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ORIGINAL ARTICLE

Genoarchitecture of the rostral hindbrain of a shark: basis for understanding the emergence of the cerebellum at the agnathan–gnathostome transition

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Abstract The cerebellum is present in all extant gnathostomes or jawed vertebrates, of which cartilaginous fishes represent the most ancient radiation. Since the isthmic organizer induces the formation of the cerebellum, comparative genoarchitectonic analysis on the meso-isthmo-cerebellar region of cartilaginous fishes with respect to that of jawless vertebrates could reveal why the isthmic organizer acquires the ability to induce the formation of the cerebellum in gnathostomes. In the present work we analyzed the expression pattern of a variety of genes related to the cerebellar formation and patterning (*ScOtx2*, *ScGbx2*, *ScFgf8*, *ScLmx1b*, *ScIrx1*, *ScIrx3,* *ScEn2*, *ScPax6* and *ScLhx9*) by in situ hybridization, and the distribution of Pax6 protein in the developing hindbrain of the shark *Scyliorhinus canicula*. The genoarchitectonic code in this species revealed high degree of conservation with respect to that of other gnathostomes. This resemblance may reveal the features of the ancestral condition of the gene network operating for specification of the rostral hindbrain patterning. Accordingly, the main subdivisions of the rostral hindbrain of *S. canicula* could be recognized. Our results support the existence of a rhombomere 0, identified as the *ScFgf8*/*ScGbx2*/*ScEn2*-positive and mainly negative *ScIrx3* domain just caudal to the midbrain *ScIrx1*/*ScOtx2*/*ScLmx1b*-positive domain. The differential *ScEn2* and Pax6 expression in the rhombomere 1 revealed anterior and posterior subdivisions. Interestingly, dissimilarities between *S. canicula* and lampreys (jawless vertebrates) were noted in the expression of *Irx, Lhx and Pax* genes, which could be part of significant gene network changes through evolution that caused the emergence of the cerebellum.

Keywords Chondrichthyan · Neural genoarchitecture · Isthmus · Midbrain–hindbrain boundary · Rhombomeres

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Introduction

In vertebrates, two prominent external constrictions initially divide the rostral part of the neural tube into three main vesicles: hindbrain (or rhombencephalon), midbrain (or mesencephalon), and forebrain (or prosencephalon). The isthmic organizer (IsO), located at midbrain–hindbrain boundary or MHB, is a secondary organizer that controls the formation of optic tectum rostrally and the cerebellum caudally (Marín and Puelles 1994; Martínez 2001; Aroca and Puelles 2005; Nakamura et al. 2008; Martínez et al. 2013). A complex network of genes is involved in this process. Firstly, the transcription factors *Otx2* and *Gbx2* determine the correct positioning of the IsO at MHB (Hidalgo-Sánchez et al. 1999, 2005a, b; Simeone 2000) and *Lmx1b* is responsible for the initiation and maintenance of *Fgf8* expression, which in turn is the main signaling molecule of the IsO (O'Hara et al. 2005; Guo et al. 2007). The *Iroquois* and *Engrailed* genes are also related to the isthmic territory and are involved in the early patterning of rostral hindbrain (for review, see Gómez-Skarmeta and Modolell 2002; Hidalgo-Sánchez et al. 2005a). These genes have intricate mutual relationships, activating and/or inhibiting

each other (for review, see Liu and Joyner 2001), which appear roughly conserved throughout evolution, as revealed in studies in *Drosophila* (Urbach 2007).

Genetic programs homologous to that found in vertebrate signaling centers as the IsO have been reported in hemichordates, although such programs have been considered components of an ancient genetic regulatory scaffold for deuterostome body patterning, which was retained to pattern divergent structures in hemichordates and vertebrates (Pani et al. 2012; Robertshaw and Kiecker 2012). On the other hand, cephalochordates appear to have a MHB-like, but genes with organizing activity are not expressed in place at this boundary (Holland and Short 2008; Holland 2013). Conversely, urochordates showed the genetic machinery for specification of an IsO, but they have secondarily lost components of those gene programs (Ikuta and Saiga 2007; Holland et al. 2013). Thus, the emergence of an IsO with competence to induce the formation of cerebellum must have occurred during the agnathan–gnathostome transition. The cerebellum appears to be an evolutionary innovation of the common ancestor of gnathostomes or jawed vertebrates, as agnathans or jawless vertebrates do not have cerebellum (for review, see Northcutt 2002; Villar-Cerviño et al. 2010; Wullimann et al. 2011): they lack the cell types that define the cerebellum, i.e., they lack Purkinje cells (Lannoo and Hawkes 1997) and granule cells (for review, see Kuratani et al. 2002), and cerebellar connections have not been demonstrated. Owing to the fact that cartilaginous fishes represent the most ancient radiation of extant gnathostomes, they are closer to the ancestral condition of jawed vertebrates and the study of this group may shed light on the emergence of the cerebellum at the agnathan–gnathostome transition. However, in cartilaginous fishes information about the isthmic territory mainly comes from developmental studies in *Scyliorhinus* focused on non-nervous structures in which the expression of isthmus-related genes as *Fgf8*, *En-1* and *En-2* is circumstantially shown (Tanaka et al. 2002; Adachi et al. 2012; Compagnucci et al. 2013). Our current knowledge about the isthmic territory in gnathostomes mainly comes from studies in mammals (for review, see Joyner et al. 2000), birds (Hidalgo-Sánchez et al. 2005a), amphibians (Glavic et al. 2002) and bony fishes (Jászai et al. 2003).

The term genoarchitecture refers to the descriptions of neural structures in terms of discrete gene expression patterns (Puelles and Ferrán 2012). Because understanding the phylogenetic and ontogenetic aspects of the neural genoarchitecture of the rostral hindbrain and, in particular, of the IsO, is fundamental to advance our knowledge on the cerebellum emergence, we have analyzed the genoarchitecture of the rostral hindbrain of the shark *Scyliorhinus canicula* or lesser spotted dogfish, considered a model species in Evo-Devo studies (see Coolen et al. 2009). For it, we studied the expression pattern of several genes as *ScOtx2*, *ScGbx2*, *ScFgf8*, *ScLmx1b*, *ScIrx1*, *ScIrx3*, *ScEn2*; *ScPax6* and *ScLhx9*, as well as the distribution of Pax6 protein at pharyngula stages. We additionally monitored the meso-isthmo-cerebellar region during later embryonic development. To analyze the degree of evolutionary conservation of the gene expression patterns found in the meso-isthmo-cerebellar area of this basal jawed vertebrate, a comparison of gnathostomes (including our new data) and agnathans was made. Similarities among gnathostomes will allow the establishment of subdivisions and early patterning of the rostral hindbrain in this shark. On the other hand, dissimilarities with respect to agnathans and non-vertebrate chordates could correspond with the causes of the cerebellar evolutionary innovation in the common ancestor of all gnathostomes.

Materials and methods

Experimental animals and tissue preparation

Embryos of the lesser spotted dogfish (*Scyliorhinus canicula*) were supplied by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France) and the Estación de Bioloxía Mariña da Graña (Galicia, Spain). Additional embryos and juveniles were kindly provided by the Aquaria of Gijón, O Grove and A Coruña (Spain). A total of 37 embryos, ranging from pharyngula stages (stages 19–27) to stage 31, were analyzed. Embryos were staged on the basis of their external features according to Ballard et al. (1993). Adequate measures were taken to minimize animal pain or discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal Decree 53/2013 for animal experimentation, and were approved by the Ethics Committee of the University of Santiago de Compostela.

Specimens were anaesthetized with 0.5 % tricaine methane sulphonate (MS-222; Sigma) in seawater. Embryos were fixed by immersion in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) containing 1.75 % urea (elasmobranch PB). Then, the fixative was removed with PB saline. Some embryos were cryoprotected with 30 % sucrose in PB, embedded in NEG 50™ (Thermo Scientific, Kalamazoo, MI, USA), frozen with liquid nitrogen-cooled isopentane and cut on a cryostat. Parallel series of transverse and sagittal sections (18–20 µm thick) were mounted on Superfrost Plus slides (Menzel-Glässer®, Madison, WI, USA).

In situ hybridization on whole embryos and on sections

We applied in situ hybridization with cDNA probes for RNA of *ScFgf8* (Compagnucci et al. 2013), *ScPax6* (Rodríguez-Moldes et al. 2011); *ScEn2*, *ScGbx2*, *ScIrx1*, *ScIrx3*, *ScLmx1b*, *ScLhx9* and *ScOtx2* (Germot et al. 2001; Plouhinec et al. 2005) genes. These probes were selected from a collection of *S. canicula* embryonic cDNA library (mixed stages, S9–22), submitted to high-throughput EST sequencing (coord. Dr. Sylvie Mazan at the Station Biologique de Roscoff, France). The cDNA fragments were cloned in pSPORT vectors. Sense and antisense digoxigenin-UTP-labeled and fluorescein-UTP-labeled probes were synthesized directly by in vitro transcription using as templates linearized recombinant plasmid DNA (*ScOtx2* and *ScPax6* probes) or cDNA fragments prepared by PCR amplification of the recombinant plasmids (*ScFgf8, ScEn2, ScGbx2, ScIrx1, ScIrx3*, *ScLmx1b* and *ScLhx9* probes).

In situ hybridization in whole embryos and on cryostat sections was carried out following standard protocols (for details, see Coolen et al. 2007; Ferreiro-Galve et al. 2012a). Briefly, sections were permeabilized with proteinase K, hybridized with sense or antisense probes overnight at 65 °C (in sections) or 70 °C (whole mount) and incubated with the alkaline phosphatase-coupled anti-digoxigenin and anti-fluorescein antibody (1:2,000, Roche Applied Science, Manheim, Germany) overnight at 4 °C. The color reaction was performed in the presence of BM-Purple and FastRed tablets (Roche). Control sense probes did not produce any detectable signal.

Immunohistochemistry

Combination of in situ hybridization on sections with immunohistochemistry for the rabbit polyclonal anti-Pax6 (Covance) and single anti-Pax6 labeling was also carried out at stages 24 and 25 following standard protocols (for details see Rodríguez-Moldes et al. 2011). For details about the specificity of Pax6 antibody in *Scyliorhinus canicula,* tested by pre-adsorption with the respective blocking peptide, see Ferreiro-Galve et al. (2012b).

Imaging

In toto hybridized embryos were analyzed in a Leica MZ16F stereo microscope fitted with a Leica DFC490 camera. Photomicrographs were taken with an Olympus DP70 color digital camera fitted to a Provis photomicroscope equipped for fluorescence with appropriate filter combinations. For presentation, some color photomicrographs were converted to gray scale, and brightness and contrast adjusted using Adobe Photoshop 7.0. Plate photomontage, schemes and lettering were made with Corel Draw X6 and Adobe Photoshop 7.0.

Results

We have studied the genoarchitectonic patterns at the meso-isthmo-cerebellar region during the early regionalization of the hindbrain in the lesser spotted dogfish. We have mainly focused the study on embryos at 19/20 and 24/25 stages, because they correspond to the period just before and after that the protuberance of the cerebellar primordium become anatomically evident, respectively. Of note, according to those features described by Ballard et al. (1993), we consider stages 19–23 (when pharyngeal pouches and branchial arches are forming and pharyngeal clefts start to open) and stages 24–27 (when all pharyngeal clefts become opened and opening and shaping of the mouth takes place) as early and late pharyngula stages, respectively. Equivalences between these developmental stages and those of other vertebrates are based on similarities in the reported gene expression patterns and external morphological features. Here we present the expression pattern of the *ScOtx2*, *ScGbx2*, *ScFgf8*, *ScLmx1b*, *ScIrx1*, *ScIrx3, ScEn2*, *ScPax6* and *ScLhx9* genes and the distribution of Pax6 protein in the caudal midbrain and rostral hindbrain at pharyngula stages.

The results obtained from in situ hybridization experiments did not yield significant differences at pharyngula stages (stages 19/20, 24/25 and 27), and even at further developmental stages (stages 29–31) as regards the expression pattern in the rostral hindbrain of most genes studied in the present work (Figs. 1, 2, 3).

Expression pattern of isthmus and cerebellum-related genes in whole embryos

At stages 20 and 24, *ScOtx2* and *ScGbx2* positive domains appeared to be complementary. Strong *Otx2* expression was found in the forebrain and midbrain (Fig. 1a, b), while *Gbx2* was expressed in the hindbrain (Fig. 1c, d). The interface between both domains delimited the midbrain–hindbrain boundary or MHB (black arrows in Fig. 1). Caudal to this line, a conspicuous band of *ScFgf8* expression was observed at stages 20 and 24 (Fig. 1e, f), similarly to that reported by Compagnucci et al. (2013). In an early pharyngula, *ScLmx1b* gene was also expressed in a transverse band of cells in the caudal midbrain, though a longitudinal band at dorsal-most levels was also extended towards rostral (up to the forebrain) and caudal (along the hindbrain) regions, where it partially overlapped with the *ScFgf8* positive domain (Fig. 1g). Later, at stage 24, both the transverse and longitudinal *ScLmx1b* positive domains appeared thinner, though weak *Lmx1b* expression continued to be rostro-caudally extended and still partially overlapped with the *ScFgf8* band in the most rostral hindbrain (compare Fig. 1f and h; see details in Fig. 2).

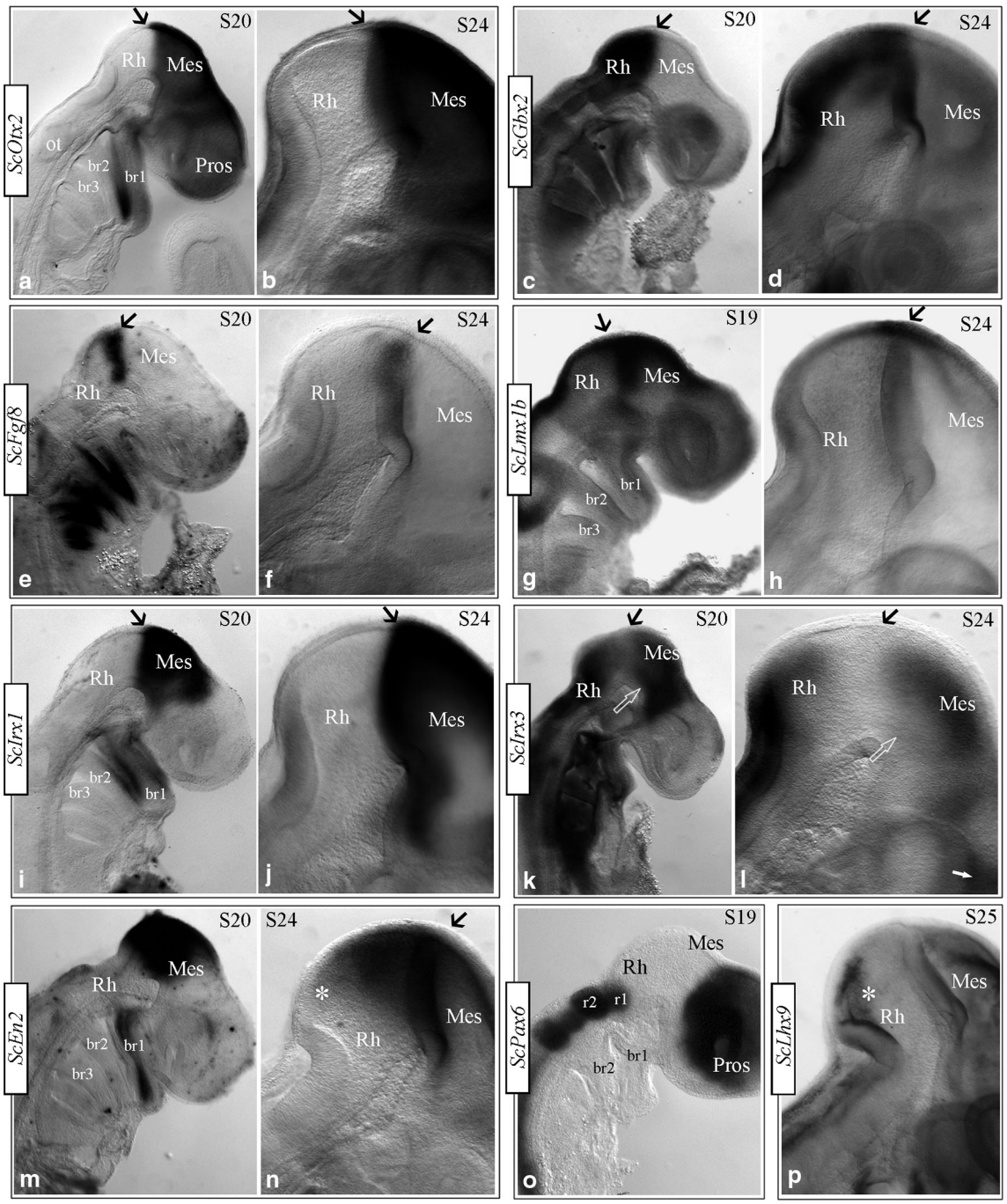


Fig. 1 Major transcription and signal factor gene expression characterizing embryonic midbrain–hindbrain region in whole mount brains of *Scyliorhinus canicula* at early (stages 19–20) and late (stages 24–25) pharyngula stages. Panoramic and details of lateral views showing in situ hybridization reactions for: *ScOtx2* (**a**, **b**), *ScGbx2* (**c**, **d**), *ScFgf8* (**e**, **f**), *ScLmx1b* (**g**, **h**), *ScIrx1* (**i**, **j**), *ScIrx3* (**k**, **l**), *ScEn2* (**m**, **n**), *ScPax6* (**o**) and *ScLhx9* (**p**) gene expression in the meso-isthmo-cerebellar region. *Black arrows* in **a**–**l**, **n** indicate the midbrain–hindbrain boundary. *Outlined arrows* in **k** and **l** indicate the mesencephalic tegmentum. *White arrow* in **l** points the rostral extension of the *Irx3* positive domain in the prosencephalon. *Asterisks* in **n**, **p** indicate the cerebellar primordium. *br1–3* branchial archs 1–3, *Mes* mesencephalon, *ot* otic vesicle, *Pros* prosencephalon, *r* rhombomere, *Rh* rhombencephalon

The gene *ScIrx1* was expressed rostrally to the MHB. At stages 20 and 24, the caudal limit of the positive domain appeared to coincide with that of *ScOtx2*, while it extended rostrally up to the caudal forebrain (Fig. 1i, j). The expression pattern of *ScIrx3*, showed a conspicuous negative gap at midbrain–hindbrain domain and two positive domains, one caudal along the hindbrain, and other rostral, which extended up to the caudal forebrain at stage 20

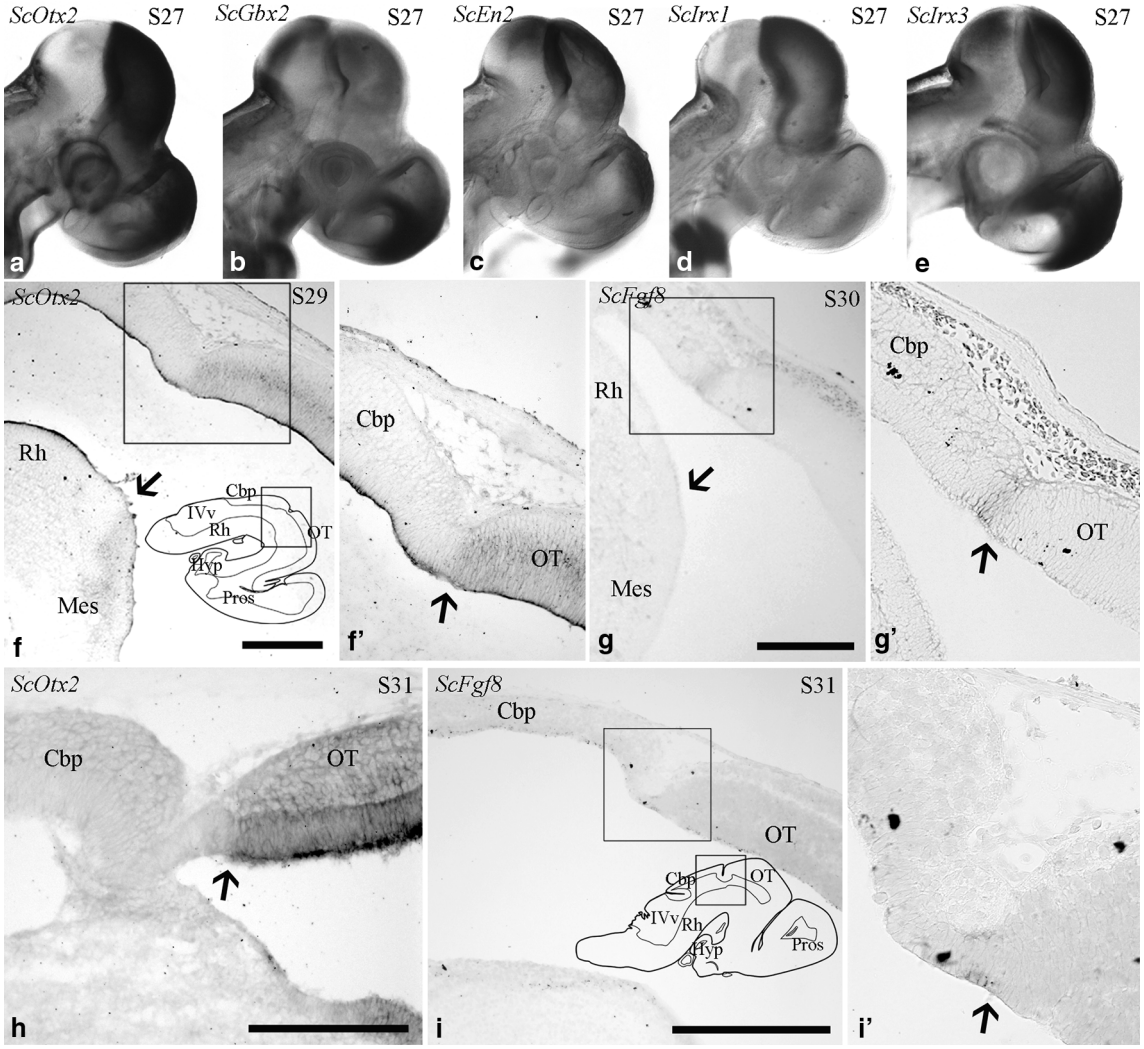
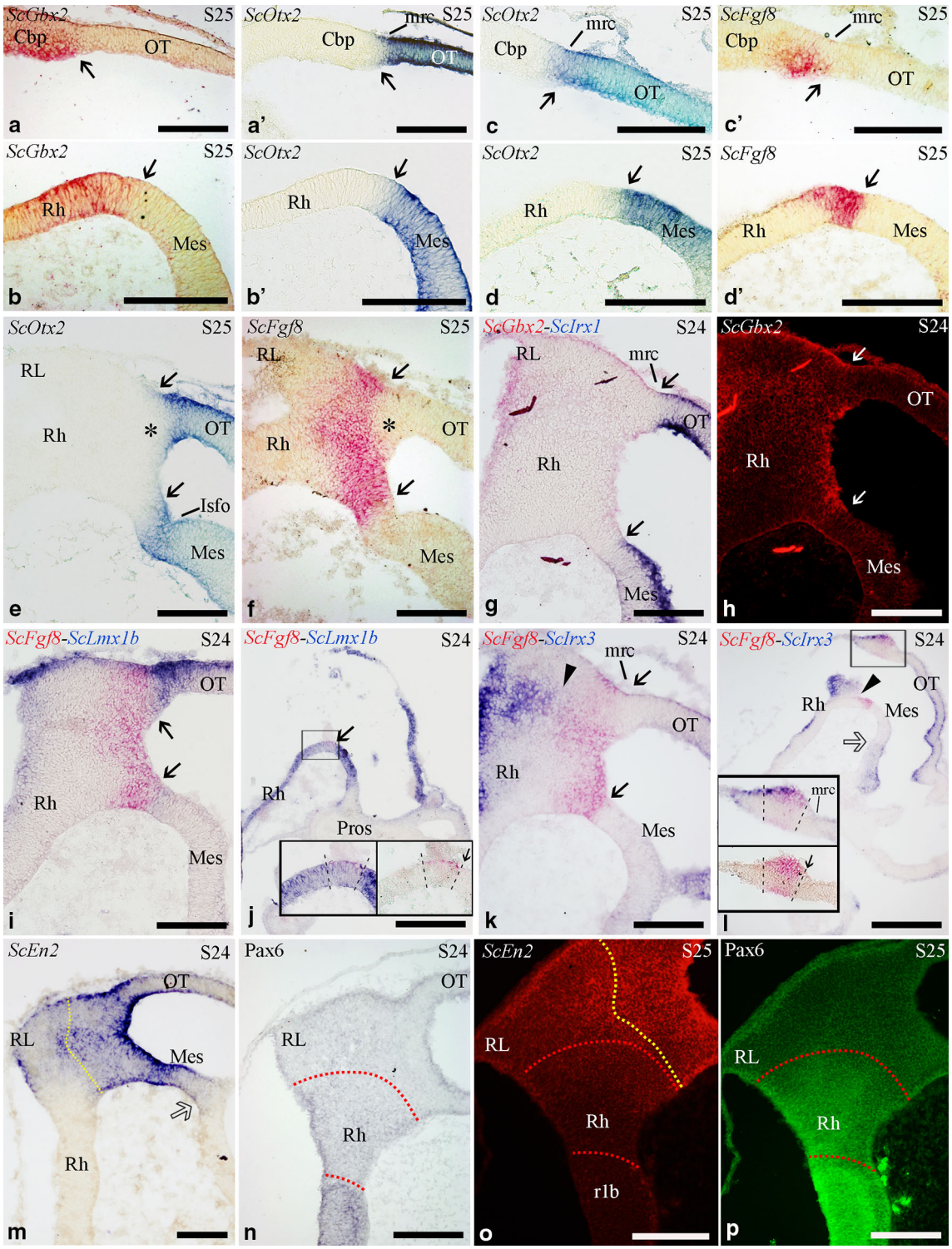


Fig. 2 Major transcription and signal factor gene expression characterizing embryonic midbrain–hindbrain region in whole mount and sagittal brain sections of *Scyliorhinus canicula* at the end of the pharyngula period (stage 27) and later on development (stages 29–31). Panoramic of lateral views at stage 27 showing the expression pattern of *ScOtx2* (**a**), *ScGbx2* (**b**), *ScEn2* (**c**), *ScIrx1* (**d**) and *ScIrx3* (**e**). Panoramic and details of parasagittal sections at the level indicated in the schemes, at stages 29 (**f**), 30 (**g**) and 31 (**h**, **i**) showing the expression pattern of *ScOtx2* and *ScFgf8*. *Black arrows* indicate the midbrain–hindbrain boundary. *Cbp* cerebellar plate, *Hyp* hypothalamus, *IVv* fourth ventricle, *Mes* mesencephalon, *OT* optic tectum, *Pros* prosencephalon, *Rh* rhombencephalon. *Scale bars* 200 μm (**f**–**h**); 500 μm (**i**)

(Fig. 1k) and more rostrally at stage 24 (white arrow in Fig. 1l). Furthermore, at stage 24 the expression in the midbrain tegmentum decreased and consequently the negative gap appeared enlarged at this level (outlined arrows in Fig. 1k, l).

The gene *ScEn2* was highly expressed in the midbrain–hindbrain domain and extended in a decreasing gradient both rostrally and caudally (Fig. 1m, n). The caudal edge of the *En2* positive domain was extended into the cerebellar primordium (probably corresponding to the upper rhombic lip, asterisk in Fig. 1n), which is located rostrally to the lateral recess of the fourth ventricle. The rostral edge approximately coincided with the rostral limit of the mesencephalic tegmentum (Fig. 1n).

In the rostral hindbrain, the *ScPax6* gene was expressed up to the rhombomere (r) 1 at early (stage 19; Fig. 1o), and late (stage 25; Fig. 1h in Rodríguez-Moldes et al. 2011) pharyngula stages. Worth mentioning is that the r1–r2 boundary was identified on the basis of the rostral edge of the *ScHoxA2* expression domain, as illustrated by Rodríguez-Moldes et al. (2011). Regarding to the *ScLhx9* gene, a faint expression was observed in the cerebellar primordium in late pharyngulas (asterisk in Fig. 1p).


ScGbx2
S25
Cbp
OT
a
ScOtx2
mrc
S25
Cbp
OT
a'
ScOtx2
mrc
S25
Cbp
OT
c
ScFgf8
mrc
S25
Cbp
OT
c'
ScGbx2
S25
Rh
Mes
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ScOtx2
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S24
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RL
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n
ScEn2
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◀**Fig. 3** Major transcription and signal factor gene expression characterizing embryonic midbrain–hindbrain region in sagittal brain sections of *Scyliorhinus canicula* at late pharyngula stages. Midsagittal (**a**–**d**, **j**, **l**) and parasagittal (**e**–**i**, **k**, **m**–**p**) sections of *S. canicula* embryos at 24 (**g**–**n**) and 25 (**a**–**f**, **o**, **p**) stages hybridized for the indicated gene markers (*upper left*). *Black* and *white arrows* indicate the midbrain–hindbrain boundary. **a**–**f** Details of *ScGbx2*, *ScOtx2* and *ScFgf8* positive domains. *Asterisk* (in **e**, **f**) indicates a negative gap for the expression of *ScFgf8* and *ScOtx2*. **g**–**m** Details (**g**–**i**, **k**, **m**) and panoramic views (**j**, **l**) of double (**g**–**l**) or single (**m**) labeling of *ScGbx2-ScIrx1* (**g**), *ScGbx2* (**h**), *ScFgf8-ScLmx1b* (**i**, **j**), *ScFgf8-ScIrx3* (**k**, **l**), and *ScEn2* (**m**) genes. *Insets* in **j** and **l** show overlapping domains of expression. *Dashed lines* in **j** and **l** indicate the rostral and caudal edges of the *Fgf8* expression domain. *Arrowhead* in **k**, **l** indicates the interface abutting *ScFgf8* and *ScIrx3* positive domains. *Outlined arrows* in **l**, **m** indicate the interface approximately abutting *ScIrx3* and *ScEn2* positive domains. Single labeling of the Pax6 protein (**n**) and double labeling of the expression of the *ScEn2* gene and Pax6 protein showing *colors* separately (**o**, **p**). Double labeled sections were photographed after the first *color* development (*red*) and also after the second one (*blue*). Some double labeled sections (**a′**, **b′**, **c**–**e**) appear single labeled because of the accidental removal of *red* color at the end of the experiment. Note also the thin expression domain of *ScFgf8* at midsagittal levels of both alar (**c′** and *inset* in **l**) and basal (**d′** and *inset* in **j**) plates of the isthmic domain. *Yellow dashed lines* in **m** and **o** tentatively indicates the caudal edge of the *Fgf8* positive domain. *Red dashed lines* in **n**–**p** indicate the rostral edge of two areas discernible by the intensity of Pax6 labeling (showing intense labeling within rhombomere r1b and weak labeling within rhombomere r1a). *Cbp* cerebellar plate, *Isfo* isthmic fovea, *Mes* mesencephalon, *mrc* meso-rhombencephalic constriction, *OT* optic tectum, *Pros* prosencephalon, *Rh* rhombencephalon, *RL* rhombic lip. *Scale bars* 200 μm (**a**–**i**, **k**, **m**–**p**); 500 μm (**j**, **l**)

The meso-isthmo-cerebellar area later in development

We also aimed to advance knowledge into the conservative degree of neural genoarchitecture and to identify the rostral edge of cerebellar region throughout development. Therefore, we also studied the meso-isthmo-cerebellar region during later development. At the end of the pharyngula period, at stage 27, the expression pattern of the isthmus-related genes still appeared the same as observed from earlier pharyngula stages. The caudal and rostral edges of *ScOtx2* and *ScGbx2* positive domains, respectively, abutted at the MHB (Fig. 2a, b). The domain of *Fgf8* expression was also observed in a band caudally to MHB (Compagnucci et al. 2013). Furthermore, though the domain of expression of *ScEn2* appeared to extend more rostrally, its caudal edge remained extended into the cerebellum (Fig. 2c). Likewise, the expression of *ScIrx1* and *ScIrx3* genes (Fig. 2d, e) also appeared very similar to that observed at earlier stages (compare Figs. 1i–l and 2d, e). In subsequent developmental stages 29–31, the *ScOtx2* and *ScFgf8* expression domains still appeared to abut at MHB (arrow in Fig. 2f–i). However, contrary to that observed at pharyngula stages, the *Fgf8* expression became restricted to the alar area and was excluded from the basal plate (Fig. 2g; see details in Fig. 3).

Boundaries among the expression domains of isthmus-related genes

To better discern the degree of overlapping among the expression domains of these genes at the meso-isthmo-cerebellar area, we performed single and double labeling on sections. Comparison of the expression patterns of *ScGbx2* and *ScOtx2* revealed that they do not overlap but just abut at MHB both at alar (compare Figs. 3a and 3a′) and basal (compare Figs. 3b and 3b′) plates, although a few cells apparently expressing both *ScOtx2* and *ScGbx2* genes were observed in the neuroepithelium (not shown). Likewise, *ScOtx2* domain abutted with *ScFgf8* domain at MHB both at alar (compare Figs. 3c and 3c′) and basal (compare Figs. 3d and 3d′) plates, except for a few weakly labeled cells in the neuroepithelium which appeared to overlap (not shown) and a conspicuous negative gap at parasagittal level (asterisk in Fig. 3e, f; see also Fig. 4b). Interestingly, the sequential analysis of sagittal sections from medial to lateral levels revealed that at some levels the isthmic or meso-rhombencephalic constriction (mrc, in Fig. 3) appeared to be located rostrally to the *ScFgf8* positive area (Fig. 3l).

The *ScGbx2* and *ScIrx1* domains were also abutting at MHB (Fig. 3g, h). The *ScFgf8* and *ScLmx1b* transverse domains abutted at parasagittal levels (Fig. 3i). The thin longitudinal domain of *Lmx1b* expression, which in parasagittal sections is located at dorsal-most levels along the hindbrain and up to the forebrain (Fig. 3i; see also Fig. 1g, h), appeared dorso-ventrally extended at midsagittal levels, clearly overlaying the whole *ScFgf8* domain (Fig. 3j). Interestingly, the *ScFgf8* domain was narrower at midsagittal (Fig. 3c′, d′, j, l) than at lateral (Fig. 3f, i, k) levels. The *ScFgf8-ScIrx3* combination showed that the rostral limit of the *ScIrx3* domain in the hindbrain roughly coincided with the caudal edge of *ScFgf8* domain (arrowhead in Fig. 3k, l) except for the median-alar portion, where both genes overlapped (inset in Fig. 3l). On the other hand, the caudal limit of the *ScIrx3* domain in the midbrain appeared adjacent to the anterior limit of *ScEn2* expression (outlined arrows in Fig. 3l, m). The *ScEn2* gene showed that the limit of expression extended beyond the MHB (compare Figs. 3k and 3m) both partially in the midbrain and hindbrain. Of note, the Pax6 labeling in the hindbrain showed two discernible areas (red dashed lines in Fig. 3n–p): the most intense labeling was occupying the caudal half of r1 (see also Fig. 1h in Rodríguez-Moldes et al. 2011), while less intense labeling was observed within the rostral half of r1 (very weak at stage 24 and more patent at stage 25, compare Figs. 3n and 3p). The caudal edge of *ScEn2* appeared approximately abutting with the rostral edge of Pax6 expression, as opposite gradients of expression (Fig. 3m–p). Ventrally at parasagittal levels, the *ScEn2*-Pax6

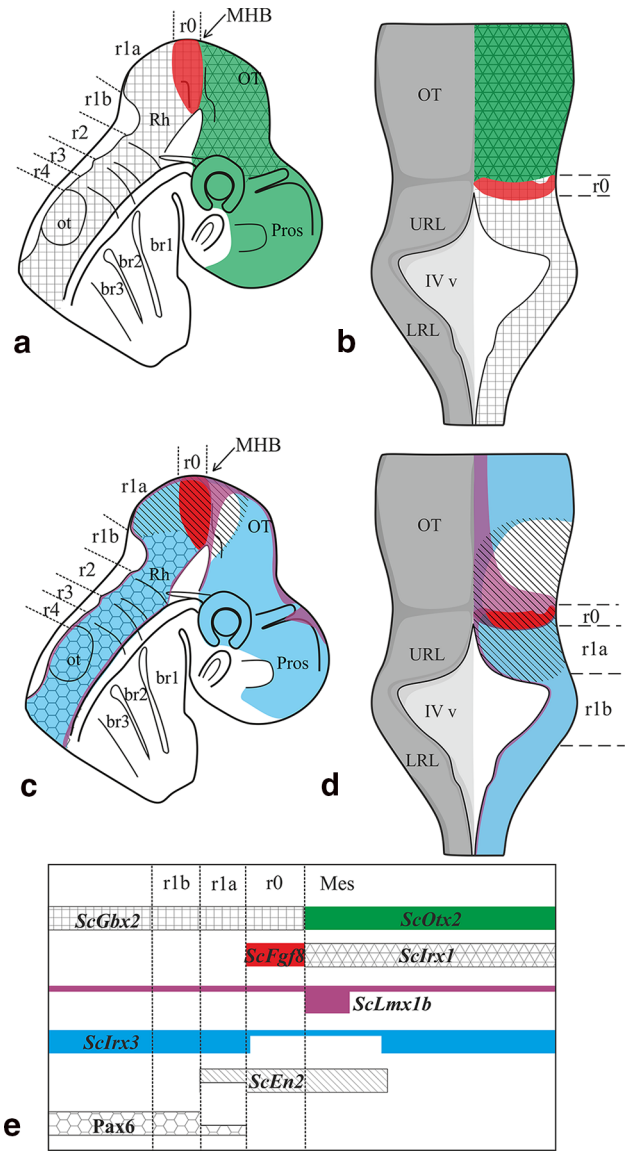

Fig. 4 Schematic drawings of rostral hindbrain genoarchitecture in *S. canicula* early embryos. **a–d** Schematic drawings of sagittal (**a**, **c**) and dorsal (**b**, **d**) views of *S. canicula* embryos at stages 24/25 showing the expression pattern of the *ScOtx2*, *ScGbx2*, *ScFgf8*, *ScLmx1b*, *ScIrx1*, *ScIrx3* and *ScEn2* genes and the Pax6 protein. **e** Diagram summarizing the distribution of domains of expression in *S. canicula* at midbrain, midbrain–hindbrain boundary, and rostral hindbrain. *Bars* indicate the extension of the expression domains. *Thin line* in *Lmx1b* and *Irx3 bars* indicate the rostro-caudal extension of the expression pattern in the dorsal-most zone. Anterior levels correspond to the right side. *br1–3* branchial archs 1–3, *IVv* fourth ventricle, *LRL* lower rhombic lip, *Mes* mesencephalon, *MHB* midbrain–hindbrain boundary, *ot* otic vesicle, *OT* optic tectum, *Pros* prosencephalon, *r0–4* rhombomeres 0–4, *Rh* rhombencephalon, *URL* upper rhombic lip

boundary appeared to coincide with the caudal edge of the *ScFgf8* positive area (illustrated by the yellow dashed line in Fig. 3m, o; see also Rodríguez-Moldes et al. 2011), but not at the dorsal hindbrain area (RL in Fig. 3m–p).

Discussion

General considerations on the regionalization of the meso-isthmo-cerebellar area

The MHB and the most caudal midbrain and rostral hindbrain areas (or meso-isthmo-cerebellar area) express genes involved in the IsO activity, according to Hidalgo-Sánchez et al. (2005a, b), O'Hara et al. (2005) and Guo et al. (2007). Nevertheless, Fgf8 is considered as the main signaling molecule in the IsO (for review, see Nakamura et al. 2008). The position of the *Fgf8*-positive domain in the most rostral area of the *S. canicula* hindbrain has allowed us the identification (at least during part of the embryonic period) of the rhombomere 0 (r0), a region that has been defined as corresponding to the isthmus or isthmic territory (Martínez et al. 2013). This region has been considered as a pseudorhombomere or *cryptorhombomere* because it does not exhibit all the features of a true rhombomere (for review, see Marín et al. 2008; Watson et al. 2012; Martínez et al. 2013). According to our results, the secondary organizer in *S. canicula* might correspond to both the caudal midbrain region expressing *ScLmx1b* and the rostral hindbrain region expressing *ScFgf8*. However, taking into account the main organizing activity of Fgf8 and that the expression domain of *ScLmx1b* is rather dynamic throughout development, we consider for convenience the isthmic area in *S. canicula* as that expressing *ScFgf8*.

Evolutionary conservation of rostral hindbrain neural genoarchitecture among gnathostomes

To know the degree of evolutionary conservation in the early cerebellar development, we carried out a comparison between the neural genoarchitecture observed in *S. canicula* and that described in other gnathostomes. The striking morphological resemblance between early embryos of the lesser spotted dogfish (stages 19–25), and those of chick (HH14–HH19) and mouse (8.5 and 9.5/10.5), greatly facilitates the comparison at these stages. Therefore, pharyngula stages would correspond to the phylotypic stage, when the embryos of all vertebrates are more similar one each other and show a basic common bauplan (reviewed in: Slack et al. 1993; Kimmel et al. 1995; Kuratani and Horigome 2000; Kuratani et al. 2001; Mueller et al. 2006). Comparison to other anamniota, as bony fishes and amphibians, was also carried out. In general, we observed that the expression pattern of the isthmus-related genes in the lesser spotted dogfish is quite similar to that reported in mouse (see Allen Developing Mouse Brain Atlas [Internet] 2009) and other gnathostomes (see below). Accordingly, we have accurately identified the MHB and the main subdivisions of the rostral-most hindbrain by

identifying the r0 as the *ScFgf8*/*ScGbx2*/*ScEn2*-positive and mainly negative *ScIrx3* domain, just caudal to the midbrain *ScIrx1*/*ScOtx2*/*ScLmx1b*-positive domain (see Fig. 4).

Regarding to the MHB, it is defined as the interface between the *Otx2*-*Gbx2* expression domains (Simeone 2000). In the lesser spotted dogfish, we observed that the caudal edge of *ScOtx2* positive domain abutted with the rostral edge of *ScGbx2* positive domain (see Fig. 4, and also see Fig. 3 in Mazan et al. 2000 and Fig. 2 in Plouhinec et al. 2005), as has been described in other gnathostomes [*Xenopus* (Glavic et al. 2002), zebrafish (Jászai et al. 2003; Kikuta et al. 2003; Rhinn et al. 2003), chick (Hidalgo-Sánchez et al. 2005a) and mouse (for review, see Joyner et al. 2000)]. Another evidence for the identification of the MHB was the gene *Irx1*, which is also required in the formation of the MHB (for review, see Aroca and Puelles 2005). In fact, the caudal edge of *ScIrx1* coincided with that of *ScOtx2*. Similarly, in most jawed vertebrates this gene is not expressed at isthmic area (Cohen et al. 2000; Cheng et al. 2001, 2007; present results). However, the *Irx1* positive domain of expression described in the hindbrain of mouse and zebrafish (Cohen et al. 2000; Cheng et al. 2001, 2007) was absent in the lesser spotted dogfish. This dissimilarity might be related to secondarily derived features.

In the lesser spotted dogfish, during early development, the isthmic constriction appeared to be located more rostrally than the anterior edge of the *ScFgf8* expression domain. However, later in development, the isthmic constriction coincided with the MHB, which would imply that, in this species, the isthmic constriction changes its position caudalwards as development proceeds, and not rostralwards as in amniotes (Puelles et al. 1995; for review, see also Martínez et al. 2013).

The r0 is recognized by the *Fgf8* positive band in the rostral hindbrain abutting with the MHB at the caudal edge of *Otx2* (as described Aroca and Puelles 2005), which is clearly identified in the lesser spotted dogfish at pharyngula stages and even later on development (Compagnucci et al. 2013; present results) and matches well with what was described in all groups of gnathostomes (Glavic et al. 2002; Inoue et al. 2008; Hidalgo-Sánchez et al. 2005a; Joyner et al. 2000). Additionally, the apparent colocalization of a few *ScFgf8*-*ScOtx2* expressing cells we observed in the lesser spotted dogfish (not shown) was also previously described in chick (Hidalgo-Sánchez et al. 2005a). Furthermore, the negative gap between *ScFgf8* and *ScOtx2* (asterisk in Fig. 2e, f) could either correspond to that observed in chick, where it has been related with the morphogenesis of the isthmic constriction (Adams et al. 2000; see Fig. 2 in Sotelo 2004), or could be equivalent to the transient area for free cell intermixing between midbrain and hindbrain described in mouse (Zervas et al. 2004). The interface abutting *ScIrx3* and the caudal edge of the *ScFgf8* domain observed in *S. canicula* (present results), also supports the r0 existence. A negative gap for *Irx3* expression in the isthmic area was also found in other gnathostomes (Fig. 2 in Kobayashi et al. 2002; see also; Bosse et al. 1997; Tan et al. 1999; Cohen et al. 2000). Contrary, in *Xenopus*, the ortholog *Xiro3* was expressed in the isthmic area at early stages, but its expression becomes reduced in this area later in development (Bellefroid et al. 1998; Rodríguez-Seguel et al. 2009).

The maintenance of *Fgf8* expression in *S. canicula* throughout development could be crucial, as in amniotes, for the specification of different structures at the meso-isthmo-cerebellar region at different developmental stages (Sato and Joyner 2009). The apparent decrease of the extension of the *ScFgf8* domain later in development (after pharyngula stages), which became excluded from the basal portion and reduced to a thin ring close to the MHB in the alar plate, also occurs in amniota species (Aroca and Puelles 2005). According to Vaage subdivisions of the rostral hindbrain (isthmus, plus r1 and r2; Vaage 1969, 1973) and its current interpretation in chick embryos (Aroca and Puelles 2005), the *Fgf8* positive domain might also correspond in *S. canicula* to certain extension of the pro-rhombomere A1, but larger at pharyngula stages than at late developmental stages (when it became reduced to its rostral-most part). Moreover, the *ScFgf8* expression domain would be a reliable marker of r0 only early on development, as described in chick (Aroca and Puelles 2005). Nevertheless, the topographic boundary between the *ScFgf8* and *ScOtx2* expression domains allows the truthful identification of the MHB also at late developmental stages, and consequently, the accurate location of diverse structures in the correct brain subdivisions according to the segmental model of the brain (for review, see Nieuwenhuys 2011). This may be useful for further evolutionary studies on the cerebellum and/or hindbrain in this basal gnathostome.

Other remarkable isthmus-related gene is the *Lmx1b*, which is important in the maintenance of the secondary organizer. It is involved in the regulation of *Wnt1* and *Fgf8* expression and is coexpressed with the *Wnt1* positive domain in the caudal-most midbrain (see Aroca and Puelles 2005; Guo et al. 2007), and therefore, it abuts with the *Fgf8* domain. Furthermore, it is involved in the formation of roof plate structures (Chizhikov and Millen 2004) and in the process of morphogenesis of the isthmic constriction (Adams et al. 2000). This gene in *S. canicula* could play the same roles, because the expression pattern of *ScLmbx1b* also appears similar to that of its orthologs in other jawed vertebrates (Adams et al. 2000; Haldin et al. 2003; O'Hara et al. 2005; Cheng et al. 2007; Mishima et al. 2009; Liu

et al. 2010). Moreover, in chick and mouse, the *Lmx1b* positive domain temporarily covers the MHB and isthmic area and progressively becomes approximately restricted to the caudal midbrain (Adams et al. 2000; Guo et al. 2007; Mishima et al. 2009). Similarly, a reduction in the extension of *Lmx1b* positive domain throughout development was also observed in *S. canicula* (present results).

These findings not only show the existence of the MHB and r0 in a basal lineage of gnathostomes, but also show the high degree of evolutionary conservation of topographic relationships among the expression patterns of a broad set of isthmus-related genes. Therefore, it supports the hypothesis that it would correspond to the gene basic and basal network necessary for the formation of the isthmic territory, and consequently the cerebellum.

Subdivision of the rhombomere 1 in *S. canicula*

Once the signaling activity from the IsO starts, the onset of the formation of the cerebellum, which is derivative from r1 plus part of r0, takes place (for review, see Martínez et al. 2013). A more detailed analysis of r1, which presents larger size and greater degree of complexity than other rhombomeres, has led to the identification of two sub-rhombomeres (r1a and r1b) in amniotes, which would be nearly consistent with that firstly described by Vaage (1969) in chick embryos (reviewed in Aroca and Puelles 2005; Moreno-Bravo et al. 2014). On the other hand, subdivisions of r1 identified in early embryos were associated with prospective different cerebellar domains at mature developmental stages (Sgaier et al. 2005).

In *S. canicula* we identified r1 as the area in between the caudal edge of the *ScFgf8* expression domain (present results) and the rostral edge of the *ScHoxA2* expression domain (see Rodríguez-Moldes et al. 2011). Searching for the existence of sub-rhombomeres in the r1 in this species, we have thoroughly analyzed the expression pattern of *ScEn2* at pharyngula stages, as in chick and mouse the caudal edge of *En2* expression domain seemed to coincide with the limit between r1a and r1b (Aroca and Puelles 2005; see also Fig. 13 in Alonso et al. 2013). The expression pattern of *Engrailed* genes at the meso-isthmo-cerebellar area appeared highly conserved among cartilaginous fishes and with respect to other gnathostomes (Liu et al. 1999; Tanaka et al. 2002; Lekven et al. 2003; Hidalgo-Sánchez et al. 2005a; Koenig et al. 2010; Adachi et al. 2012; present results). Moreover, the caudal limit of the *ScEn2* positive domain allowed us to distinguish rostral region in the r1 (r1a) at dorsal-most levels, while differential expression of Pax6 allow us to distinguish it at ventral-most levels (see Fig. 4). In zebrafish, *En2* also appears approximately abutting with *Pax6* (Scholpp et al. 2003). Other evidence for r1 subdivision are the GAD-DCX double labeled cells previously described at the most rostral part of r1 (or r0–r1 boundary), which may correspond to migrating neuroblasts from upper rhombic lip or part of the r1 (Rodríguez-Moldes et al. 2011; Pose-Méndez et al. 2014), as they appear to abut with the caudal edge of *ScFgf8* expression domain and to overlap with the *ScEn2* positive domain in the rostral half of the r1 (present results).

The identification of these two sub-rhombomeres (r1a and r1b) in *S. canicula* may help us to determine in further studies whether this subdivision would be related to the formation of different regions within the cerebellum. In that case, it might be associated with different functional domains of the cerebellum also in basal gnathostomes.

Basis for understanding the emergence of the cerebellum by comparing with non-vertebrates and agnathans

Despite the portions of gene networks related to a IsO-like regulatory program have been described in hemichordates (Pani et al. 2012; Robertshaw and Kiecker 2012; Holland et al. 2013), a true IsO inducing the cerebellar formation appears to be present only in gnathostomes. Therefore, dissimilarities on the gene expression patterns between the lesser spotted dogfish with respect to non-vertebrates and agnathans could explain why this secondary organizer acquired the ability of inducing the formation of the cerebellum, for the first time in evolution, probably in the common ancestor of all jawed vertebrates (see Fig. 5).

Differently from that observed in the lesser spotted dogfish and other vertebrates (see above), the edge of the *Fgf8* positive band and the *Otx* and *Gbx* boundary are reversely located in hemichordates (Pani et al. 2012). Conversely, in cephalochordates (amphioxus) and tunicates (ascidians), the topographic boundaries of certain isthmus-related genes show higher resemblance to those of vertebrates (including *S. canicula*) than in the case of hemichordates (see Pani et al. 2012; Holland et al. 2013; present results). The amphioxus show an *Otx2-Gbx2* interface as in *S. canicula* and other vertebrates, but other isthmus-related genes are not expressed at this boundary. On the other hand, urochordates show more of the machinery for an IsO-like in place than cephalochordates, but they have lost the *Gbx2* gene. So, our results would support the hypothesis proposed by Pani et al. (2012) and Holland et al. (2013) that at least a partial IsO-like signaling center predates vertebrates despite that it is not entirely clear whether it occurred in the ancestor of deuterostomes (Pani et al. 2012) or at the base of the chordates lineage (Holland et al. 2013). A summary of comparative aspects in relation to the MHB and IsO throughout evolution is shown in Fig. 5.

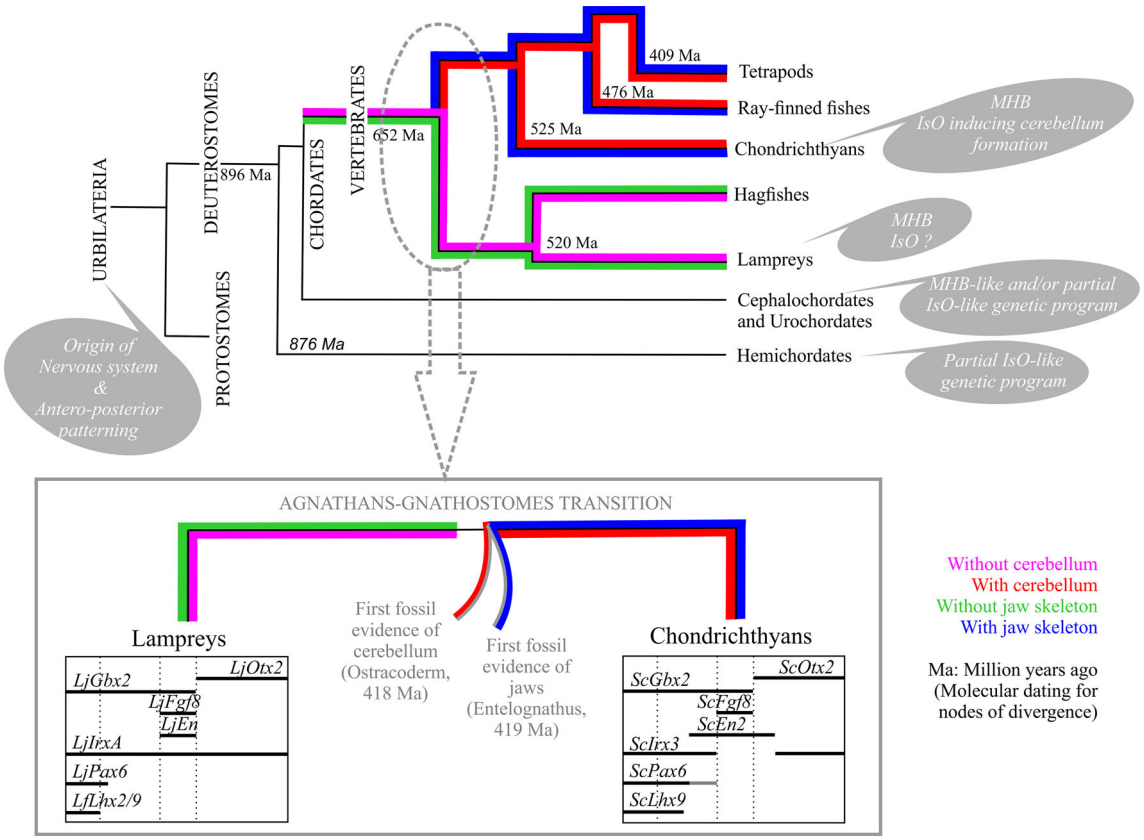

Fig. 5 Comparative aspects in relation to the midbrain–hindbrain boundary and isthmic organizer throughout evolution. Cladogram summarizing the main aspects of the potential evolutionary origin of isthmus-related genes and their genetic program in different groups of extant animals. Information about urbilateria and prochordates is based on data from: Hirth et al. (2003), Urbach (2007), Irimia et al. (2010), Steinmetz et al. (2011), Pani et al. (2012), Holland et al. (2013). Topographic similarities and dissimilarities in the expression pattern of different isthmus-related genes between agnathans (in lampreys) and gnathostomes (in the lesser spotted dogfish, see text for details) are also illustrated. Presence/absence of the cerebellum and jaws in the different animal groups were taken into account. Scheme of gene expression patterns in lampreys was elaborated based on Kuratani et al. (2002), Suda et al. (2009), Takio et al. (2007), Sugahara et al. (2011), Matsuura et al. (2008), Jiménez-Guri and Pujades (2011), Osório et al. (2005), Murakami et al. (2005). Data about millions of years in the divergence nodes of different taxa and time of evolutionary emergence of diverse groups (based on the molecular dating) were taken from: Blair and Hedges (2005), Lu et al. (2012). On the other hand, data about fossil dating in millions of years (in the *box* below the cladogram) were taken from: Janvier (2008), Friedman and Brazeau (2013), Zhu et al. (2013)

In agnathans (see Fig. 5), the expression pattern of an array of genes related to the MHB appears consistent with that of other vertebrates (Kuratani et al. 2002) including the lesser spotted dogfish (present results). This is the case for the expression domains of *Otx* (Tomsa and Langeland 1999; Murakami et al. 2001; Murakami and Watanabe 2009; Suda et al. 2009), *Gbx* (Takio et al. 2007), *Fgf8* (Rétaux and Kano 2010; Sugahara et al. 2011) and *En* (Matsuura et al. 2008; Hammond et al. 2009) genes in lampreys. Nevertheless, the *IrxA* gene in lampreys, ortholog to the *Irx1/3* of gnathostomes, does not show a negative gap of expression at MHB and r0 (Fig. 2s–x in Jiménez-Guri and Pujades 2011), differently from that observed in the lesser spotted dogfish (present results). In fact, mice deficient for the expression of *Irx2* (which is involved in the formation of the cerebellum, Matsumoto et al. 2004), did not present the negative gap of *Irx3* expression at MHB (Lebel et al. 2003). Besides, *ziro3* in zebrafish appears expressed in the MHB, but only after the formation of the cerebellum (Tan et al. 1999). Additionally, a close observation of the ortholog to *Lhx9* gene in lampreys reveals that, differently from *S. canicula* and other gnathostomes (Wang et al. 2005; Sun et al. 2008; present results), it does not appear expressed in the r1 (see Figs. 2, 3, 6 in Osório et al. 2005). Of note, in mouse *Lhx9* is a marker of deep cerebellar nuclei. However, in chick and *Xenopus* this gene is not expressed in the cerebellum, though some of *Lhx9*-expressing structures may be rhombic lip derivatives (Moreno et al. 2005; Liu et al. 2010; Green and Wingate 2014; Green et al. 2014). Therefore, the possibility that this gene was not so relevant for the evolutionary emergence of the cerebellum cannot be ruled out.

The findings obtained in the present work would support several potential causes to explain the emergence of the cerebellum, probably in the common ancestor of all gnathostomes. Firstly, the fact that in the lesser spotted dogfish, as in other gnathostomes, Pax6 is expressed in the rhombic lip and cerebellum (Rodríguez-Moldes et al. 2008, 2011; present results) together with the absence of Pax6 in the dorsal part of r1 in lampreys, supports previously proposed hypothesis that Pax6 was co-opted in this area (Kuratani et al. 2002; Murakami et al. 2001, 2005; Murakami and Watanabe 2009). A relationship between co-option of genes and innovation of structures was also described by Holland (2013). Furthermore, the possibility cannot be ruled out that the induction of the cerebellum may be directly or indirectly related to the appearance of new *Iroquois* or *Lhx* isoforms due to genetic duplications in gnathostomes (Kerner et al. 2009) and/or to the down-regulation of *Irx3* at the MHB and r0. Genetic duplications and/or changes in developmental programs that result in the duplication of a structure are considered necessary for evolutionary innovations (Wagner 2008; Montgomery et al. 2012). However, some authors have proposed that the novelty in evolution is more influenced by changes in regulatory mechanisms than by genetic duplications (Carroll 2008). Alternatively, the novelty in the evolution of the cerebellum may depend on the threshold of expression of particular genes. Thus, some genes must reach a high threshold of expression and must be expressed long time enough to activate the pathways involved in the formation of the cerebellum, as in the case of *Fgf8* (Sato and Nakamura 2004; Sato and Joyner 2009) or *Gbx2* (Waters and Lewandoski 2006) genes. Therefore, though the expression pattern of some isthmus-related genes appears very similar in agnathans and gnathostomes, it could be possible that the level of expression of these genes was not high enough before the evolutionary innovation of the cerebellum. Certainly, the cause of the evolutionary emergence of the cerebellum could be a sum of all the factors cited above.

Conclusions

Similarities observed between the lesser spotted dogfish and other gnathostomes show the high degree of conservation of the expression pattern of isthmus-related genes. Furthermore, due to so divergent lineages, features in common between them might correspond to those already present in the common ancestor of all gnathostomes, which supports the hypothesis that the chondrichthyans bauplan might reveal the ancestral condition of the cerebellar formation. Additionally, this comparative analysis allowed the accurate recognition of the boundaries between midbrain–hindbrain, r0–r1 and r1a–r1b. On the other hand, the dissimilarities found with respect to other gnathostome species reveal some features that could be secondarily derived.

While non-vertebrates present particular combinations of various isthmus-related genes, only vertebrates present a whole set of isthmus-related genes with conserved expression patterns. Finally, though more neural genoarchitectonic studies in basal gnathostomes and agnathans would be necessary, small dissimilarities we observed between them might give a clue to clarify why the isthmic organizer acquired the ability to induce the formation of the cerebellum in the common ancestor of all jawed vertebrates.

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Conflict of interest The authors declare that they have no conflict of interest

Ethical standard The manuscript does not contain clinical studies or patient data.

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