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Original Article

Neural development in the marsupial frog *Gastrotheca riobambae*

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ABSTRACT The expression pattern of the tyrosine kinase gene *Pag* in whole-mount preparations of *Gastrotheca riobambae* embryos and the immunostaining of embryos against the proteins vimentin, NCAM, Pax-2, Hoxd9, and antigen 2G9 allowed detection of migrating streams of cranial neural crest (NC) cells, the isthmus, the hindbrain boundaries, rhombomeres, cranial nerves, and the developing spinal cord. Expression patterns of these genes and the basic neural morphology of *Gastrotheca* have been conserved in comparison with other vertebrates. However, as in *Xenopus*, a prominent stream of migrating cranial NC cells from rhombomere 5 was found in *Gastrotheca* embryos. By contrast, in chick embryos, premigratory NC cells from rhombomeres 3 and 5 undergo extensive apoptosis, which suggests that in anurans, apoptosis of the cranial NC may deviate from the chick pattern. The branchial-anterior and branchial-posterior masses of cranial NC cells, that populate the gill arches, are very large in *G. riobambae*. We cannot distinguish whether this feature corresponds to an anuran trait related to development of the tadpole pharyngeal skeleton, or is related to development of the peculiar external bell gills of this frog. This work provides the descriptive groundwork for lineage studies of the NC in *G. riobambae* embryos. *Gastrotheca* embryos are large and flat, have prominent streams of cranial NC cells, and develop very large external bell gills. These unique characteristics may facilitate future comparative analysis of the role of apoptosis in patterning the amphibian NC cell streams, and the contribution of the NC to development of the gills.

KEY WORDS: *branchial arches, cranial nerves, cranial neural crest, rhombomeres*

Introduction

The cranial NC (neural crest), a prominent and conserved feature of vertebrate embryos, is involved in development of the head (Hörstadius, 1950; Gans and Northcutt, 1983; Noden, 1991; Le Douarin, *et al.*, 1993). In the chick, the cranial NC moves in a highly patterned fashion and has been investigated extensively in comparison with other vertebrates (reviewed in Le Douarin, 1982; Bronner-Fraser, 1994; Le Douarin *et al.*, 1994). Streams of cranial NC cells at the mid- and hindbrain levels and the cranial paraxial mesoderm contribute to the branchial arches (reviewed in Lumsden and Graham, 1996). Based solely on morphological criteria, it is very difficult to distinguish the cranial NC from the paraxial mesoderm. However, current methodologies allow identification of gene expression domains and of their protein expression patterns in whole embryos, highlighting morphological features such as the dorsal blastopore lip of the gastrula or the NC (reviewed in Dent *et al.*, 1989; Le Douarin *et al.*, 1994; Steinbeisser, 1996; Schilling and Kimmel, 1997). In addition, the comparison of genes, their expression domains, and molecular pathways demonstrate high levels of molecular conservation in organisms as diverse as *Drosophila* and *Xenopus* (reviewed in Gilbert *et al.*, 1996).

Several genes are differentially expressed in the hindbrain (reviewed in Lumsden and Krumlauf, 1996). In particular, expression of the highly conserved *Hox* genes in the developing CNS (central nervous system) and associated NC contribute to the mechanisms that underlie antero-posterior neural patterning in different vertebrates (Hunt *et al.*, 1991a,b; Godsave *et al.*, 1994; Krumlauf, 1994; Burke *et al.*, 1995; Kolm and Sive, 1995; Lumsden and Krumlauf, 1996; Vieille-Grosjean *et al.*, 1997). Analysis of cranial NC morphology and gene expression patterns in anurans is of interest given the diversity of anuran reproductive strategies (reviewed in Lamotte and Lescure, 1977; Duellman and Trueb, 1986). In addition, the cranial NC of amphibian embryos is prominent (reviewed in Epperlein and Löfberg, 1993; Mayor *et al.*, 1995). However, the comparative potential is restricted by the limited information available on developmental mechanisms for amphib-

Abbreviations used in this paper: CNS, central nervous system; NC, neural crest; Pag, Pagliaccio; r, rhombomere

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ians, with the exception of *Xenopus* (reviewed in Elinson, 1997).

Some aspects of development have been studied in the terrestrial frog *Eleutherodactylus coqui* and in the egg-brooding marsupial frog *Gastrotheca riobambae* (reviewed in Elinson, 1997). *Gastrotheca* and *Eleutherodactylus* not only belong to different families (Hylidae and Leptodactylidae, respectively), but also have major differences in their developmental and reproductive adaptations (reviewed in Elinson *et al.*, 1990; Elinson, 1997). Embryos of *E. coqui* develop very fast, whereas *G. riobambae* has a slow developmental rate (del Pino, 1989; del Pino and Loor Vela, 1990). In spite of the large egg (3 mm in diameter) of both frogs, gastrulation in *E. coqui* resembles the pattern of other anurans (del Pino and Elinson, 1983), whereas in *G. riobambae* gastrulation results in the formation of an embryonic disk, and the subsequent planar development of embryos (del Pino and Elinson, 1983; Elinson and del Pino, 1985; del Pino, 1996). Moreover, eggs of *E. coqui* develop terrestrially (reviewed in Elinson *et al.*, 1990), whereas in *Gastrotheca* the mother carries the developing eggs in a specialized pouch until an advanced tadpole stage (reviewed in del Pino, 1989). (In other species of *Gastrotheca*, small frogs emerge from the maternal pouch; del Pino and Escobar, 1981). *E. coqui* does not produce tadpoles (reviewed in Elinson *et al.*, 1990). In spite of the precocious appearance of adult cranial morphology, cranial NC morphology and craniofacial patterning have been conserved in *E. coqui* (Moury and Hanken, 1995; Fang and Elinson, 1996; Hanken *et al.*, 1997).

In *G. riobambae*, the morphology of the chondrocranium, head ligaments and musculature, and the changes at metamorphosis have been analyzed in detail (Haas, 1996). The larval chondrocranium of *G. riobambae* and 4 additional species of *Gastrotheca* (including *G. orophylax*, a species that gives birth to small frogs) have similar features. In *Gastrotheca*, the tectum parietale develops in advanced larval stages (Haas, 1996). This character is known exclusively from larvae of the Hylidae. The time and sequence of metamorphosis events of *G. riobambae* are comparable to other members of the Neobatrachia, and the ossification sequence is similar to other Bufonoidea taxa (Haas, 1996). Earlier aspects of craniofacial patterning in *Gastrotheca*, including the shape of cranial NC cell streams, have not been studied previously.

Prominent features of *G. riobambae* early embryos are the planar orientation of the embryo on top of an embryonic disk and the development of very large streams of cranial NC in almost the same plane as the developing body (reviewed in del Pino, 1989). These features allow identification of the developing CNS and cranial NC in *G. riobambae* more easily than in other vertebrate embryos. In this work, the expression patterns of the neural markers given in Table 1 were combined with the characteristic planar morphology of *G. riobambae* embryos to describe regions of the developing CNS, the cranial NC, and the relationship of the cranial NC with the hindbrain. This work provides comparative data on neural patterning mechanisms. It provides also the descriptive groundwork for studies of NC cell derivatives and of the role of apoptosis in patterning the NC cell streams of *G. riobambae*.

Results

Neural morphology and gene expression patterns in the Gastrotheca neurula (stage 12)

Embryos of *G. riobambae* were hybridized with antisense transcripts of the *Xenopus* receptor tyrosine kinase gene *Pag* (Winning and Sargent, 1994) and immunostained with neural antibodies to determine the pattern of neural development. Of the 20 antibodies tested, only 5 gave specific signals in embryos of *Gastrotheca*. The neural regions detected by these markers in other vertebrates are given in Table 1.

In this study we used vimentin as a generalized marker of *Gastrotheca* neural development, since other markers become differentially expressed in the developing CNS and in the NC (Table 1). In early embryos of *Xenopus*, vimentin marks the migrating NC cells and developing CNS. At later stages, vimentin marks neural tissues and non-neural structures, such as the somites and pronephros (Dent *et al.*, 1989; Herrmann *et al.*, 1989a). At stage 12 (late neurula) of *G. riobambae*, the embryo lays flat on the embryonic disk (del Pino and Escobar, 1981). Immunostaining of stage 12 *Gastrotheca* embryos against vimentin revealed the neural folds, the prominent mandibular, hyoid, and branchial streams of cranial NC cells (Fig. 1A).

Antigen 2G9 is an extracellular neural antigen that has been detected in the embryonic and adult CNS of *Xenopus*. In addition, antigen 2G9 has been detected in mice and axolotls, suggesting that it has been conserved during vertebrate evolution (Jones and Woodland, 1989). In stage 12 embryos of *Gastrotheca*, the emerging hyoid stream, branchial-posterior cranial NC cell mass, and the vagal NC cells were positive for antigen 2G9. In contrast, the alternate cranial NC cells of the mandibular stream and branchial-anterior NC cell mass, and the closing neural tube were only weakly stained (Fig. 1B). The branchial-posterior cranial NC cell mass became visible by the antigen 2G9 expression pattern in an undivided branchial stream of cranial NC cells (as detected by the vimentin pattern in whole-mount preparations, compare Fig. 1A,B).

TABLE 1

MOLECULAR MARKERS AND THE NEURAL REGIONS RECOGNIZED BY THEIR EXPRESSION PATTERNS IN VERTEBRATE EMBRYOS

Molecular marker	Neural region	Probes	References
Antigen 2G9	Regions of the CNS, some streams of cranial NC cells, trunk NC cells, cranial nerves	A	7
Hoxd9	Developing spinal cord	B	3,5,10
NCAM	Developing CNS, cranial nerves	C	1, 11,13
Pag	Rhombomeres 3 and 5, the branchial-anterior cranial NC cell mass, regions of the forebrain	D	14
Pax2	Developing isthmus	E	4,8,9,12
Vimentin	Developing CNS, NC, cranial nerves	F	2,6

Probes: [A]Antibody 2G9, Jones and Woodland (1989). [B]Antibody HU6 (Oliver *et al.*, 1989). [C]Antibody 4d (Watanabe *et al.*, 1986). [D]Digoxigenin labeled *Xenopus* riboprobe (Winning and Sargent, 1994). [E]Anti-Pax2 (Dressler and Douglass, 1992). [F]Antibody 1413.8 (Herrmann *et al.*, 1989 a,b).

References: [1]Balak *et al.*, (1987). [2]Dent *et al.*, (1989). [3]Doniach *et al.*, (1992). [4]Dressler *et al.*, (1990). [5]Godsave *et al.*, (1994). [6]Herrmann *et al.*, (1989a). [7]Jones and Woodland (1989). [8]Krauss *et al.*, (1991). [9]Nornes *et al.*, (1990). [10]Oliver *et al.*, (1988). [11]Ruiz i Altaba and Jessell, (1991). [12]Torres *et al.*, (1996). [13]Watanabe *et al.*, (1986). [14]Winning and Sargent (1994).

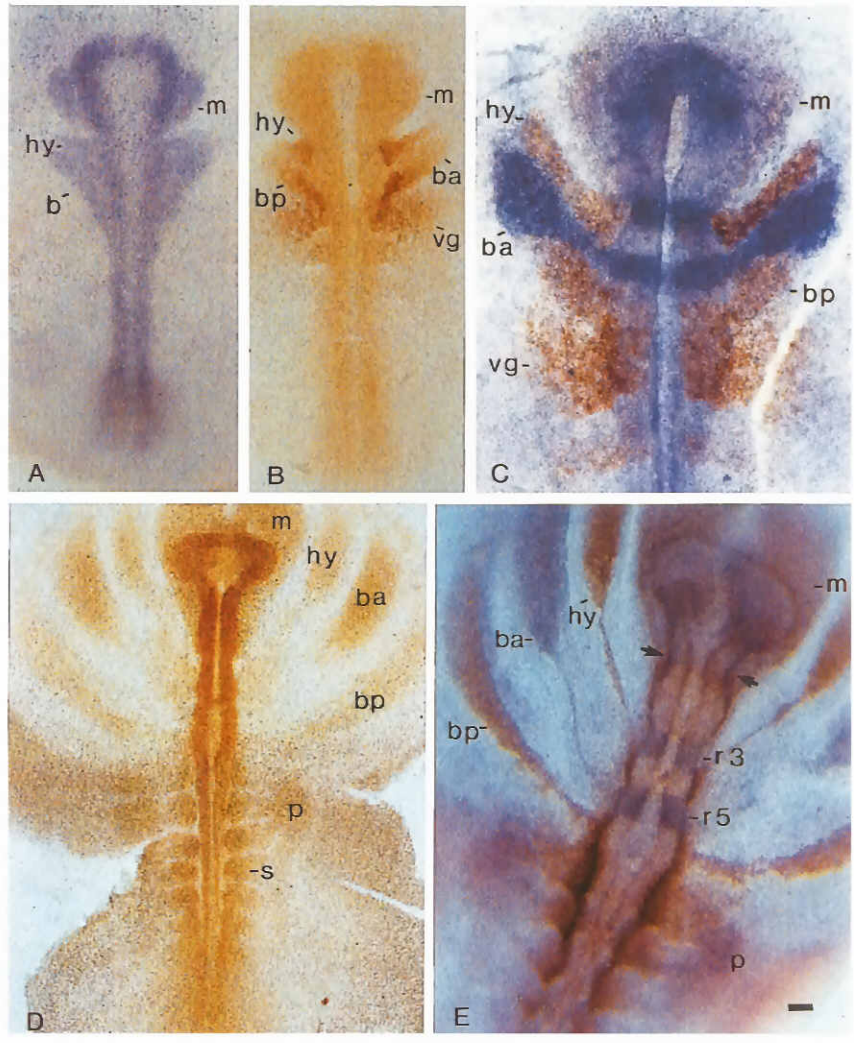

Fig. 1. Neural development in *Gastrotheca riobambae* embryos of stages 12 (A-C) and 13 (D, E). *All embryos are oriented with the anterior end toward the top. Embryos shown in* **(A)** *and* **(D)** *were immunostained against vimentin. The different color reaction in these embryos is due to the use of different secondary antibodies and color substrates. For the embryo in* **(A)**, *an alkaline phosphatase coupled secondary antibody was used, which gives a blue signal under the conditions used. In contrast, for the embryo in* **(D)**, *a horseradish peroxidase coupled secondary antibody was used, which gives a brown signal with the corresponding substrate. The embryo in* **(B)** *was immunostained against antigen 2G9. Embryos in* **(C)**, *and* **(E)** *were immunostained against antigen 2G9 (brown signal) and cross-hybridized against transcripts of the receptor tyrosine kinase gene Pag (blue signal).* **(A)** *Emergence of the NC in a stage 12 embryo immunostained against vimentin. The closing neural tube, the emerging mandibular, hyoid, and branchial streams of cranial NC cells are vimentin positive.* **(B)** *A stage 12 embryo immunostained against antigen 2G9. The emerging NC cells of the hyoid stream, branchial-posterior cranial NC cell mass, and the vagal NC cells are strongly positive for antigen 2G9. The developing CNS, and the emerging cranial NC cells of the mandibular stream and branchial-anterior cranial NC cell mass are weakly stained.* **(C)** *Differential expression of antigen 2G9 and Pag RNA in a stage 12 embryo. Alternate streams of cranial NC cells were positive for antigen 2G9 and for Pag RNA. The cranial NC cells of the mandibular stream and branchial-anterior cranial NC cell mass are Pag positive. In contrast, the hyoid stream and the branchial-posterior cranial NC cell mass, and the vagal NC are positive for antigen 2G9. The two Pag positive bands in the neural tube correspond to prospective r3 and r5. Apparently antigen 2G9 and Pag transcripts are present in the prospective mandibular arch. Pag marks regions of the forebrain, as in Xenopus embryos (Winning and Sargent, 1994). The antigen 2G9 pattern of this embryo is comparable to that seen in (1B).* **(D)** *Morphology of a stage 13 embryo immunostained against vimentin. The developing CNS, streams of cranial NC cells, somites, and the pronephros are vimentin positive.* **(E)** *Stage 13 embryo of Gastrotheca double labeled against antigen 2G9 (in brown) and Pag transcripts (in blue). The constriction between mid- and hindbrain is indicated by arrows. The isthmus became strongly stained against antigen 2G9. Regions of the forebrain, r3, and r5 are Pag positive. Cranial NC cell streams positive for antigen 2G9 or for Pag RNA alternate, as described in the younger embryo shown in* **(C).** *b, morphologically undivided branchial stream of cranial NC cells; ba, branchial-anterior cranial NC cell mass; bp, branchial-posterior cranial NC cell mass; hy, hyoid stream of cranial NC cells; m, mandibular stream of cranial NC cells; p, pronephros; s, somite; vg, vagal NC. Bar, 100 μm for A,B,D,E, and 50 μm for C.*

The *Eph*-related receptor tyrosine kinase gene, *Pag* (Winning and Sargent, 1994), is the *Xenopus* allele of *Xsek-1* (Xu *et al.*, 1995). In *Xenopus* embryos, *Pag* is expressed in prospective rhombomere (r) 3 and r5, before morphological segmentation of the hindbrain. In addition, *Pag* is expressed in the branchial-anterior cranial NC cell mass, which in *Xenopus* emerges from prospective r5 (Bradley *et al.*, 1992; Winning and Sargent, 1994). *Pag* has a complex pattern of expression in other tissues of *Xenopus* embryos (Winning and Sargent, 1994). Similarly in the *Gastrotheca* neurula, *Pag* transcripts were detected in two conspicuous bands of the closing neural tube, which were considered to be prospective r3 and r5 by analogy with the *Xenopus* expression pattern. As in *Xenopus*, these prominent expression bands were visible before morphological segmentation of the hindbrain (Fig. 1C). In addition, *Pag* transcripts were detected in the branchial-anterior cranial NC cell mass (in the still undivided branchial stream of cranial NC cells, evidenced in light microscopic whole-mount preparations, compare Fig. 1A,C). As in *Xenopus*, the branchial-anterior cranial NC cell mass is associated with the *Pag* positive prospective r5 (stage 12, Fig. 1C).

The *Pag* expression pattern is complementary to that of antigen 2G9 (compare Fig. 1B,C) and reveals differential molecular patterning in the nascent branchial-anterior and branchial-posterior cranial NC cell masses in *Gastrotheca* embryos (Fig. 1B,C). Identification of the NC by the patterns of vimentin, antigen 2G9, and *Pag* (Fig. 1A-C) reveals that in *Gastrotheca*, migration of the cranial NC commences before closure of the neural tube.

Neural morphology and gene expression patterns in Gastrotheca stage 13 embryos

In stage 13 embryos of *G. riobambae*, the streams of cranial NC cells that surround the developing head are prominent (Fig. 1D). The

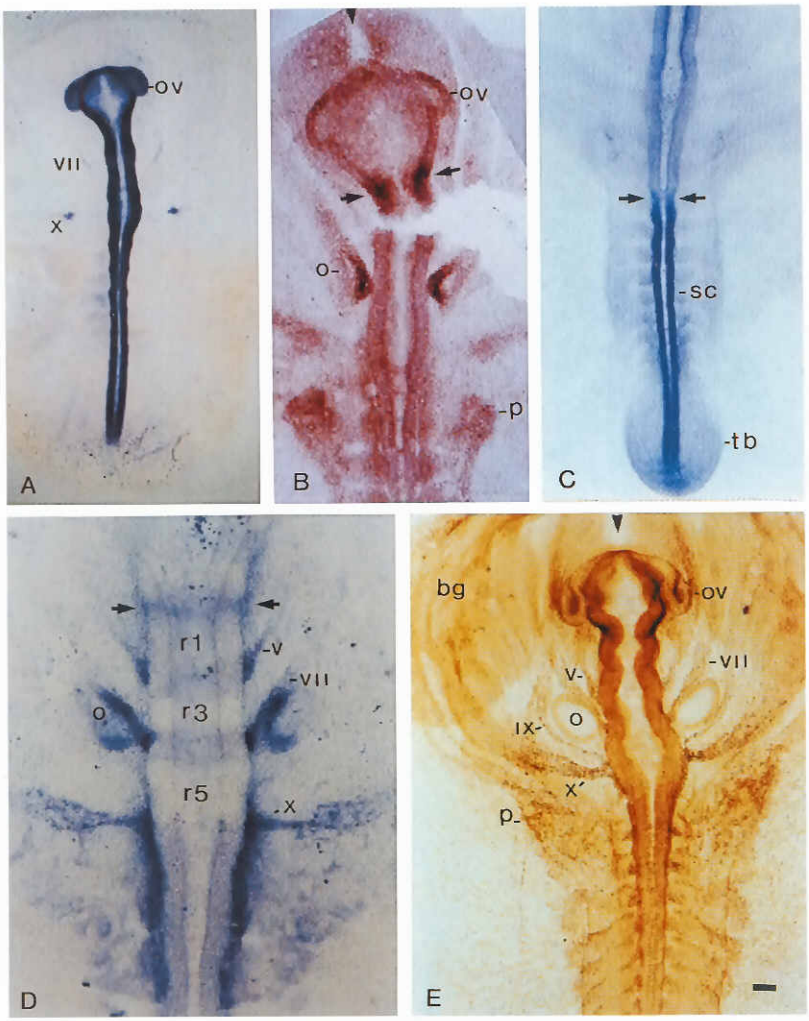

Fig. 2. Neural development in *G. riobambae* embryos of stages 14 (A-D) and 16 (E) marked against NCAM (in A), Pax2 (in B), Hoxd9 (in C), antigen 2G9 (in D), and vimentin (in E). *All embryos are oriented with the anterior end toward the top.* **(A)** *The developing CNS of a stage 14 embryo immunostained against NCAM. The developing CNS, optic vesicles, the ganglia of the facial (VII), and vagus (X) nerves are NCAM positive.* **(B)** *Stage 14 embryo immunostained against Pax2. A Pax2 positive band of cell-nuclei, at the level of the isthmus, marks the boundary between mid-and hindbrain (arrows). Otic vesicles, pronephros, and the pronephric duct are Pax2 positive.* **(C)** *Stage 14 embryo immunostained against Hoxd9. The spinal cord is Hoxd9 positive and allows detection of the boundary between the hindbrain and the spinal cord (arrows).* **(D)** *Embryo of Gastrotheca (stage 14) immunostained against antigen 2G9. The isthmus (arrows), all rhombomeres (except for r1, r3 and r5), and the trigeminal (V), facial (VII) (running parallel to the rostral limit of otocysts), and vagus (X) nerves are positive for antigen 2G9. The glossopharyngeal (IX) nerve is not visible in this embryo. Otic vesicles are located opposite r4. The blue signal in this embryo is due to the use of an alkaline phosphatase coupled secondary antibody, which gives a blue signal with the substrate used.* **(E)** *Morphology of a stage 16 embryo. The developing CNS, optic cups, otocysts, pronephros, NC of the trunk, somites, and the trigeminal (V), facial (VII), glossopharyngeal (IX), and vagus (X) nerves are vimentin positive. Otocysts are located between facial and glossopharyngeal nerves as in Xenopus (Hemmati-Brivanlou et al., 1992). Bell gills develop on each side of the head. The stomodeal depression is rostral to the head (arrowhead in B,E), because the embryo has a flat orientation on the embryonic disk until these advanced stages. In slightly more advanced embryos (stage 17), the head fold begins to separate the head (not shown). bg, bell gill; o, otocyst; ov, optic vesicle; sc spinal cord; tb, tailbud. Other abbreviations as in Figure 1. Bar, 200 μm for A, 100 μm for B,C,E, and 50 μm for D.*

body of the embryo, the developing CNS, optic vesicles, NC, somites, pronephros, and tailbud are oriented flat on the embryonic disk, as revealed by morphological criteria and by the vimentin expression pattern (del Pino and Escobar, 1981; Fig. 1D). The developing CNS was segmentally organized. However, neural regions could not be detected in embryos immunostained against vimentin (Fig. 1D).

Regional differences of the stage 13 *Gastrotheca* developing CNS and emerging cranial NC became visible by the complementary expression patterns of the receptor tyrosine kinase gene *Pag* and antigen 2G9 (Fig. 1E). *Pag* transcripts were found in r3 and r5, as in *Xenopus* (Winning and Sargent, 1994). In addition, *Pag* transcripts were found in the branchial-anterior cranial NC cell mass (Fig. 1E). This cranial NC cell mass is connected with the *Pag* positive r5 (Fig. 1E). *Pag* expression in r3 and r5 was transient and disappeared later in development (by stage 16). As in *Xenopus*, Pag was expressed faintly in the developing forebrain (Fig. 1E). In the late gastrula, *Pag* transcripts were detected in the caudal region of the notochord and in the tailbud, as in *Xenopus* embryos (not shown).

In stage 13 *Gastrotheca* embryos, r3 and r5 were negative for antigen 2G9. In contrast, the isthmus (a midbrain region located rostral to the constriction between midbrain and hindbrain), r1, r2, r4, r6 and following rhombomeres stained strongly for antigen 2G9. Rhombomeres were identified in reference to r3 and r5, which are positive for *Pag* RNA and negative for antigen 2G9 (Figs. 1E, 2D). The prominent mandibular and hyoid NC cell streams, the branchial-posterior cranial NC cell mass, the vagal, and trunk NC cells were strongly positive for antigen 2G9 (Fig. 1E). In contrast, the alternate and equally prominent branchial-anterior cranial NC cell mass became faintly stained for antigen 2G9 (not shown). The antigen 2G9 pattern suggested a relationship between the hyoid stream of cranial NC cells and r4 and between the branchial-posterior cranial NC cell mass and r6 (Fig. 1E).

Neural morphology and gene expression patterns in Gastrotheca embryos of stages 14-16 and boundaries of the hindbrain

The developing CNS of *Gastrotheca* embryos was observed by immunostaining against the neural cell adhesion molecule, NCAM. In *Xenopus* embryos, NCAM marks the neural plate, the developing CNS, cranial nerves, and ganglia. In contrast, the migrating NC is NCAM negative (Balak *et al.*, 1987). As in *Xenopus*, the developing CNS and some cranial nerves were NCAM positive in *Gastrotheca* embryos of stage 14, whereas the migrating NC cells were NCAM negative (Fig. 2A). Segmentation of the developing CNS is pronounced in *Gastrotheca* stage 14 embryos. The optic vesicles have a planar orientation (Fig. 2A) and are less conspicuous than the protruding optic vesicles of *Xenopus* stage 21 embryos (not shown). NCAM expression in *Gastrotheca* embryos was transient, as in *Xenopus* (Balak *et al.*,

1987), since it could be detected from the neural plate stage (stage 11, not shown) until stage 14.

The paired-box gene *Pax2* was used as a marker of the rostral hindbrain boundary. In mouse and zebrafish embryos, *Pax2* is expressed in the isthmus. In addition, *Pax2* is expressed in otocysts and pronephros (Dressler *et al.*, 1990; Nornes *et al.*, 1990; Krauss *et al.*, 1991; Torres *et al.*, 1996). In stage 14 *Gastrotheca* embryos, the developing isthmus, otocysts, and pronephros were Pax2 positive (Fig. 2B). A strong Pax2 positive band in the prospective isthmus is present earlier in the neurula (stage 12, not shown), before visible segmentation of the developing brain, as in other vertebrates.

Hoxd9 was used as marker of the caudal hindbrain boundary. In *Xenopus* and mouse embryos, *Hoxd9* is expressed in the developing spinal cord (Oliver *et al.*, 1988; Doniach *et al.*, 1992; Godsave *et al.*, 1994). A similar pattern of expression was found in *Gastrotheca* embryos (stages 13-15, Fig. 2C). The Hoxd9 signal was transient and disappeared in stage 16 embryos of *Gastrotheca*. The *Hoxd9* expression is also transient in embryos of the mouse and *Xenopus*.

The expression pattern of antigen 2G9 changed during development. In *Gastrotheca* embryos of stage 14, the isthmus, r2, r4, and rhombomeres caudal to r5 were positive for antigen 2G9. In contrast, rhombomeres 1,3, and 5 were negative for antigen 2G9 (Fig. 2D), whereas in younger embryos, r1 was antigen 2G9 positive (compare Figs. 1E, 2D). The antigen 2G9 signal in the streams of cranial NC cells could not be detected in embryos of stage 14. In contrast, the emerging trigeminal (V), facial (VII), glossopharyngeal (IX), and vagus (X) nerves, and the otic vesicles gave a strong antigen 2G9 signal (Fig. 2D). Cranial nerves were detected by their position in regard to otocysts and by comparison with *Xenopus* (Hemmati-Brivanlou *et al.*, 1992). In stage 16 embryos, cranial nerves became conspicuous and were detected by immunostaining against antigen 2G9 (not shown) and vimentin (Fig. 2E).

A notable feature of stage 14-16 embryos of *Gastrotheca* is their planar orientation. Structures located ventrally in embryos of *Xenopus* and other vertebrates are positioned dorsally on the *Gastrotheca* embryonic disk. For example, the prospective stomodeal depression (located between the mandibular arches; Huettner, 1949, Fig. 2B,E) and the small optic cups have a planar orientation on the embryonic disk of stage 16 embryos of *Gastrotheca* (Fig. 2E). Similarly, the otocysts and cranial nerves have a flat orientation (Fig. 2A-E). The heart develops rostral to the mandibular arch in the same plane of the body. Blood circulation begins when the heart occupies this position (stage 15, not shown). Only later do the body folds displace these structures to their typical locations.

Discussion

The planar orientation of Gastrotheca embryos

At the onset of emergence, the cranial NC has a planar orientation in vertebrate embryos, as demonstrated by the gene expression patterns in the NC of the 9 somite chick embryo (Sechrist *et al.*, 1995), the stage 18 *Xenopus* embryo (Mayor *et al.*, 1995), and the early embryo of *E. coqui* (Hanken *et al.*, 1997). Similarly, the emerging streams of cranial NC cells have a flat orientation in embryos of *G. riobambae* (stage 12, Fig. 1A-C). However, the planar condition of *Gastrotheca* embryos remains until onset of organogenesis (stages 14-16, Figs. 1D-E, 2A-E, 3), whereas in other vertebrates, the streams of cranial NC cells become shifted to the sides of the embryo as development proceeds (Fig. 4A-B). *Xenopus* eggs are smaller than those of *G. riobambae* and the elongation of the *Xenopus* neurula may contribute to shift the cranial NC cell streams to the sides of the head (as illustrated in

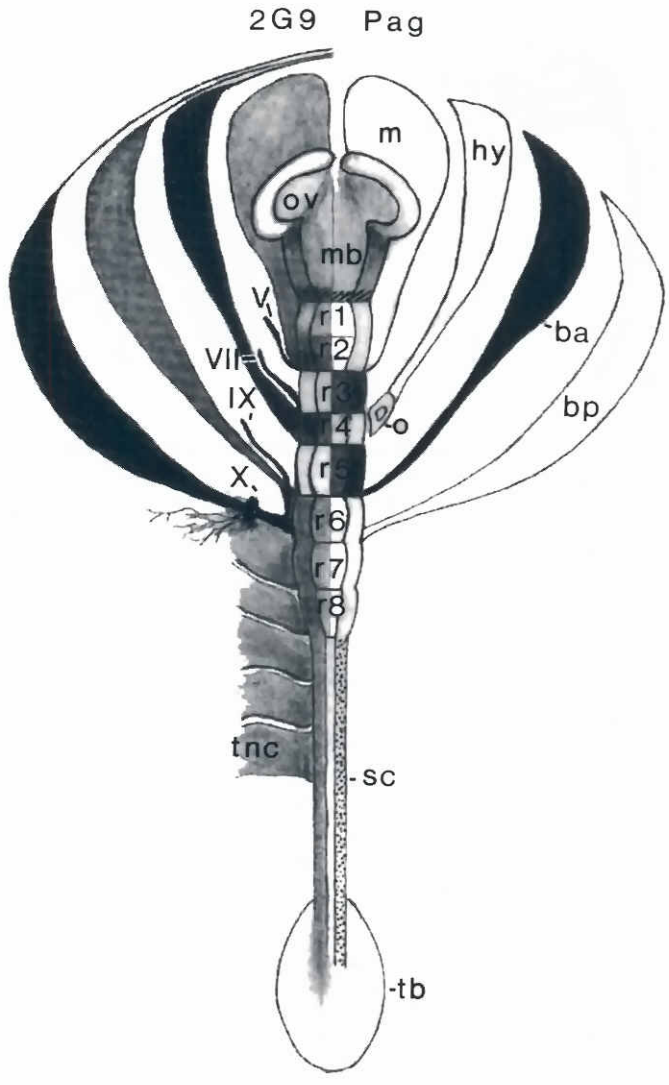

Fig. 3. Composite diagram of neural marker expression in *G. riobambae* embryos, which combines features of stages 13 and 14 embryos. *The pattern of antigen 2G9 is shown on the left and the pattern of the receptor tyrosine kinase Pag on the right. The intensity of marker expression is shown with different grades of shading. The weak expression of Pag in the cranial NC cell stream of the mandibular arch is not shown. The patterns of Pax2 (hatched) and Hoxd9 (stippled), which correspond to stage 14 embryos, are shown on the right. The streams of cranial NC cells are shown as in embryos of stage 13, including thin rostral extensions (shown on the left). Cranial NC cell streams of stage 13 embryos positive for Pag RNA alternate with cranial NC cell streams positive for antigen 2G9. Similarly r3 and r5 are positive for Pag RNA and negative for antigen 2G9. In stage 14 embryos cranial nerves (shown on the left), and otic vesicles (shown on the right) become visible. The number of trunk NC segments is shown for stage 13 embryos and increases in embryos of stage 14 (not shown). Marker expression in otic vesicles and pronephros are not shown. mb, midbrain; r1 to r8, rhombomeres 1 to 8; tnc, neural crest of the trunk; V, trigeminal nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagus nerve. Other abbreviations as in Figures 1-2.*

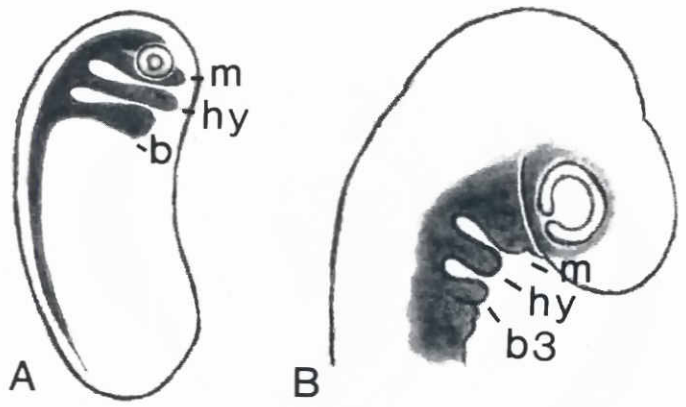

Fig. 4. Location of cranial NC cell streams in embryos of *Xenopus* (A), and the chick (B). *The anterior end is towards the top.* **(A)** *The cranial NC cell streams of a stage 25 embryo of Xenopus are represented according to the expression pattern of Xslug given in Mayor et al., (1995). The branchial stream of cranial NC cells is still undivided.* **(B)** *Schematic drawing of the location of branchial arches in chick embryos. b3, third branchial arch. Other abbreviations as in Figures 1-3.*

Sadaghiani and Thièbaud, 1987; Brändli and Kirschner, 1995; Mayor *et al.*, 1995; Fig. 4A).

The planar orientation of *Gastrotheca* embryos may relate to the developmental restrictions imposed by the large egg size. *G. riobambae* eggs measure 3 mm in diameter, and other *Gastrotheca* species have even larger eggs (ranging from 2.5 to 10 mm in diameter, del Pino and Escobar, 1981). However, planar development does not depend solely on egg size since the eggs of *E. coqui*, which measure also 3 mm in diameter, lack planar development (Townsend and Stewart, 1985; Moury and Hanken, 1995). Moreover, the chick also lacks planar development (Fig. 4B), although the embryo develops from a disk of cells on top of a very large egg (reviewed in Huettner, 1949). The planar condition of *Gastrotheca* embryos may relate to the delayed formation of the head fold, which separates the rostral region of the embryo from the yolk. The flat orientation of the *Gastrotheca* embryo on the surface of the egg may be advantageous for exchanges with the surrounding tissues of the maternal pouch. Moreover, to facilitate exchanges with the pouch, embryos of *Gastrotheca* precociously develop the heart and external, so-called bell gills, which serve as an embryonic organ for metabolic exchange with the mother during the four months of incubation in the maternal pouch (reviewed in del Pino, 1989). The planar morphology of the developing CNS, streams of cranial NC cells, and cranial nerves of *Gastrotheca* embryos, as revealed by cross-specific detection of neural markers (Fig. 3), may facilitate comparison of *Gastrotheca* neural development and NC cell migration with other vertebrates.

Comparison of neural development patterns

Comparison of gene expression patterns in whole embryos provides a molecular complement to morphological descriptions and are valuable tools for the analysis of development. However, under the conditions of low stringency needed for cross-specific in situ hybridization, only certain highly conserved genes give reliable patterns in whole-mount preparations. For example, expression of the receptor tyrosine kinase gene *Pag* in *Gastrotheca* embryos coincides with the *Xenopus* pattern described in Winning and Sargent (1994). In contrast, hybridization of *Gastrotheca* embryos with a *Xenopus* riboprobe against *Xlim-1* unspecifically marked all cell nuclei (unpublished). Similarly, comparison of neural protein expression patterns in whole embryos is restricted by the limited number of antibodies available and by the fact that numerous antibodies do not cross-react with other species (Roth and Gall, 1987, and this work). In embryos of *Gastrotheca*, the expression patterns of the neural markers (Table 1; Fig. 3) coincide with the patterns described for other vertebrates, suggesting that these genes and their expression patterns have been conserved in *Gastrotheca*.

Gastrotheca embryos have specific patterns of neural gene expression before overt segmentation of the hindbrain and cranial NC, as in embryos of other vertebrates (Hunt *et al.*, 1991b; Lumsden and Guthrie, 1991; Bradley *et al.*, 1992; Selleck *et al.*, 1993; Winning and Sargent, 1994; Brändli and Kirschner, 1995). The complementary expression patterns of the receptor tyrosine kinase gene *Pag* with antigen 2G9 reveal associations of the mandibular stream of cranial NC cells with r1 and r2, the hyoid cranial NC cell stream with r4, and the branchial-anterior cranial NC cell mass with r5 (Fig. 3). This pattern is equivalent to that seen in *Xenopus* embryos (Bradley *et al.*, 1992; Godsave *et al.*, 1994). Otic vesicles develop opposite r4 (Fig. 3), as in *Xenopus* (Hartenstein, 1993). *Gastrotheca* embryos may also develop 8 rhombomeres (Fig. 3), as in *Xenopus* (Hartenstein, 1993), however, the borders between rhombomeres 6-8 could not be detected by the expression of the neural markers used in this work.

The relationship of rhombomeres with the emerging streams of cranial NC cells of *Xenopus* and *Gastrotheca* differs from the chick. In *Xenopus* embryos, gene expression patterns in the hindbrain and associated cranial NC reveal prominent cranial NC cell streams from r3 (Brändli and Kirschner, 1995) and r5 (Bradley *et al.*, 1992; Winning and Sargent, 1994; Xu *et al.*, 1995). As in *Xenopus*, a prominent stream of cranial NC cells from r5 was detected in *Gastrotheca* embryos (Fig. 3). In contrast, the premigratory NC from r3 and r5 in the chick undergoes extensive apoptosis (Couly *et al.*, 1996; Köntges and Lumsden, 1996; Lumsden and Graham, 1996). These differences suggest that apoptosis of the anuran cranial NC may deviate from the pattern observed in the chick.

Patterning of the branchial cranial NC

The NC origin of the pharyngeal skeleton is a conserved feature of vertebrates including the zebrafish, amphibians, and the chick (reviewed in Hörstadius, 1950; Sadaghiani and Thièbaud, 1987; Le Douarin *et al.*, 1993; Köntges and Lumsden, 1996; Olsson and Hanken, 1996; Schilling and Kimmel, 1997). In anurans, the morphology of the cranial NC cell streams is basically similar to the pattern observed in *Bombina* (Olsson and Hanken, 1996). Likewise, the number of cranial NC cell streams in *G. riobambae* embryos resembles the basic anuran pattern exemplified by *Bombina* (Olsson and Hanken, 1996). However, the pharyngeal skeleton is morphologically diverse in anurans, in spite of its conserved NC-origin. Similarly, the external gills of anuran embryos are morphologically diverse. These features relate to the diverse ecological niche and life-cycle adaptations of anuran tadpoles (reviewed in Duellman and Trueb, 1986; Haas, 1996).

The external gills of many anurans function only transiently after hatching, are absent in some species, or are highly conspicuous in

others. In addition, anuran embryos develop internal gills, which function in the tadpole (reviewed in Balinsky, 1970; Duellman and Trueb, 1986). A correlation may exist between the morphology of the cranial NC cell streams and the patterns of gill development. The presence of NC cells, derived from the branchial stream of cranial NC, in the external gills of *Bombina* (Olsson and Hanken, 1996) and in the mesenchyme of the gills in *Xenopus* embryos (Sadaghiani and Thiébaud, 1987) lend support to this hypothesis. Moreover in older literature, the connective tissue of external gills is reported to be a derivative of the NC (Stone, 1922,1926,1929,1932, according to Hörstadius, 1950). In addition, the morphology of the cranial NC cell streams varies notably in *E. coqui* and *G. riobambae*, frogs that have extremely different patterns of gill development.

Frogs of the genus *Eleutherodactylus* lack tadpoles and in *Eleutherodactylus nubicola* neither external nor internal gills develop at all (Lynn, 1942). However, *E. coqui* embryos develop rudimentary external gills (Townsend and Stewart, 1985). In contrast, *G. riobambae* develops prominent external gills, the bell gills, and internal gills (del Pino and Escobar, 1981). Accordingly, the branchial-anterior and branchial-posterior cranial NC cell masses are very small in *E. coqui* (as illustrated in Moury and Hanken, 1995; Fang and Elinson, 1996; Hanken *et al.*, 1997), in comparison with *G. riobambae* (Fig. 3). In *Gastrotheca* embryos, the branchial-anterior and branchial-posterior cranial NC cell masses, that populate gill arches, are particularly large (Fig. 3). This feature may correspond to a characteristic anuran trait related to the development of the very large branchial cartilaginous tadpole skeleton and possibly also to the development of the specialized bell gills. Lineage tracing of the cranial NC in different amphibians may indicate whether there is indeed a relationship between size of cranial NC cell streams and the patterns of gill development.

Concluding remarks

Outstanding developmental features of *Gastrotheca* embryos are the planar condition of embryos, the development of prominent streams of cranial NC cells, and the large external bell gills. These features are advantageous for analysis of the potential relationship between the prominent cell masses of branchial NC and the development of the external bell gills. Moreover, the *Gastrotheca* planar early neural development provides a comparative advantage for the study of apoptosis in the NC. In the chick, apoptosis of most of the premigratory NC cells from r3 and r5 patterns the remaining cranial NC into non-mixing streams (reviewed in Lumsden and Graham, 1996). This pattern may differ in anurans, as in *Xenopus* conspicuous streams of NC cells emerge from r3 and r5 (Bradley *et al.*, 1992; Winning and Sargent, 1994; Brändli and Kirschner, 1995). Moreover, different levels of apoptosis may occur in the r3 premigratory NC cells of anuran embryos, as suggested by the presence, in *Bombina*, of a NC-free zone in the CNS. This NC-free region separates the mandibular from the hyoid cranial NC cell streams, and varies in size among anurans (Olsson and Hanken, 1996). In *Gastrotheca* embryos, a stream of cranial NC emerges from r5 (Fig. 3), and we do not know whether a crest-free zone occurs in the region of prospective r3 (Fig. 1A-C). The cranial NC cell patterning of anuran embryos, not only may differ from the chick, but also may vary among anurans. The *Gastrotheca* distinctively planar orientation of embryos is advantageous for analysis of the patterning mechanisms that allow separation of the cranial NC cell streams.

Materials and Methods

Embryos and fixation procedures

Embryos of the marsupial frog *Gastrotheca riobambae* were obtained from spontaneous matings and fixed in MEMFA for 2 h at room temperature (Harland, 1991), or in Dent fixative (Dent *et al.*, 1989). Lipids were removed according to Coutinho *et al.* (1992). Afterwards embryos were stored in methanol at -20°C. Embryos of *Xenopus* were processed similarly. Procedures for the handling of embryos, jelly removal, fixation, and lipid extraction are given in del Pino (1996). Staging of *G. riobambae* embryos was determined according to del Pino and Escobar (1981). *Xenopus* embryos were staged according to Nieuwkoop and Faber (1967). Branchial arches are named according to Noden (1991). The streams of cranial NC in anurans are the mandibular (or rostral) stream, which populates the first branchial (or mandibular) arch; the hyoid (or rostral otic) stream, which populates the second (or hyoid) arch; and the branchial (or caudal otic) stream, which consists of parallel masses of cells (including the branchial-anterior and branchial-posterior cranial NC cell masses) that populate branchial arches 3-6 (Olsson and Hanken, 1996).

Cross-species whole-mount in situ hybridization

The protocol of Harland (1991) was modified to allow in situ hybridization of *Gastrotheca* embryos with a digoxigenin-labeled riboprobe for the *Xenopus* receptor tyrosine kinase gene, *Pag* (Winning and Sargent, 1994). Embryos fixed in MEMFA and xylene-extracted were treated for 30 min in 5:1 methanol:hydrogen peroxide, washed twice in methanol for 5 min, and rehydrated. Treatment with proteinase K (5 (μg/ml) was reduced to 10 min. After treatment with triethanolamine and refixation, embryos were washed 5 times (5 min each) in phosphate-buffered saline with 0.1% Tween and 50 mM EDTA. Embryos were incubated in the hybridization buffer for one h at 65°C to inactivate endogenous phosphatases. The hybridization buffer contained 10 mM EDTA.

The conditions for cross-species hybridization included lower hybridization temperatures and washing stringency. Hybridization temperatures of 40-53°C were tested. Specific signals for *Pag* were obtained at 53°C. After hybridization, embryos were washed 3 times (10 min each) in 50% formamide, 5xSSC, 0.1% CHAPS, and 0.1% Tween, and washed twice for 20 min at 37 °C in 2xSSC with 0.3% CHAPS. The wash in 0.2xSSC and the RNase treatment of the Harland (1991) protocol were omitted.

Incubation with the anti-digoxigenin antibody (Boehringer Mannheim), color reaction, and fixation steps were done as described in Harland (1991), except that the antibody was preabsorbed for 2 h at 4°C with *Gastrotheca*-inactivated tadpole powder (del Pino, 1996). The blue hybridization signal became visible after 8-20 h of incubation in the color reagent (Protoblot NBT and BCIP color development system, Promega). Embryos of *Xenopus* were processed simultaneously as a control. The embryonic disk of *Gastrotheca* embryos was dissected from the segmented yolk, cleared in benzyl benzoate:benzyl alcohol (2:1), and photographed (del Pino, 1996). Embryos were then stored in glycerol at -20°C.

Whole-mount immunocytochemistry

Embryos of *Gastrotheca* fixed in MEMFA and extracted with xylene were immunostained according to Hemmati-Brivanlou and Harland (1989) and del Pino (1996). The monoclonal antibody 2G9 against a neural antigen from *Xenopus* (Jones and Woodland, 1989) was used without dilution. The monoclonal antibody (4d) against NCAM from chicken brain membranes was diluted 1:10 (Watanabe *et al.*, 1986). The monoclonal antibody 1413.8 against vimentin from *Xenopus* was diluted 1:4 (Herrmann *et al.*, 1989a,b). The polyclonal antibody HU6 against human HOXD9 was diluted 1:100 (Oliver *et al.*, 1989). The polyclonal antibody against Pax2 was diluted 1:500 (Dressler and Douglass, 1992). For immunostaining with 2G9 and anti-NCAM, embryos were fixed in Dent fixative. In addition, the following 15 antibodies against neural antigens were tested in embryos of *Gastrotheca*: anti-vimentin (14h7), anti-neurofilament 200, anti-NCAM (5e), anti-axonin1 (23,4-5), anti-neurofilament (2H3), anti-tyrosine hydroxylase (αTH), anti-

bursa of Fabricius (BEN), anti-retinoic acid receptor γ2 (DEF), anti axonal filament (LINC), anti-middle neurofilament (XC10C6), anti-engrailed (4D9), anti-snail1, anti-acetylcholine receptor (mAb35), anti-β-tubulin (Tub 2.1), and the neural antibody HNK-1.

Secondary antibodies: sheep anti-rabbit IgG conjugated to alkaline phosphatase (Boehringer Mannheim), donkey anti-rabbit, and sheep anti-mouse conjugated to horseradish peroxidase (Amersham) were diluted 1:500. Goat anti-mouse IgG conjugated to alkaline phosphatase (Boehringer Mannheim) was diluted 1:2500. For peroxidase-conjugated antibodies, the chromogenic reaction was done with DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma), which gives a brown color. In contrast, for alkaline phosphatase-conjugated antibodies the Protoblot NBT and BCIP color development system (Promega) was used according to instructions of the manufacturers. This reaction gives a blue color. For double labelling of embryos, the *in situ* hybridization was applied first, followed by fixation in MEMFA for 30 min, extensive washes in phosphate-buffered saline, and immunostaining.

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