomy of the brain, including its anatomical boundaries, in terms of discrete gene expression domains (Puelles and Ferran, 2012; Schredelseker and Driever, 2020). Here, we used whole-mount *in situ* hybridization (WISH) to profile the spatial and temporal expression of selected marker genes (Table 1) in the bitterling fish (*Rhodeus ocellatus*). Our aim is to visualize early brain development and regionalization. The expression patterns have been compared with those in the zebrafish in order to detect temporal shifts in the gene expression (transcriptional heterochrony (Richardson et al., 2009). We then examine whether transcriptional heterochrony might provide an explanation for phenotypic evolution as suggested by (Bickelmann et al., 2012).

Materials and Methods

Rhodeus ocellatus embryos

Rhodeus ocellatus embryos, synchronized in developmental age, were produced by the Martin Reichard Lab of the Institute of Vertebrate Biology in Brno, Czech Republic by IVF (*in vitro* fertilization). After fixation of the embryos of various developmental stages in 4% paraformaldehyde (pFA) in phosphate buffered saline (PBS), they were dehydrated in graded methanol and stored in 100% methanol (MeOH) at -20°C. The developmental staging of the embryos was based on Nagata and Miyabe (Nagata and Miyabe, 1978) and on the staging table in Chapter 2 of this thesis.

Zebrafish embryos

Zebrafish (*Danio rerio*) embryos (AB/TL line) were collected from the fish facility of the Institute of Biology Leiden. The eggs were fertilized by 1:1 spawning (single crossing) at the beginning of the light period (14 hr light/10 hr dark). The fertilized eggs were collected and incubated in egg water (containing 60 µg/mL "Instant Ocean" sea salts) at 28.5 °C. Embryos were collected at 12, 24, 36 and 48 hpf (hours post fertilization). After collection, the embryos were immediately fixated in 4% pFA in PBS. Fixed embryos were dehydrated step-wise in graded methanol and stored in 100% methanol at -20 °C. Developmental stages were determined according to Kimmel *et al*. (Kimmel et al., 1995).

Whole-mount *in situ* hybridization

The digoxigenin-labeled antisense RNA probes used on *R. ocellatus* embryos were synthesized from plasmids containing PCR products of a major part of the coding sequences of genes *shha*, *fgf8a*,

pax6a, *msx3*, *sox9b*, *dlx2a* of *D. rerio*, using cDNA retro-transcribed from embryonic mRNA of *R. ocellatus* template. Whole-mount *in situ* hybridization (WISH) was performed according to (Thisse and Thisse, 2014).

Table 1 List of developmental marker genes used in this research

Gene	Name	Family	Expression pattern	Reference
dlx2a	Distal-less homeobox 2a	Homeo-box genes	Forebrain segmental boundaries	(Mueller, Wullimann, & Guo, 2008; Renz et al., 2011; Stock, 2006; C. Thisse & Thisse, 2005; Wullimann, 2009)
fgf8a	Fibroblast growth factor 8a	Growth factors	Midbrain/hindbrain boundary (MHB) or isthmus	(Albertson & Yelick, 2005; Nechiporuk, Linbo, Poss, & Raible, 2006; Ornitz & Itoh, 2015; Rhinn & Brand, 2001; B. Thisse et al., 2001; Wurst, Bally-Cuif, & Bally-Cuif, 2001)
msx3	Muscle segment polarity homeobox 3	Homeo-box genes	Dorsal part of central nervous system	(Bendall & Abate-Shen, 2000; Ekker et al., 1997; C. Thisse & Thisse, 2005)
pax6a	Paired box 6a	Pair rule genes	Eye and forebrain development; segmental organization of the hindbrain	(B. Thisse et al., 2001; C. Thisse & Thisse, 2005; Zhang, Boa-Amponsem, & Cole, 2017)
shha	Sonic hedgehog a	Growth factors	Floorplate and zona limitans intrathalamica (Zli)	(Jeong et al., 2007; Zhang et al., 2017)
sox9b	SRY (sex determining region Y) box 9b	Transcription factors	Early neural crest	(Chiang et al., 2001; Li, Zhao, Wang, Zhao, & Meng, 2002; B. Thisse & Thisse, 2004; Yan et al., 2005)

Results

Bitterling expression pattern

Stage 10-somite: early brain regionalization

At 10-somite stage (30 hpf), the bitterling embryo has just hatched out from the chorion and has 10-12 somites. The neural tube is differentiated into a brain primordium and narrower spinal cord primordium. The optic primordia extend laterally from the future diencephalon so that the outline of the head in dorsal view is arrowhead shaped. In the bitterling, there is strong expression of f*gf8a* at this stage, indicating onset of forebrain regionalization and the induction of the telencephalon (Figure 1A and D). The *pax6a* gene is expressed in the diencephalic part of the forebrain, non-overlapping with the telencephalic *fgf8a* expression (Figure 1B and E). The region from midbrain to r2 is devoid of *pax6a* expression (Figure 1B and E), and so the forebrain-midbrain boundary is indicated by the posterior margin of the *pax6a* diencephalic expression domain. There is strong expression of *fgf8a* in the MHB (isthmus organizer).

In the hindbrain, expression of *msx3* is located laterodorsally of the neural ectoderm (Figure 1C and F), in contrast to the ventromedial expression of *pax6a* and *fgf8a*. These three genes all present a segmental expression pattern in the hindbrain. *pax6a* has the highest intensity at r2, *fgf8a* is strongly expressed at r1 and r4, and *msx3* is expressed most strongly from the MHB to the posterior hindbrain, with weaker expression in r3.

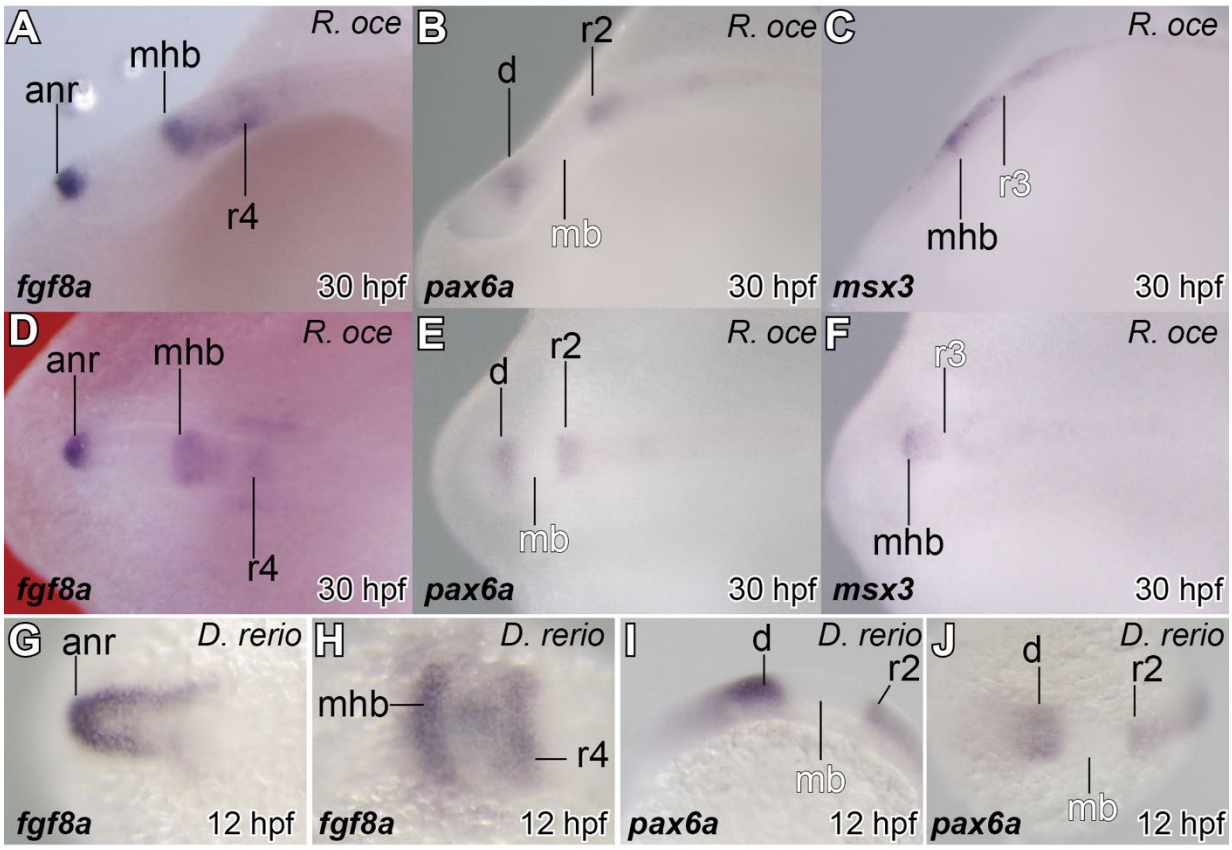

Figure 1 **Gene expression patterns deomonstrate early brain regionalization in the rosy bitterling (*R. oce*) and zebrafish (*D. rerio*)**. A to C, and I, lateral view, head to the left. D to H, and J, dorsal view, head to the left. Key for all figures in this chapter: anr, anterior neural ridge; fb, forebrain; mb, midbrain; hb, hindbrain; mhb, midbrain-hindbrain boundary; t, telencephalon; d, diencephalon; sp, subpallium; r1-7, rhombomeres; pr, preotic; po, postotic; re, retina; le, lens; ot, otic vesicle; tg, tegmentum; zli, zona limitans intrathalamica; hp, hypothalamus; pt, pretectum; ce, cerebellum; s1-5, cell streams; op, olfactory placode; of, optic fissure; fp, floor plate.

Stage 30-somite: specification of neural crest cells

At this stage (which is approximately 50 hpf) the somite number is 30 (the maximum number in this species is 35), the optic rudiment is distinctly cup-shaped, and the rhombomeres are visible as seven bulges. Compared with the previous stage, *fgf8a* expression is limited to the telencephalic regions and the MHB, and this gene is no longer expressed in the rhombomeres (Figure 2A and D). The expression of *pax6a* persists in the diencephalon and the proximal part of the retina (Figure 2B and E). The gene *sox9b* has four bilateral expression domains at the axial level of forebrain, anterior hindbrain, preotic, and postotic region of the hindbrain respectively (Figure 2C and F). The expression of *dlx2a* at the preotic level marks an early stage of neural crest migration (Figure 2G and H).

Stage 5-ovl: migration of neural crest cells

This stage (approximately 70 hpf) is the end of somitogenesis period. The somite has reached the maximum number. The hindbrain neural tube shows a cavity, the fourth ventricle. The expression of *fgf8a* is limited to the MHB, in the region where the tectum forms a fold that deeply invaginates towards the midline (Figure 3A and D). The gene *pax6a* shows strongest hybridization in the dorsal diencephalon and the proximal retina (Figure 3B and E). The expression domains of *sox9b* and *pax6a* in the forebrain and midbrain are adjacent but non-overlapping. For example, *pax6a* is not expressed in the midbrain, while *sox9b* is expressed in the ventral region of the midbrain, marking out the tegmentum (Figure 3C and F). In the optic region, *pax6a* is expressed not only in the retina, but also in the lens placode; by contrast, *sox9b* is expressed only in the proximal portion of the retina. The hindbrain expression of *sox9b* is located on the ventral boundaries of r4/r5, r5/r6, and r6/r7. Expression of *sox9b* in the neural crest cells disappears. The anterior boundary of *pax6*a expression in hindbrain is still located at r2.

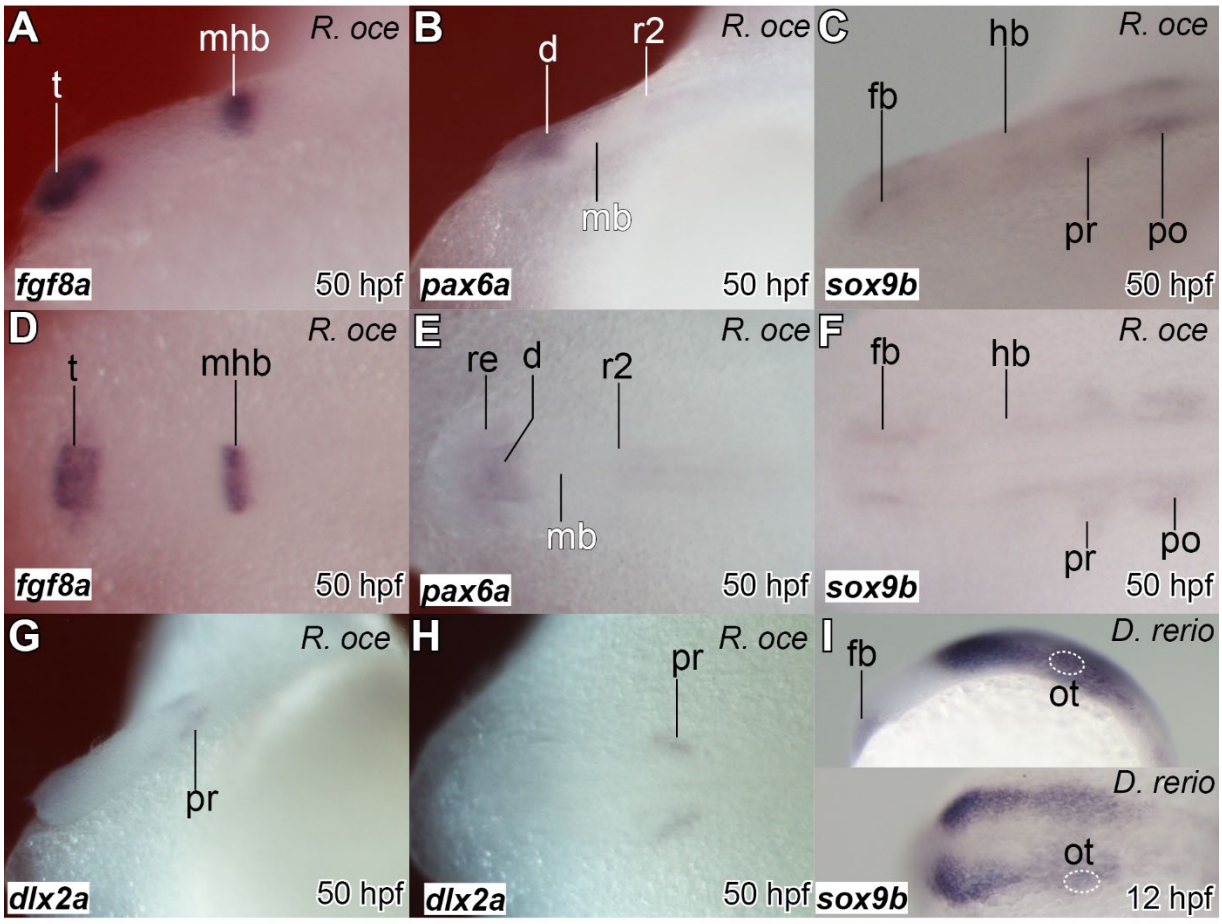

Figure 2 **Gene expression and early neural crest specification in the rosy bitterling (*R. oce*) and zebrafish (*D. rerio*)**. A to C, G, and I (upper), lateral view, head to the left. D to F, H and I (lower), dorsal view, head to the left. See Fig. 1 for abbreviations.

At this stage, *dlx2a* has a telencephalic expression domain in the subpallium; and a diencephalic domain in the periventricular zone of the prethalamus (Figure 3G and J). The gene *shha* is expressed in the hypothalamus, and its floor plate expression bifurcated rostrally in the diencephalic region, marking the Zli boundary between the thalamus and the prethalamus (Figure 3H and K). The gene

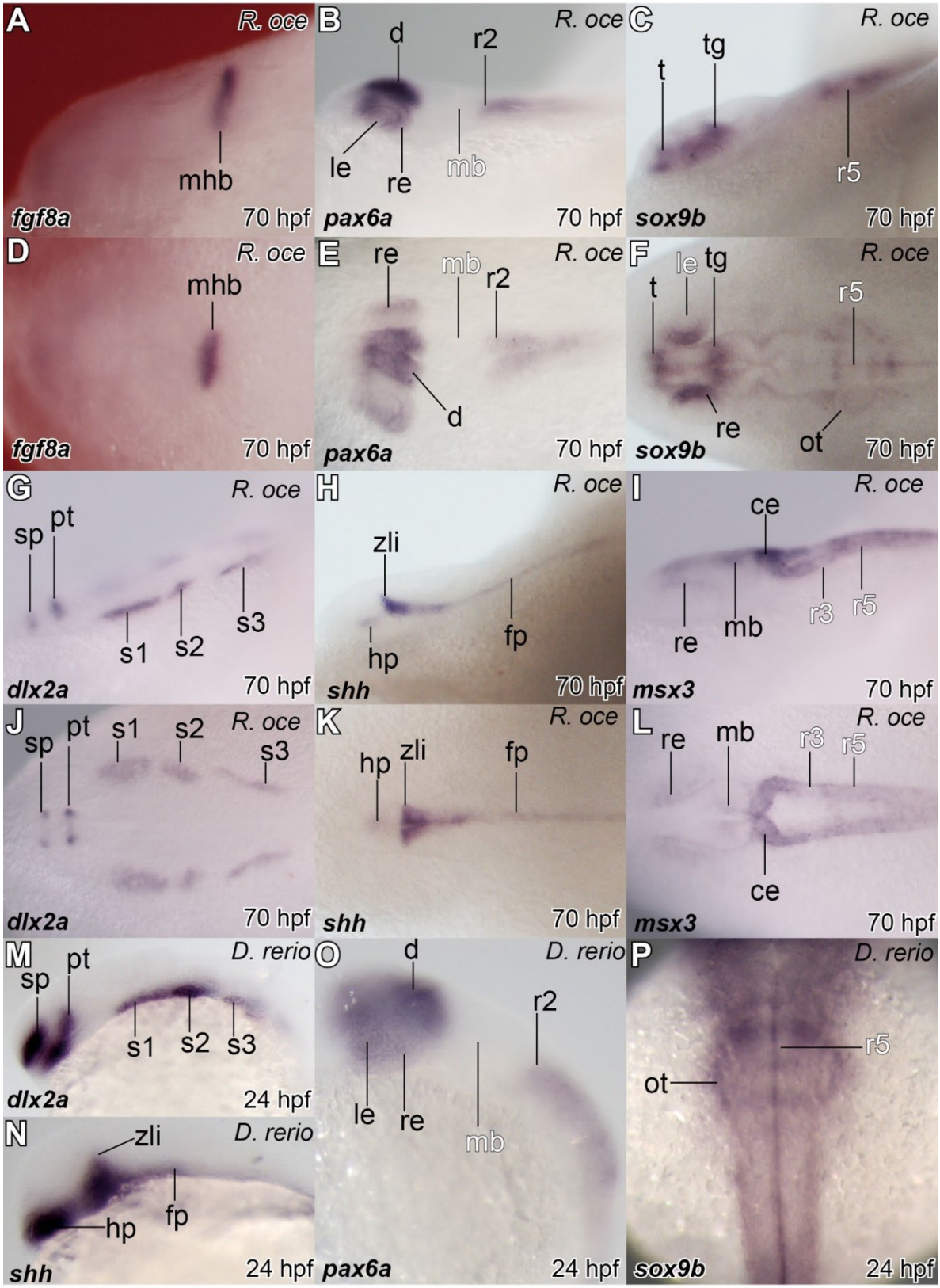

Figure 3 **Gene expression and early neural crest migration in the rosy bitterling (*R. oce*) and zebrafish (*D. rerio*)**. A to C, G to I, M to O, lateral view, head to the left. D to F, J to L, and P, dorsal view, head to the left. See Fig. 1 for abbreviations.

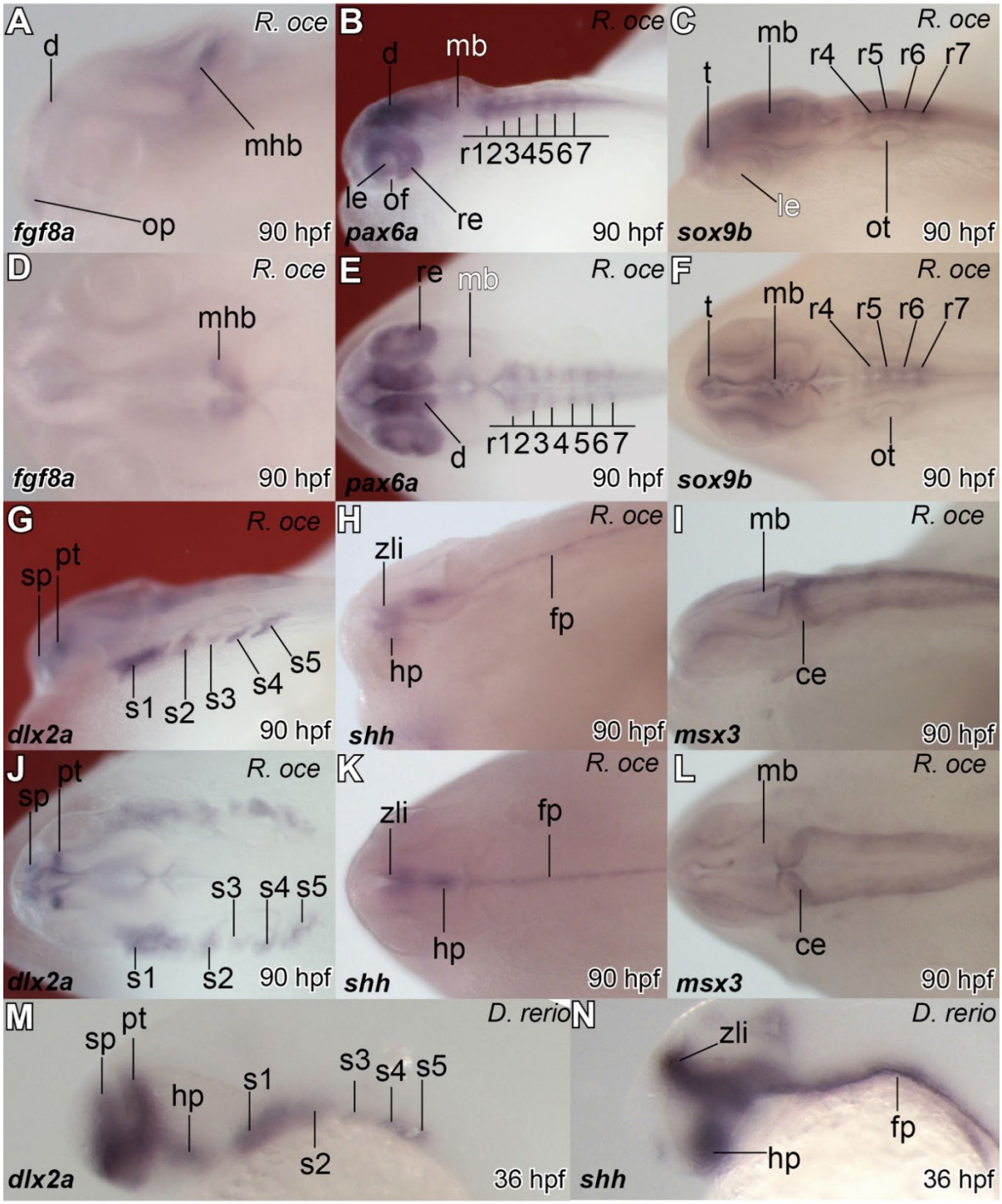

Figure 4 **Gene expression and flexure of the neuraxis in the rosy bitterling (*R. oce*) and zebrafish (*D. rerio*)**. A to C, G to I, M and N, lateral view, head to the left. D to F, J to L, dorsal view, head to the left. See Fig. 1 for abbreviations.

msx3 showed sporadic expressions on the dorsal side of the midbrain and retina. In the hindbrain, *msx3* probe hybridizes to the entire dorsal rhombencephalon except r3 and r5 where hybridization is weak or absent (Figure 3I and L).

Stage prim-3: cephalic flexure

At 90 hpf, the leading posterior end of the posterior lateral line primordium is at the axial level of myotome 3, and the stage is named accordingly as prim-3. New expression domains of *fgf8a* appear in the olfactory placode and the dorsal diencephalon (Figure 4A and D). The expression of *pax6a* persists in the retina and lens. In the hindbrain, *pax6a* has a new expression pattern, now marking intersegmental boundaries of the rhombomeres from r1 to r7 (Figure 4B and E). The alar plate of the rhombencephalon and basal plate of the r3 and r5 show hybridization with *msx3* (Figure 4F and I).

With the advance of the cephalic flexure, the longitudinal *shha* expression domain is displaced ventrad (Figure 4H and K). There are five streams of neural crest cells as indicated by expression of *dlx2a* (Figure 4G and J). The first and second streams are migrating to the mandibular and hyoid arches, respectively. The posterior three postotic streams are migrating to the 3rd, 4th, and the common primordium of the 5-7th pharyngeal arches.

Comparison between bitterling and zebrafish expression patterns

Comparing the expression patterns between the bitterling and zebrafish, we find that the zebrafish expression domain of *fgf8a* (Figure 1G and H) and *pax6a* (Figure 1I and J) at 6-somite stage (12 hpf) is similar to those of the bitterling at the 10-somite stage (30 hpf). However, the ANR expression of the zebrafish was more extensive (Figure 1G) than in the bitterling (Figure 1D). Expression of *sox9b* in the zebrafish at the 6-somite stage (Figure 2I) and in the bitterling at the 30-somite stage (50 hpf, Figure 2C and F) are similar; located bilaterally along the neural tube but is not yet subdivided into distinct domains (Figure 2I)

The expression of *dlx2a* in the migratory neural crest is similar between the zebrafish and bitterling. At the prim-5 stage in the zebrafish (24 hpf; Figure 3M and N) and 5-ovl stage of bitterling (70 hpf; Figure 3G and J) both species present three streams: two preotic streams migrating to the mandibular and hyoid arches, and one postotic stream migrating to 3-7th pharyngeal arches. At later stages, *dlx2a* shows expression in five NCC streams in both species (at the prim-25 stage in zebrafish, which is approximately 36 hpf; Figure 4M) and at prim-3 in the bitterling, which is approximately 90 hpf; Figure 4G and J). Expression of *pax6a* and *sox9b* is similar in zebrafish (at the prim-5 stage; Figure 3O and P) and the bitterling (at the 5-ovl stage). The longitudinal expression of *shha* has become displaced ventrad in the zebrafish at prim-25 stage (36 hpf; Figure 4N) and the bitterling at prim-3 stage (90 hpf; Figure 4H and K).

Discussion

Early brain development in the bitterling

This is the first study of brain development in a bittering analyzed through gene expression patterns. At the 10-somite stage, there are no discernible anatomical boundaries in the brain. The MHB at this stage is discernible by microCT, but is distinctly marked by *fgf8a* expression (Figure 5). At this stage, the non-overlapping expression domains of *fgf8a* and *pax6a* indicate that the subdivision of the prosencephlon into telencephalon and diencephalon is underway.

Similarly, while there are no rhombomeres discernible by microCT in the 10-somite bitterling, *fgf8a* expression at this stage demarcates r1 and r4, and *msx3* demarcates r3; segmentation of the hindbrain is in therefore already in progress. At the 30-somite stage, diminished expression of *fgf8a* and *pax6a* suggest that the segmentation of the rhombencephalon along the AP axis is complete. The appearance and initial migration of neural crest cells indicated by *sox9b* and *dlx2a* expression indicates that the differentiation of the hindbrain region on the DV axis has started at the 30-somite stage.

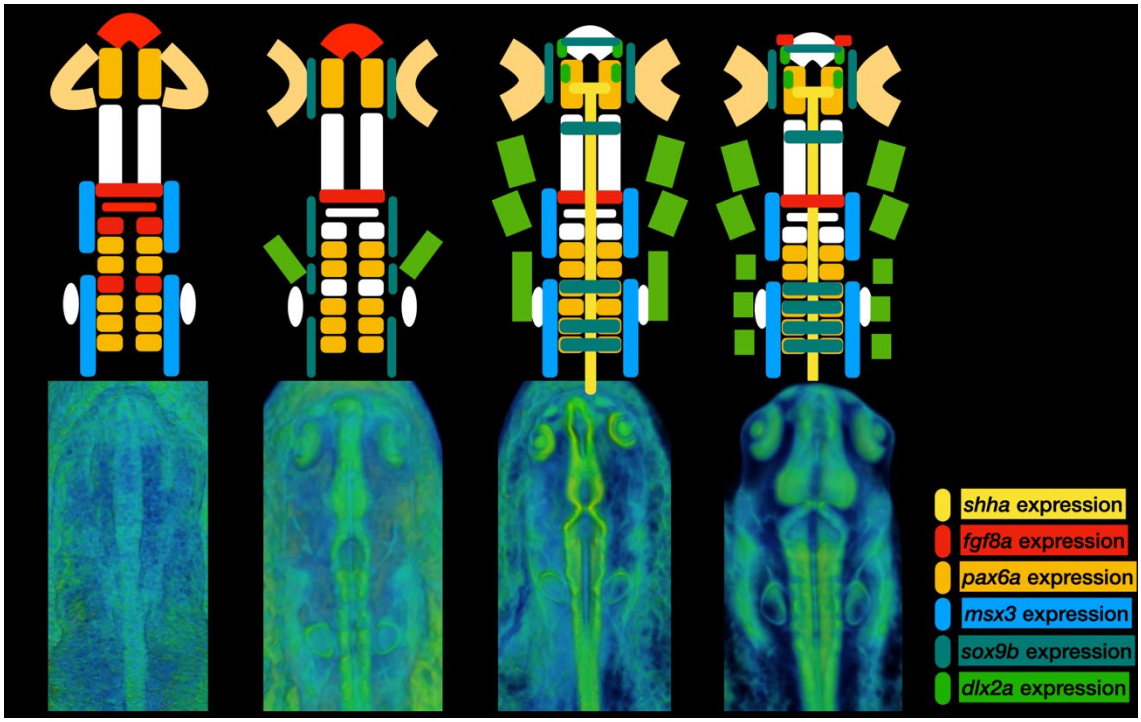

Figure 5 **Gene expression patterns and brain regionalization in the bitterling**. From left to right: stage 30 hpf, 50 hpf, 70 hpf, and 90 hpf. The upper images are color-coded schematics of gene expression domains. The lower images are volume renderings from microCT of embryos at the same developmental stage as the corresponding schematic. Dorsal views, rostral is to the top.

Transcriptional heterochrony between bitterling and the zebrafish

We identify three developmental periods of brain development that are extremely similar in the bitterling and zebrafish, namely: 10-somite to 6-somite, 5-ovl to prim-5, and prim-3 to prim-25 periods. Especially noteworthy in this comparative context is the expression of *dlx2a*. Its expression changes from demarcating 3 NCC streams to 5, and this represents a particulary useful comparative landmark because it is a transition that can be quantitatified. In the zebrafish, the 5 NCC streams do not appear until prim-17 (30 hpf), but in the bitterling they appear as early as prim-3. The prim number is based on the migration of the posterior lateral line primordium in the trunk. Therefore, the brain of a prim-3 bitterling corresponds to the development degree of a prim-17 zebrafish, while the trunk corresponds to an earlier prim-3 zebrafish.

In the zebrafish, truncal expression of *dlx2a* is apparent in pectoral fin bud at the prim-17 stage. A similar expression of dlx2a in the pectoral fin bud appears does not appear in the bitterling until the 1-ovl stage (150 hpf). A possible explanation for this discrepancy in relative timing is that

development is modular, such that each module develops autonomously and can evolve its own independent timing in different species. Just as there are different time zones on the earth, the time zone of the brain region could be a few hours earlier than the trunk region between zebrafish and bitterling.

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