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Cloning and expression analysis of cadherin7 in the central nervous system of the embryonic zebrafish

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Abstract

Cadherin cell adhesion molecules exhibit unique expression patterns during development of the vertebrate central nervous system. In this study we obtained a full-length cDNA of a novel zebrafish cadherin using reverse transcriptase-polymerase chain reaction (RT-PCR) and 5′ and 3′ rapid amplification of cDNA ends (RACE). The deduced amino acid sequence of this molecule is most similar to the published amino acid sequences of chicken and mammalian cadherin7 (Cdh7), a member of the type II cadherin subfamily. *cadherin7* message (*cdh7*) expression in embryonic zebrafish was studied using in situ hybridization and RT-PCR methods. *cdh7* expression begins at about 12 hours post fertilization (hpf) in a small patch in the anterior neural keel, and along the midline of the posterior neural keel. By 24 hpf, *cdh7* expression in the brain shows a distinct segmental pattern that reflects the neuromeric organization of the brain, while its expression domain in the spinal cord is continuous, but confined to the middle region of the spinal cord. As development proceeds, *cdh7* expression is detected in more regions of the brain, including the major visual structures in the fore- and midbrains, while its expression domain in the hindbrain becomes more restricted, and its expression in the spinal cord becomes undetectable. *cdh7* expression becomes reduced in 3-day old embryos. Our results show that *cdh7* expression in the zebrafish developing central nervous system is both spatially and temporally regulated.

Keywords

zebrafish; development; cell adhesion molecules; brain; spinal cord; visual system

1. Results and Discussion

Zebrafish has become as an important model system to study vertebrate neural development, due to its numerous experimental advantages including external embryonic development, transparency of the embryos, and its demonstrated utility as a genetic model. Similar to other vertebrate animals, development of zebrafish central nervous system (CNS) involves partitioning of the anterior portion of the neural tube into three vesicles called the forebrain, midbrain and hindbrain. Subsequently, the forebrain is further divided to smaller units, the telencephalon and diencephalon (reviewed by Kimmel, 1993).

The molecular mechanisms underlying the regionalization of the vertebrate CNS have been under intense investigation. Specific regulatory molecules (Puelles and Rubenstein, 1993; Macdonald et al., 1994; Mastick et al., 1997; Hauptmann and Gerster, 2000; Andermann and Weinberg, 2001) and cell adhesion molecules including members of the cadherin superfamily

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(Inoue et al., 1997, 1998, 2001; Redies, 1997, 2000; Heyers et al., 2003) have been implicated in the formation of these structures.

Cadherins are a large family of cell adhesion molecules that include the classic cadherins (type I), atypical cadherins (type II), protocadherins, desmosomal cadherins, and Flamingo cadherins (Nollet et al., 2000). Most cadherins are transmembrane proteins that mediate cell adhesion mainly through homotypic interactions (Takeichi, 1991; Gumbiner, 1996). The type I classic cadherins have a conserved His-Ala-Val (HAV) sequence in their extracellular domain one (EC1), and share a high degree of amino acid sequence similarity to E-cadherin (cadherin1), while the type II cadherins do not contain HAV in their EC1, and they are more similar in protein sequence to cadherin11 (reviewed by Nollet et al., 2000). Studies of several classic cadherin molecules (e.g. cadherin2 and cadherin4, also known as N- and R-cadherin respectively) reveal that these molecules play essential roles in the development of the vertebrate CNS (Radice et al., 1997; Lele et al., 2002; Treubert-Zimmermann et al., 2002; Malicki et al., 2003; Masai et al., 2003; Babb et al., 2005). Cdh7 is a member of the type-II cadherin subfamily (Redies, 1997; Nollet et al., 2000). So far, Cdh7/*cdh7* expression pattern has been examined in chicken central nervous system (Becker and Redies, 2003; Heyers et al., 2003; Ju et al., 2004; Luo et al., 2004), and in human total RNA from various tissue samples (Kools et al., 2000). There are no published reports on Cdh7/*cdh7* expression in fish. Zebrafish *cdh7* cDNA sequence and deduced amino acid sequence, predicted by automated computational analysis, were recently published in GenBank (accession number: **XM 691001**).

To confirm the predicted GenBank zebrafish *cdh7*, we performed RT-PCR, 5′ and 3′ RACE using total RNA from 50 hours post fertilization (hpf) zebrafish embryos and zebrafish *cdh7* specific primers (see Experimental procedures). These experiments revealed that our sequence could only be aligned with the nucleotides from 5892 to 7780 of the GenBank sequence (accession No. **DQ 411036**). The GenBank sequences before and after this region showed no similarity to any cadherin molecules, suggesting that this sequence was incorrectly assembled with unrelated gene sequences.

The resulting open reading frame produces a protein of 787 amino acid residues containing a putative hydrophobic signal sequence, presequence, extracellular domains, transmembrane and cytoplamic domains (Fig. 1). The imputed start codon was identified because (1) it was the first methionine codon following a 484-nucleotide 5′-untranslated sequence; (2) a canonical signal sequence follows this methionine codon; (3) the presequence (including the signal sequence) found in our clone is similar in length (43 amino acid residues) to presequences from other Cdh7 and type II cadherin sequences (from mouse, chicken and human), while presequences in type I cadherins (e.g. Cdh1, Cdh2 and Cdh4) are much longer (about 150 amino acids). An alignment of related cadherin sequences shows that the zebrafish *cdh7* is most similar to human, mouse, and chicken *cdh7* at the amino acid level, while showing little similarity to *cdh1*, a type-I classic cadherin (Fig. 2A and B). The complete coding sequence for the zebrafish *cdh7* gene was deposited in GenBank (Accession No. **DQ_411036**)

Using RT-PCR and whole mount in situ hybridization methods, we analyzed expression of *cdh7* in embryonic zebrafish from 2 hpf to 70 hpf. PT-PCR experiments revealed that *cdh7* transcripts were absent from early embryos, and became detectable in older embryos (Fig. 3). Strong *cdh7* expression was detected at 24 hpf and 50 hpf, and it became reduced in 3-day old embryos (70 hpf). Whole mount in situ hybridization experiments showed that at 12 hpf, *cdh7* expression was detected in a small patch located immediately caudal to the eye primordium in the anterior neural keel (Fig. 4A and B). At a distance approximately the two times the anteroposterior length of the eye primordium from the first expression domain, *cdh7* expression was found along the midline throughout the rest of the neural keel (Fig. 4B

and C). By 18 hpf, six *cdh7* expression domains were identified in the brain regions anterior to the caudal boundary of the cerebellum (Fig. 4D and E). The most anterior expression domain was in the telecephalon, anterodorsal to the optic recess. There were two closely located expression domains in the ventral diencephalon, while three expression domains almost equally spaced were found in the presumptive midbrain and mid-hindbrain regions (Fig. 4E). In the hindbrain, *cdh7* was detected throughout the ventral half of this region (Fig. 4D and F). Expression levels, judging by staining intensities, varied in the hindbrain. *cdh7* expression was weaker in the hindbrain region at the level of the otic placode (Fig. 4F–H). *cdh7* expression continued in the spinal cord (Fig. 4D and I). The notochord was also *cdh7* positive (Fig. 4I).

The major divisions of the zebrafish brain become readily discernable by 24 hpf (Kimmel, 1993; Ross et al., 1992). *cdh7* expression at this stage in the fore- and midbrains was similar to 18 hpf embryos (Fig. 5). To determine the relative positions of the *cdh7* expression domains, we performed two types of double labeling experiments. In the first type of double-labeling experiments, *cdh7* in situ hybridization was followed by immunohistochemistry using a monoclonal antibody zn12 that labels L2/HNK-1 tetrasaccharide expressed by several early differentiated neurons and their axonal processes (Trevarrow et al., 1990; Ross et al., 1992; Andermann and Weinberg, 2001). Results from these experiments showed that the *cdh7* expression domain in the telencephalon overlapped with the anterior commissure (Fig. 5B–D), and appeared to occupy the same place as the dorsorostral cluster described by Ross et al. (1992). The two closely located *cdh7* expression domains in the ventral diencephalon (asterisks in Fig. 5B and D) were situated mainly dorsocaudal to the tract of the postoptic commissure, while the two midbrain *cdh7* expression domains (arrowheads in Fig. 5B and D) were located dorsomedial to the ventrocaudal cluster described by Ross et al. (1992). Examination of sections from embryos processed for the whole mount in situ hybridization revealed that the *cdh7* expression domains in the diencephalon (Fig. 5E) had less defined borders than the first midbrain expression domain (Fig. 5F).

In the second type of double-labeling experiments, *cdh7* (digoxigenin-labeled) and *pax2a* (fluorescein-labeled) cRNA probes (*pax2a* labels the boundary between the mid- and hindbrains and the otic placodes at this stage in zebrafish; Krauss et al., 1991) were used to determine the position of the third *cdh7* expression domain (Fig. 4E, arrow in Fig. 5A, D and G) in the mid-hindbrain region. The anterior boundary of this *cdh7* expression domain coincided with the boundary between the mid- and hindbrains (Fig. 5G). This *cdh7* expression domain, separated from *cdh7* expression domain in the hindbrain of younger embryos (Fig. 4E), became continuous (viewing laterally) with the *cdh7* expression domain in the hindbrain (Fig. 5B, D and G). *cdh7* expression in the rest of the hindbrain, although continuous, was confined to the medial region along the midline and composed of six swellings of approximately equal size viewing dorsally (Fig. 5H). Positions of these swellings in relation to the rhombomeres were determined using the first type of double labeling experiments described above. zn12 positive neurons and their processes in the hindbrain occupy the central region of each rhombomere (Fig. 5I). The *cdh7* expressing swellings appeared to be located between clusters of zn12 positive cells and processes (Fig. 5J), suggesting that the swellings were situated at about half rhombomere width caudal to their corresponding rhombomeres. This was further confirmed by double-labeling the embryos using fluorescein-labeled *krox20* (detects rhombomeres 3 and 5; Oxtoby and Jowett, 1993). Slightly younger embryos (21 hpf, Fig. 5K) were used because *krox20* staining in the hindbrain is much reduced at 24 hpf compared to younger embryos (9-21 hpf, Oxtoby and Jowett, 1993). The width of the second *cdh7* expressing swelling was about ½ of the *krox20* expressing rhombomere 3 or rhombomere 5, suggesting that the *cdh7* expression swellings partially overlapped with the rhombomeres (Fig. 5K). Examination of cross sections from the *cdh7* expressing swellings (Fig. 5M) or constrictions (Fig. 5M insert) in the hindbrain revealed that *cdh7* expression was confined to the mid-central region with sharp boundaries, with no *cdh7* expression in the

medioventral and dorsolateral hindbrain regions. *cdh7* continued to be expressed throughout the entire length of the spinal cord, and similar to the hindbrain, its expression was confined to the mid-central region (Fig. 5L and N).

By 34 hpf, *cdh7*continued to be found in similar regions in the brain as in the younger embryos: in most part of the telencephalon, in distinct regions of the diencephalon (in the middle region, Fig. 6A and B), tegmentum (dorsal region, Fig. 6C), and hindbrain (see below). *cdh7* continued exhibiting a segmental expression pattern in the hindbrain (Fig. 6D), although this expression domain appeared to become more restricted to the dorsomedial hindbrain (Fig. 6F). *cdh7* was also found in a pair of small clusters of cells anterodorsal to the otic vesicle (Fig. 6D and E).

By 52 hpf, *cdh7* expression in the fore- and midbrains became increased (Fig. 6G). In addition to be detected in regions described above, *cdh7* was also expressed by visual structures, the pretectal region, the optic tectum (Fig. 6G and H), and the retina (Fig. 6K and L). All *cdh7* expression retinal cells were confined to the retinal ganglion cell layer and the inner most layer of the inner nuclear layer where retinal ganglion cells and amacrine cells, respectively reside. There appeared to be more labeling in the inner nuclear layer than the retinal ganglion cell layer (Fig. 6L). At this stage, *cdh7* expression in the dorsomedial hindbrain became more restricted (Fig. 6I and J), and no longer showing a segmental expression pattern (Fig. 6I). *cdh7* expression was also detected in the mediolateral hindbrain (Fig. 6G, I and J). By 3 days (70 hpf) of development, *cdh7* expression continued in similar brain and retinal regions as in younger embryos, but its expression levels were reduced (Figs. 3, 6M–O).

cdh7 expression in the embryonic zebrafish is similar to that in chicken and humans, where its expression is found in the embryonic brain, and is developmentally regulated (Kools et al., 2000; Heyers et al., 2003). As in the developing chicken, *cdh7* expression in the zebrafish retina is detected in the retinal ganglion cells and amacrine cells (Wöhrn et al., 1998). But unlike the chicken retina in which *cdh7* expression is also found in the outer half of the inner nuclear layer (Wöhrn et al., 1998), while *cdh7* expression in the zebrafish retina is confined to the retinal ganglion cell layer and the inner most portion of the inner nuclear layer (Fig. 6L). Similar also to the chicken (Wöhrn et al., 1998), *cdh7* is expressed by the major visual structures in zebrafish brain, the pretectum and the optic tectum (Fig. 6G, H and N).

In both chicken (Ju et al., 2004) and zebrafish (Fig. 5 M and N), *cdh7* expression in the hindbrain and spinal cord is confined to the mid-central region with clear boundaries. The dorsal boundary of the *cdh7* expression domain in the chicken hindbrain and spinal cord coincides with the boundary between the alar (dorsal) and basal (ventral) plates (Ju et al., 2004). It is possible that the dorsal boundary of the *cdh7* expression domain in zebrafish may also define the border between the dorsal and ventral hindbrain/spinal cord. Similar to the chicken hindbrain (Ju et al., 2004), *cdh7* expression in zebrafish hindbrain exhibits a neuromeric organization. Unlike the chicken in which *cdh7* expression is interrupted at the rhombomere boundaries, *cdh7* expression in zebrafish rhombomeres is continuous but with swellings and restrictions, and each swelling straddling the rhombomere boundaries.

Similar to the embryonic chicken, *cdh7* expression pattern in the embryonic zebrafish CNS was distinct from other type I and type II cadherins. Compared to Cdh2 (also called N-cadherin), a type I cadherin expressed throughout the CNS of the embryonic zebrafish (Bitzur et al., 1994), *cdh7* expression in the embryonic CNS is much more restricted. Cdh4 (also called R-cadherin), another type I cadherin, exhibits a neuromeric expression pattern in both the developing zebrafish brain and spinal cord (Liu et al., 1999, 2001). It also displays a different temporal expression profile from *cdh7* (e.g. Cdh4 expression begins after 1 day of development). The *cdh7* expression pattern is also different from *cdh6* and *cdh10*, two type II cadherins, in the developing zebrafish nervous system (Liu et al., 2006a, b). *cdh6* and *cdh10*

have more restricted expression in the brain than *cdh7*, but both cadherins are also expressed by the cranial and lateral line ganglia where *cdh7* expression is absent.

2. Experimental procedures

Zebrafish embryos, obtained from in house breeding, were maintained at 28.5°C as described in the Zebrafish Book (Westerfield, 2000). Embryos for whole mount in situ hybridization were raised in PTU (1-phenyl-2-thiourea, 0.003%) and fixed in phosphate buffered 4% paraformaldehyde.

RT-PCR was performed using total RNAs isolated from whole zebrafish embryos. The following zebrafish *cdh7* specific primers, designed according to the predicted zebrafish *cdh7* sequence in GenBank (accession number: **XM 691001**), were used for PCR for verification of the predicted *cdh7* sequence, analysis of temporal *cdh7* expression profile, generation of cDNA template for PCR in vitro synthesis of *cdh7* cRNA probes and/or 5′ and 3′RACE: cdh7Fd (5′-TGTTGGCAAGCTTCATTCTG-3′) cdh7Fd1 (5′-AACGATGCCACACAAATTGA-3′), cdh7Fd2 (5′-TGAGGGAAATGGAACAACAA), cdh7Fd3 (5′-CCCAATGCCTATCCTACCAG-3′), cdh7Fd4 (5′-TGGCCAAGATAAAAGCCTTG-3′), cdh7Fd5 (5′-AAATTGAGGCATCCAACAGG-3′), cdh7Fd6 (5′-GGTTGAGGTTAAGGCGACTG-3′), cdh7Fd7 (5′-CAGATGGGAGGACTCTCAGG-3′), cdh7Rv (5′-ACCGTGGGTCTATGTTCCTG-3′), cdh7Rv1 (5′-TCATCGATGGTGAAAATGGA-3′), cdh7Rv2 (5′-CAGAATGAAGCTTGCCAACA-3′), cdh7Rv3 (5′-TCAGGCGAGAACTCTGAGTT-3′), cdh7Rv4 (5′-AAGGGGGTCTGAGATCAGGTA-3′), cdh7Rv5 (5′-AAGGAGGAAAAGGGGGAGAT-3′). To confirm the 5′ end sequence (signal and presequence) of the predicted GenBank zebrafish *cdh7* sequence, we performed RT-PCR using primers cdh7Fd1, cdh7Fd2, or cdh7Fd3 to pair with cdh7Rv1 or cdh7Rv2. But the experiments resulted in no amplified product, suggesting that at least part of the predicted signal and presequence for the zebrafish *cdh7* gene is incorrect. To obtain the sequence of the missing 5′ end of the zebrafish *cdh7*, 5′RACE was performed using a 5′RACE kit (Invitrogen, Carlsbad, CA) and zebrafish *cdh7* specific primers (cdh7Rv1 and cdh7Rv2). This experiment yielded a nucleotide sequence (Genbank accession No. **DQ 411036**) that could be aligned with the GenBank sequence (accession No. **XM 691001**) only from nucleotide 5892 forward. The sequence suggested a likely start codon much closer to the EC1 than previously suggested.

To confirm the 3′ end sequence (end of EC3 to the end of the cytoplasmic domain) of the predicted GenBank zebrafish *cdh7* sequence, we performed RT-PCR using primers cdh7Fd4 or cdh7Fd5 to pair with cdh7Rv3, cdh7Rv4 or cdh7Rv5. But there was no amplified product, suggesting that at least part of the predicted 3′ half for the zebrafish *cdh7* gene is incorrect. To obtain the sequence of the missing 3′ half of the zebrafish *cdh7*, 3′RACE was performed using a 3′RACE kit (Invitrogen) and zebrafish *cdh7* specific primers (cdhFd5, cdh7Fd6 and cdhFd7). This experiment yielded a nucleotide sequence that could be aligned with the GenBank sequence only through nucleotide 7780 (182 nt from the end).

RT-PCR analysis of *cdh7* temporal expression profile was performed using cdh7Fd and cdh7Rv, and zebrafish *cdh1* specific primers (*cdh1* forward primer: 5′-TGTCAGAGTTGAGCGTGTCC-3′; *cdh1* reverse primer: 5′-TTGAAAAACCCACCCTTGTC-3′, GenBank accession number: **AF_364811**) bracketing a similar cadherin region (EC1 to EC3, 852 bp and 887 bp for *cdh7* and *cdh1*, respectively). *cdh1* transcripts were used as the control for the RT-PCR experiments because *cdh1* was shown to be strongly expressed by early zebrafish embryos (Babb et al., 2001). The same pair of *cdh7* primers (cdh7Fd and cdh7Rv) were used to obtain a cDNA fragment that was cloned into the pCRII-TOPO vector (Invitrogene), verified using restriction mapping and sequencing, and

used as a template for the synthesis of digoxigenin-labeled *cdh7* cRNA sense or antisense probes for in situ hybridization. For the synthesis of digoxigenin-labeled *pax2a* or fluorescein-labeled antisense probe, the zebrafish *pax2a* full-length cDNA in pCRII-TOPO vector (from Dr. Pamela Raymond, university of Michigan) was used as a template. cDNA used to generate the fluorescein-labeled antisense *krox20* cRNA probe was kindly provided by Lisa Maves (Fred Hutchinson Cancer Research Center, Seattle, WA). Detailed procedures for the cRNA probe synthesis and whole mount in situ hybridization were described previously (Liu et al., 1999). There was no staining in zebrafish embryos at 18, 24 and 52 hpf using the sense *cdh7* probe (data not shown). zn12 antibody (Zebrafish International Resource Center, Eugene, OR) was used at 1:5000. The secondary antibody was biotinylated anti-mouse IgG (Vector laboratories, Burlingame, CA). Detailed procedures for whole-mount immunocytochemistry were reported previously (Liu et al., 2001), while procedures for double labeling experiments were described by Jowett (1999).

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Sig
MTILLLAQRDVGCGGMTLNSVPLRSVSLRPLRDSVGTSLQRHKR
EC1
NWVWNQFFVLEEYTGDEPLYVGKLHSDVDKGEGKVKYVLNGEGATSIFTIDENTGDIHAT
KRLDREEQAYYTLRAQARDRATNLPVEPESEFVIKVQDINDNEPKF
EC2
LDGPYNAQVPEMSPVGTSVVEVKATDADDPTYGNSARVVYSILEGQPYFSVEPKTGIVRT
ALPNMDREARDQYLLVIQAKDMVGQMGGLSGTTSVTVTLTDVNDNPPRF
EC3
SRKSYQFAVPESLPVASVVAKIKALDSDIGPNAEMDYRIIEGDGLGVFRVTTDKDTQEGV
ITLQKNLDFETKSSYTLKIEASNRNIDPRFLSQGPFSDTAMVRLTVENVDEPPVF
EC4
SSPLSKMVVSEAAKVGTMIGTVSAMDPDTTNSPIRYSIDRNTDLERFFNIESPTGVISTA
EPLDREANAVHNITILAIESMDPSQVGKGVALITVMDINDNAPVF
EC5
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AG
TM
LSTGALIAILACIITLLVSGAAD
Cyto
RDNEEEEEGTSDLDEDRDVREDIVRYDDEGGGEEDTEAFDMVALRNLNVVRDSKARRDVT
PEVPTLYCSRPPPYKITPDNGIFREFIWDRLKDADVDPSAPPYDSLQTYAFEGSGSAAES
LSSLDSLSTDSEQNYDYLSDWGPRFRKLADLYGHGDSSNIFSS

Figure 1.
Deduced amino acid sequence of zebrafish Cdh7. The putative hydrophobic signal sequence (Sig) is underlined. Other abbreviations: cyto, cytoplasmic domain; EC1-EC5, extracellular domains 1–5; TM, transmembrane domain.

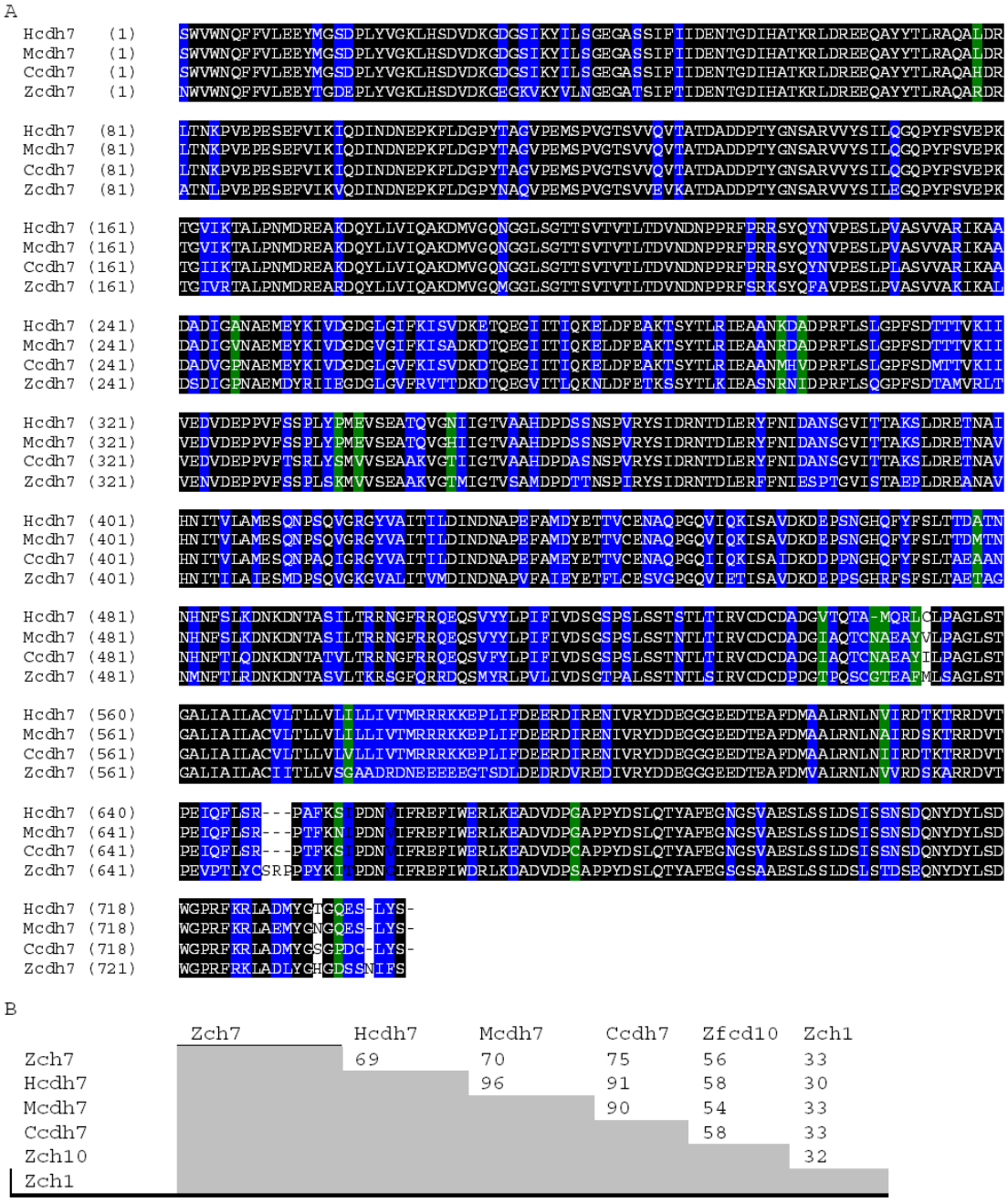

Figure 2A.

Amino acid sequence comparison between the deduced zebrafish Cdh7 amino acid sequence (Zcdh7), chicken Cdh7 (Ccdh7), human Cdh7 (Hcdh7) and mouse Cdh7 (Mcdh7). Comparisons were between published sequences from the EC1 to near the end of the coding sequences. Residues that are identical among all 4 sequences are outlined in black. Residues conserved in 3 of 4 sequences are outlined in blue, while 2 of 4 outlined in green. Figure 2B shows sequence identity percentages for pairwise comparisons between zebrafish Cdh7, several other Cdh7 sequences, cadherin10 (Zcdh10, another type II zebrafish cadherin), and a type I zebrafish cadherin, cadherin1 (Zcdh1). Sequence comparisons were performed using Clustal W (Des Higgins, EBI, Hinxton Hall, UK).

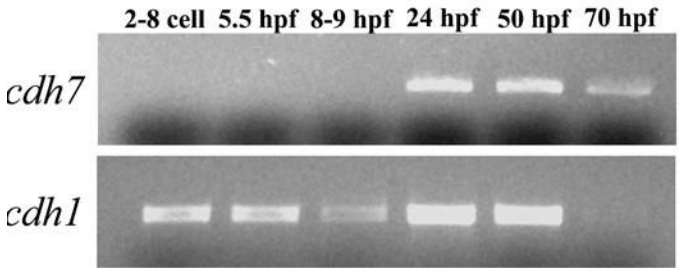

Figure 3.
RT-PCR analysis of *cdh7* expression in embryonic zebrafish using total RNAs. RT-PCR for *cdh1* was performed as loading control.

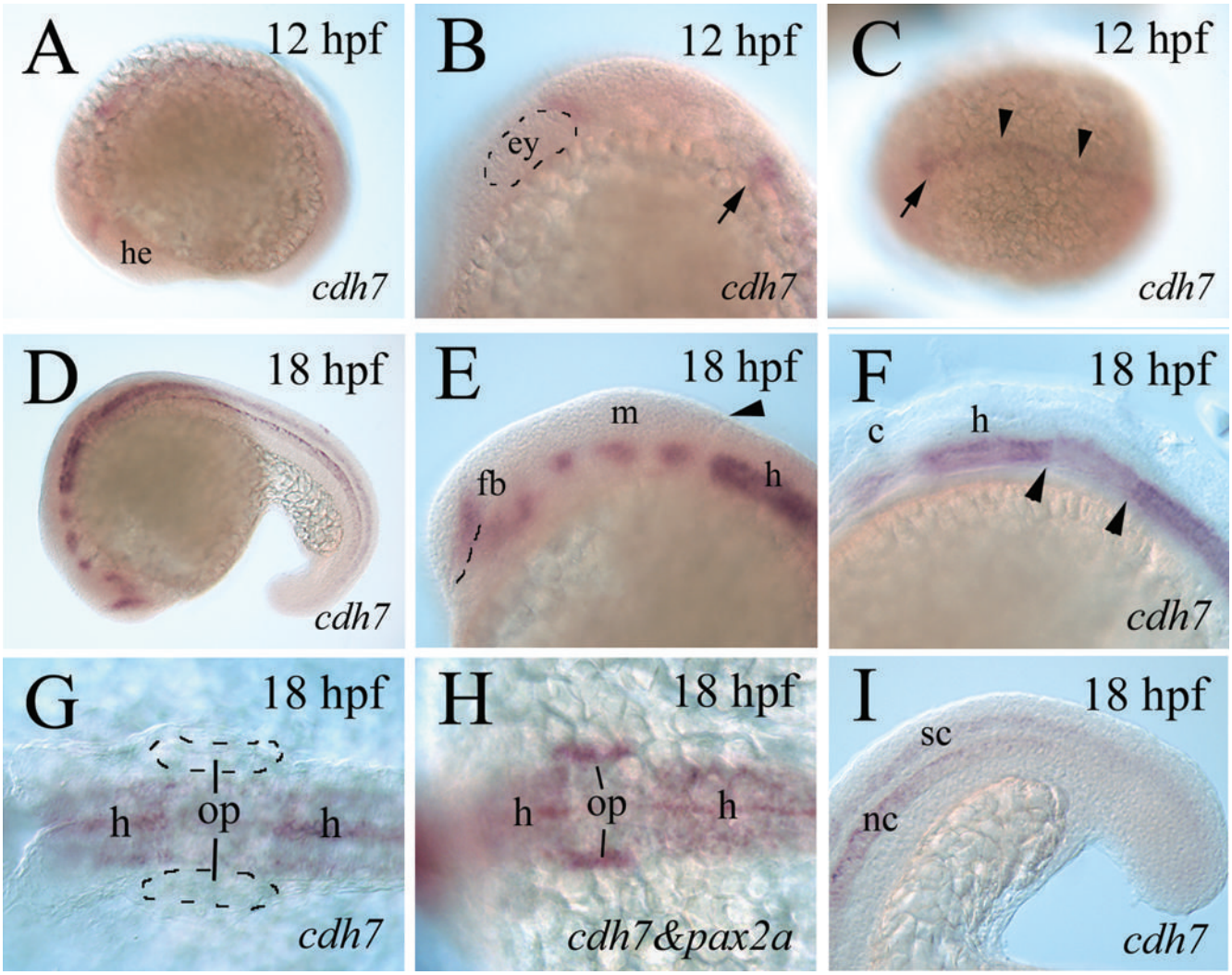

Figure 4.

cdh7 expression in 12–18 hpf zebrafish embryos. All panels, except panels C, G and H, show lateral views of whole mount embryos (anterior to the left and dorsal up) labeled with either *cdh7* cRNA (panels A–G, and I) or *cdh7* and *pax2a* cRNA probes (panel H, both probes were digoxigenin-labeled). Panel B is a higher magnification of the head region of the embryo with the eye primordium outlined by the dashed line. Panel C is a dorsal view (anterior to the left) of the embryo. The arrows in panel B and C point to the same *cdh7* expression region in the hindbrain, while arrowheads in panel C indicate *cdh7* expression at the midline. Panels E–G are higher magnifications of the head region, while panel I is a higher magnification of the tail region. The dashed line in panel E indicates the optic recess, while the arrowhead points to the caudal boundary of the cerebellum. The pair of arrowheads in panel F bracket a hindbrain region with reduced *cdh7* expression. Panels G and H are dorsal views (anterior to the left) of higher magnifications of the hindbrain region of the embryos showing that the region with the reduced *cdh7* expression is at the level of the otic placode. The otic placodes in panel G are outlined with dashed lines. Abbreviations: c, cerebellum; ey, eye premordium; h, hindbrain; he, head region; nc, notochord; op, otic placode; sp, spinal cord.

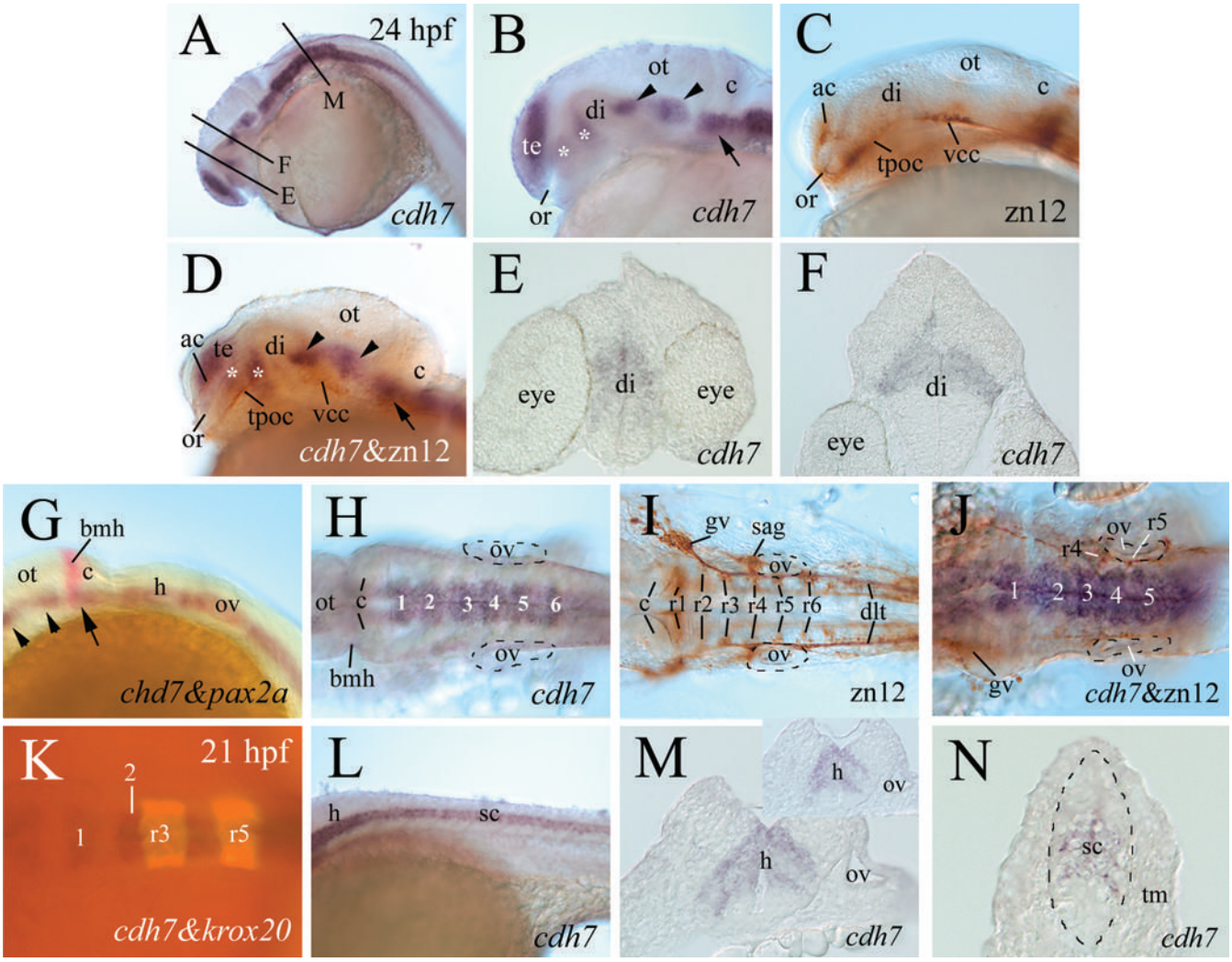

Figure 5.

cdh7 expression in 24 hpf zebrafish embryos (panel K is from 21 hpf). Zebrafish embryos processed for *cdh7* whole mount in situ hybridization are shown in panels A, B, E, F, H, L–N. Panels C and I show embryos processed for zn12 whole mount immunostaining, while panels D and J show embryos processed for double-labeling (*cdh7* in situ hybridization (purple) followed by zn12 immunostaining (brown)). Panel G is from a double-labeling experiment using digoxigenin-labeled *cdh7* (purple) and fluorescein-labeled *pax2a* (red) cRNA probes. Panel K shows an embryo processed for double-labeling experiment using the digoxigenin-labeled *cdh7* probe (dark) and fluorescein-labeled *krox20* probe (fluorescent). Panels A–D, and G are lateral views of the head region with anterior to the left and dorsal up. Panels E, F, M and N are cross sections (dorsal up) from embryos processed for *cdh7* whole mount in situ hybridization. Asterisks in panels B and D indicate *cdh7* expressing domains in the diencephalon. Arrowheads and arrows in panels B–D and G indicate corresponding *cdh7* expressing domains in the mid- and hindbrains. The section levels of panels E, F and M are indicated by corresponding letters in panel A. Panels H–K are dorsal views (anterior to the left) of higher magnification of the hindbrain region. The otic vesicles in panels H–J are outlined by dashed lines. The white numbers in panels H, J and K label swellings of *cdh7* expression in the hindbrain. Panel M shows a section from a swelling in *cdh7* expression in the hindbrain at the otic vesicle level, while the insert in panel M shows a section from a restriction in

cdh7 expression in a similar region. Panel L is a lateral view of the mid-trunk region of the embryo. The spinal cord region in panel N is outlined by the dashed line. Abbreviations: ac, anterior commissure; bmh, boundary of the mid- and hindbrains; di, diencephalon; gv, trigeminal ganglion; ot, optic tectum; ov, otic vesicle; r1–r6, rhombomeres 1–6; sag, statoacoustic ganglion; te, telencephalon; tm, trunk muscles; tpoc, tract of postoptic commissure; vcc, ventrocaudal cluster. Other abbreviations are the same as in Figure 4.

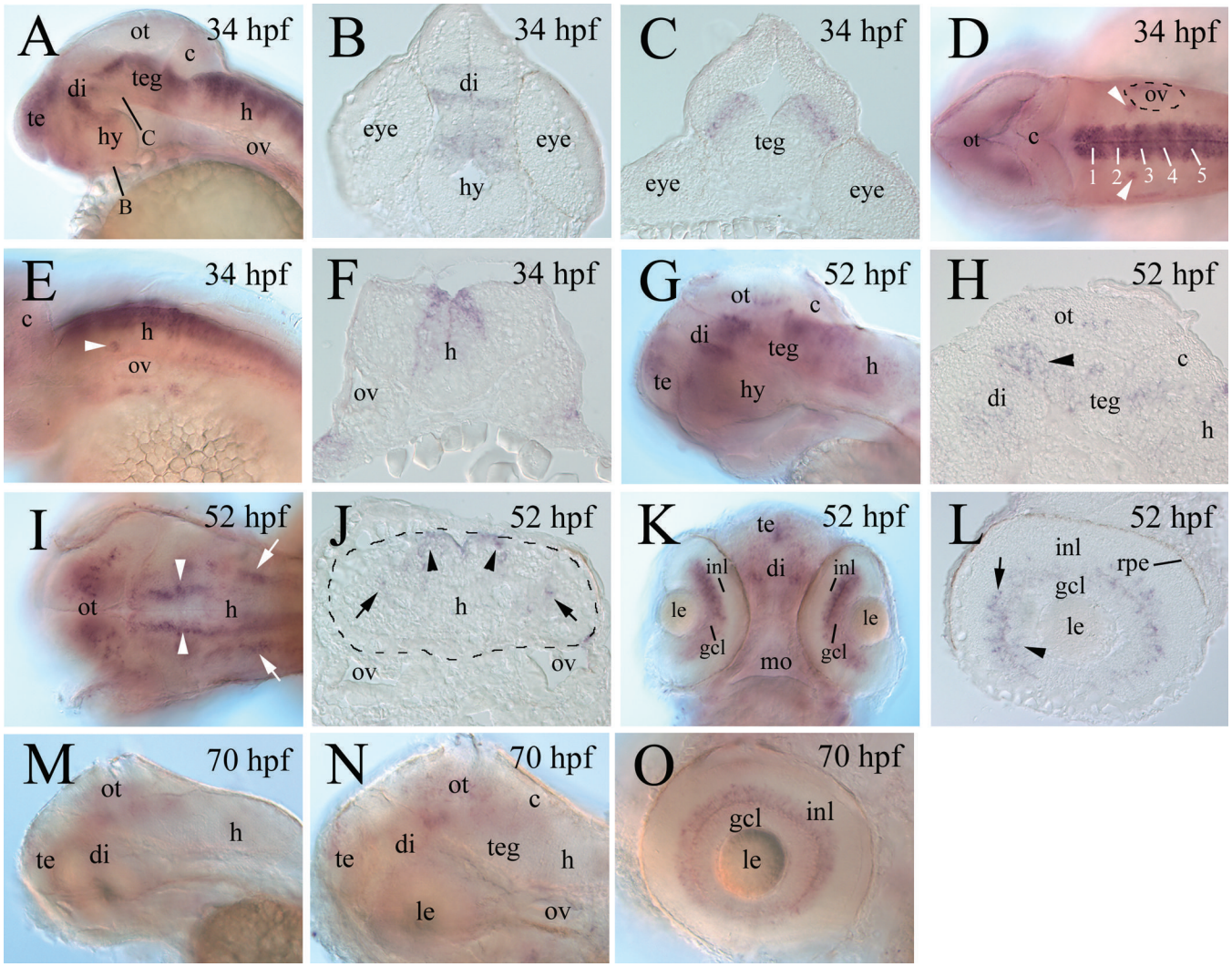

Figure 6.

cdh7 expression in 34 hpf (panels A–F), 52 hpf (panels G–L) and 70 hpf (panels M–O) zebrafish embryos. Panels A, D, E, G, I, K, M–O are from whole mount embryos processed for *cdh7* in situ hybridization, while the remaining panels are sections from embryos processed for *cdh7* whole mount in situ hybridization. Panels A, G, M and N are lateral views of the head region (anterior to the left and dorsal up). Panels B and C are cross sections (dorsal up) of the brain, with their section levels indicated by corresponding letters in panel A. Panels D and I are dorsal views (anterior to the left), while panel E is a lateral view (anterior to the left and dorsal up) of the hindbrain region. The white numbers in panel D indicate *cdh7* expression swellings in the hindbrain. The arrowheads in panels D and E point to a pair of *cdh7* expressing cell clusters in the ventral region anterodorsal to the otic vesicle. Panel F is a cross section (dorsal up) from the hindbrain. Panel H is a higher magnification of parasagittal section of the midbrain region. The arrowhead indicates *cdh7* expressing cells in the pretectal region. The pair of arrowheads in panel I point to the dorsomedially located *cdh7* expression domains, while the arrows indicate the ventrolaterally situated expression domains. Panel J is a cross section (dorsal up) from the hindbrain also showing the same *cdh7* expressing domains (indicated by corresponding arrowheads and arrows) as in panel I. The hindbrain boarder in this panel is outlined by the dashed line. Panel K is a ventral view (anterior up) of the forebrain and eyes, while panel L is a parasagittal section (anterior to the left and dorsal up) of the retina. Panel N

is a higher magnification of the head region in panel M, while panel O (anterior to the left and dorsal up) shows a lateral view of the eye. Abbreviations: gcl, retinal ganglion cell layer; hy, hypothalamus; inl, inner nuclear layer; mo, mouth; rpe, retinal pigmented epithelium; teg, tegmentum. Other abbreviations are the same as in previous figures.