Anat Embryol (2000) 202:385–400

ORIGINAL ARTICLE

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Proliferation pattern changes in the zebrafish brain from embryonic through early postembryonic stages

Accepted: 17 May 2000

Abstract Proliferating cell nuclear antigen (PCNA)-immunohistochemistry was used for demonstrating the spatiotemporal course of proliferation in the brains of embryonic (24 h) through postembryonic (5 days) zebrafish (*Danio rerio*). Parallel series of the same stages prepared according to the combined Bodian fiber silver-stain/cresyl Nissl-stain were used for improved morphogenetic analysis (i.e., in detecting critical neuroanatomical landmarks). Starting from an essentially ubiquituous proliferation throughout the neural tube before 24 h, PCNA-immunoreactive cells become successively more restricted to a subset of gray matter cells around 48 h and even more distinct proliferation zones become apparent around 72 h. Both hindbrain and forebrain reveal a segmental organization with regard to the distribution of proliferation zones, but the rhombomeric pattern of PCNA-immunoreactive cells emerging between 48 h and 72 h precedes a similar prosomeric pattern by about 48 h. Two divisions of the midbrain-hindbrain boundary are described here morphologically and both are demonstrated to show sustained proliferation throughout the investigated time frame. In contrast, proliferation in the adjacent mesencephalic and cerebellar domains is rapidly down-regulated during the first 5 days of development.

Key words Forebrain · Hindbrain · Midbrain-hindbrain boundary · Proliferating cell nuclear antigen · Prosomeres

Introduction

While it is currently commonly accepted that the vertebrate brain is segmented, the anteroposterior extent of segmentation is a matter of dispute. All segmental models agree on the reality of neuromeres in the early rhombencephalon (rhombomeres) that were demonstrated, e.g., by studies on clonal restriction, glial boundaries, early regulatory gene-expression patterns and concomitant activity (Lumsden 1990; Kimmel 1993; Puelles and Rubenstein 1993; Mcdonald et al. 1994; Guthrie 1995; Lumsden and Krumlauf 1996). However, a more extended model of brain segmentation has been proposed to include segmentation of the prosencephalon (prosomeres; Puelles and Rubenstein 1993). We have recently studied early proliferation in the zebrafish brain (*Danio rerio*; Wullimann and Puelles 1999; Wullimann et al. 1999) using an immunohistochemical approach for detecting a nuclear protein essential for mitosis, i.e., a monoclonal antibody against the proliferating cell nuclear antigen (PCNA; PC10, so-called "cyclin" of Waseem and Lane 1990), which was later demonstrated to represent the auxiliary protein of DNA polymerase-δ (Mathews et al. 1984; Bravo et al. 1987). In these two previous studies, the detailed pattern of proliferative activity in the postembryonic zebrafish forebrain at 5 days was described using PCNA as a mitotic marker. This analysis revealed highly reproducibly all major proliferative forebrain zones and, furthermore, was in strong support of a prosomeric organization of the zebrafish forebrain, at least of its posterior part. Accordingly, separate pretectal (P1, synencephalon), dorsal thalamic (P2, posterior parencephalon) and ventral thalamic (P3, anterior parencephalon) segments (i.e., prosomeres) could be observed based on the morphology and distribution of distinct alar and basal plate proliferation zones for each of those prosomeres. While the hypothalamic, preoptic and telencephalic proliferation zones could not be aligned equally easily into a prosomeric pattern, their analysis also revealed important morphogenetic findings. For example, the pattern of pallial proliferation conforms exactly to its location predicted by the process of telencephalic eversion (Nieuwenhuys and Meek 1990). Also, the two major adult subpallial divisions are foreshadowed ontogenetically by two massive proliferation centers.

While distinct proliferation zones were also described in the midbrain in these studies, the hindbrain (medulla

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oblongata) and cerebellar proliferations were not treated in these previous papers. As will be shown in this report, the postembryonic zebrafish hindbrain at 5 day shows mostly scattered proliferative cells, except for distinct proliferation zones in the cerebellar anlage, in the rhombic lip, and two more in the alar plate of the medulla oblongata.Thus, hindbrain proliferation appears to be down-regulated much earlier compared to that in mid- and forebrain.

In the present study, a more dynamic description of spatio-temporal changes in proliferative patterns in the whole zebrafish brain from embryonic through early postembryonic stages will be given. When appropriate, the data will be put in context to the neuromeric model mentioned above (Puelles and Rubenstein 1993). However, it should be clear that our description of proliferation zones may only be consistent with – but cannot be causal proof of – any segmental brain model.
In particular, the following issues will be considered:

1. How does the forebrain proliferation pattern described earlier (see above) emerge during the first 5 days of development?
2. How do more posterior proliferation patterns spatiotemporally develop during the first 5 days and what are the major proliferation zones in the zebrafish hindbrain – including midbrain-hindbrain-boundary and cerebellum?

Materials and methods

Maintenance and breeding of zebrafish (*Danio rerio*) followed Westerfield (1995). Specimens were staged according to Kimmel et al. (1995). The stages used for all investigations reported here ranged between 24 h postfertilization and 5 days postfertilization. A total of 126 zebrafish specimens was used. The research reported herein did not involve animal experiments in the sense of the "German Law on the Protection of Animals". Nevertheless, the guidelines established in the "Principles of laboratory animal care" (NIH publication NO. 86–23, revised 1985) were followed when applicable and the planned number of animals to be used in this study were reported prior to starting the investigations to the local authorities (Senate of the City of Bremen).

Bodian silver-staining/Cresyl-violet Nissl staining

The combined silver/cresyl stain procedure applied on 30 specimens used for transverse, sagittal or horizontal serial sections has been reported before (Wullimann and Puelles 1999).

PCNA immunohistochemistry

Zebrafish were collected between 24 h and 5 days at daily intervals, anesthetized with MS 222 (Sigma, Deisenhofen, Germany) and fixed in cold (4°C), buffered 4%-paraformaldehyde between two weeks and one year. The number of specimens processed for PCNA-immunohistochemistry for each stage was: 25 (24 h), 20 (48 h), 25 (72 h), 5 (4 days), 21 (5 days). A total of 96 specimens was used for immunohistochemistry and transverse, sagittal and horizontal sections were made.

After fixation, embryonic and postembryonic zebrafish specimens were dehydrated and embedded in paraffin before they were cut transversely on a rotary microtome at 6 or 8 µm. Sections were mounted serially on glass slides covered with poly-L-lysin (MW 70,000–150,000, P-1399, Sigma, Deisenhofen, Germany). Sections were then deparaffinized and rehydrated. Alternatively, five of the 24 h brains and 4 of the 48 h brains were cryostate-sectioned instead of paraffin sectioned at 10 µm. All sections were then washed in phosphate buffered saline (PBS, pH 7.2, after Romeis 1989) prior to heating in citric acid-citrate buffer (pH 6, after Romeis 1989) in the microwave at 700 W for 10 to 30 min. The further processing of sections was identical to that reported previously (Wullimann and Puelles 1999).

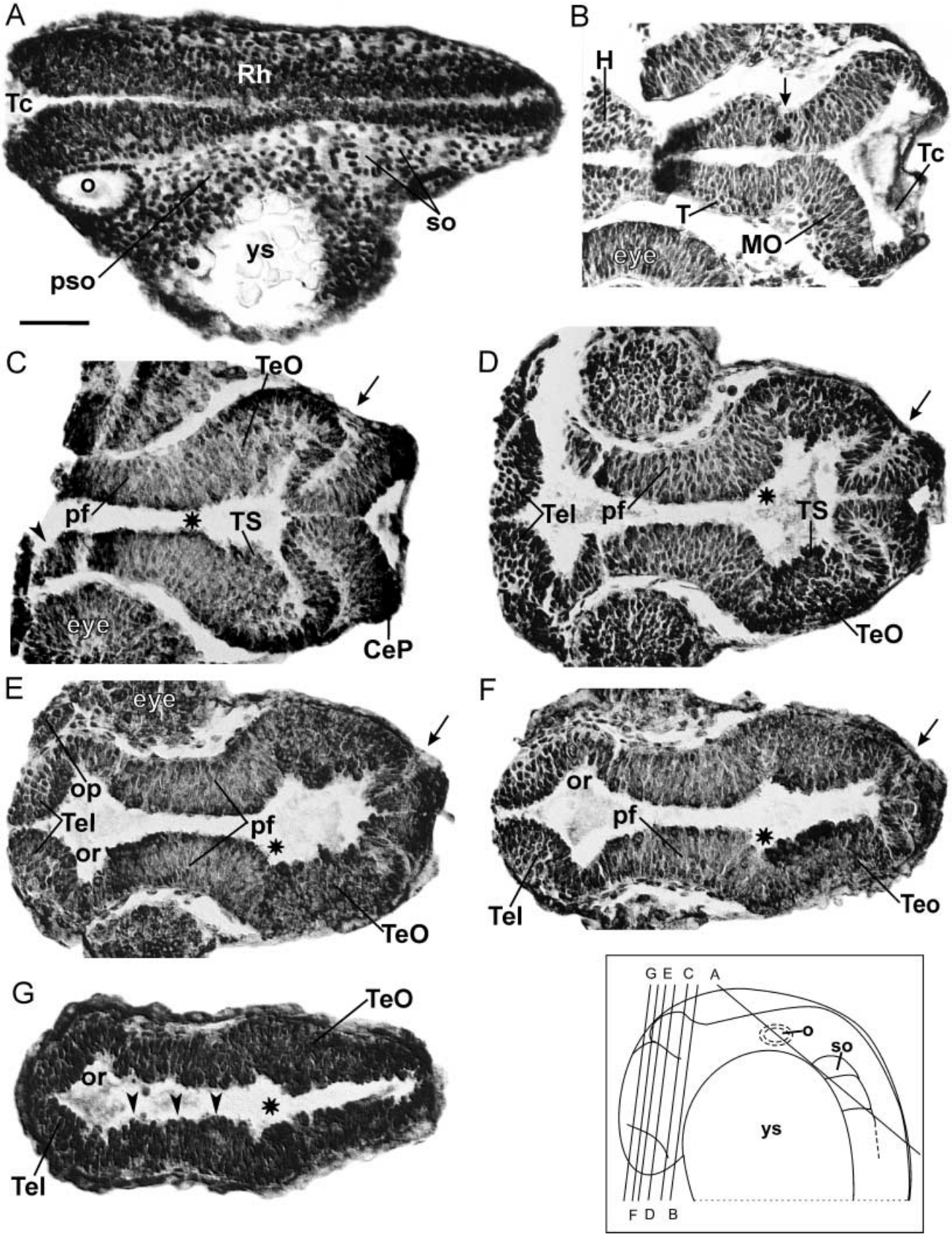
Controls using PBS instead of the primary antibody, but leaving everything else unchanged, revealed no reactivity within the central nervous system or any other tissue.

Results

The results will first deal with spatiotemporal changes of proliferation patterns during the first 5 days in the forebrain and, secondly, in the mid-/hindbrain.

Abbreviations for figures: *ac* Anterior commissure, *anc* ansulate commissure, *AP* alar plate proliferation, *BP* basal plate proliferation, *c* cerebellar proliferation, *cc* cerebellar commissure, *Ce* corpus cerebelli, *CeP* cerebellar plate, *cpt* commissure of the posterior tuberculum, *D* diffuse nucleus of inferior lobe, *DT** dorsal thalamic proliferation zone, *e* extraneural space, *E* epiphyseal complex (incl. epiphysis and dorsal sac), *f* medial longitudinal fascicle, *H* hypothalamus, *Ha* habenula, *hac* habenular commissure, *Hc* caudal hypothalamus, *Hc** proliferation of Hc, *Hi* intermediate hypothalamus, *Hi** proliferation of Hi, *Hr* rostral hypothalamus (anterior tuberal hypothalamus), *Hr** proliferation zone of Hr, *Hy* hypophysis (pituitary), *IN* interpeduncular nucleus, *l* lateral tectal proliferation zone, *m* medial tectal proliferation zone, *M* Mauthner cell axon, *md* dorsal part of m, *mhb* midbrain-hindbrain boundary (or its proliferation in Fig. 3B), *MO* medulla oblongata, *mv* ventral part of m, *N* proliferation in the region of nucleus of medial longitudinal fascicle (basal synencephalon), *NIII* Nucleus nervi oculomotorii, *o* otic vesicle, *OB* olfactory bulb, *OB** proliferation of OB, *OC* optic chiasma, *op* olfactory placode, *or* optic recess, *os* optic stalk, *P* pallium, *P** pallial proliferation zone, *pc* posterior commissure, *pf* posterior forebrain, *Po* preoptic area, *Po** preoptic proliferation zone, *poc* postoptic commissure, *Pr* pretectal area, *Pr** pretectal proliferation zone, *pso* presomitic mesoderm, *PT* posterior tuberculum area, *PTd* dorsal proliferation zone of PT, *ptm* posterior tectal membrane, *PTv* ventral proliferation zone of PT, *rct* rostral cerebellar thickening, *Rh* rhombencephalon, *RL* rhombic lip proliferation, *S* subpallium, *S** subpallial proliferation zone, *so* somite, *SR* superior raphe, *T* mesencephalic tegmentum, *T** mesencephalic tegmental proliferation, *Tc* tela chorioidea, *Tel* telencephalon, *TeO* tectum opticum, *Th* thalamus, *TM* tectum mesencephali, *TS* torus semicircularis, *v* ventricle, *Va* valvula cerebelli, *VT** ventral thalamic proliferation zone, *ys* yolk sac

Fig. 1A–G Microphotographs (Nomarski optics) of PCNA-stained zebrafish brain horizontal sections at day 1 postfertilization; sections **B** to **G** show successively more dorsal neural tube levels indicated in the sketch at the lower right end (modified from Kimmel et al. 1995). With the exception of **A**, all sections are taken from the same specimen. Midbrain-hindbrain boundary (mhb) is indicated everywhere by an *arrow*, forebrain-midbrain boundary is indicated by an *asterisk*. *Arrowhead* in **C** indicates hypothetical position of preoptic region. Black stain close to the ventricle at the isthmus in **B** is an artefact. *Bar* in **A** 50 µm, and applies to all microphotographs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Changes in forebrain proliferation patterns between the 1st and the 5th day

Day 1

At 24 h virtually every cell in the neural tube is labelled immunohistochemically with the PCNA antibody (Fig. 1), i.e., since PCNA is below detection level in quiescent cells (Bravo and Mcdonald-Bravo 1987), the overwhelming majority of cells appears to be mitotic (see Discussion).

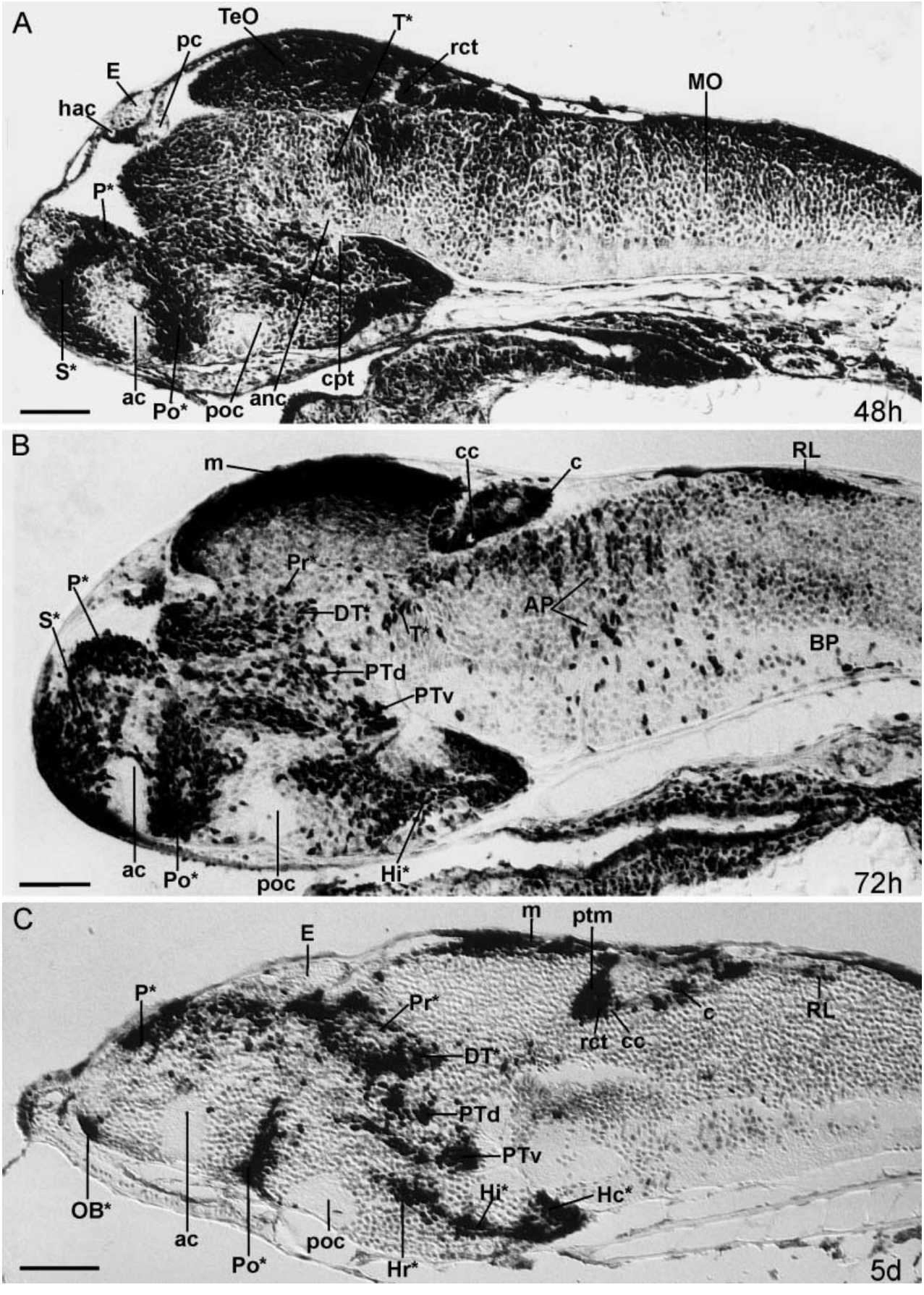
Dramatic morphogenetic processes have occurred already in the first 24 h resulting in the generation of major longitudinal and segmental entities of the neural tube (Fig. 1). The segmental organization of the forebrain (i.e., prosomeres) does not become obvious in the PCNA preparations at 24 h, although one might suspect that three intraventricular bumps (arrowheads in Fig. 1G) might represent the P1 through P3 alar plate protrusions. However, larger scale longitudinal differentiations are overtly evident. The thick wall of the telencephalon is separated by a sulcus (optic recess in much of the developmental literature) from the adjoining neural tube. As has been described previously (Ross et al. 1992; Macdonald et al. 1994; Kimmel et al. 1995), the optic stalk extends as a thin-walled cellular lining of the ventrolateral ventricular diverticulum of the optic recess and contains most distally the thicker wall of the histologically still little differentiated eye. The optic fibers have not yet formed at 24 h (Wilson et al. 1990), i.e., the first axons of retinal ganglion cells appear only at 32 h in the optic stalk and reach the optic tectum first at around 45 h (Burrill and Easter 1995). Thus, the optic stalk at day 1 marks the future entrance point of the optic nerve fibers and the optic chiasma. Logically then, the preoptic region must be present in the area between the anterior commissure lying in the ventral part of the pillow-shaped, thick piece of neural tissue, which will develop into the telencephalon, and the postoptic commissure that lies slightly posterior to the optic recess at day 1 already (commissures were demonstrated by Wilson et al. 1990, their Fig. 10D). Although these commissures can not be seen in our preparations at day 1, a piece of tissue in this general region may be hypothesized to represent the preoptic region (arrowhead in Fig. 1C). In successively more dorsal horizontal sections, one progresses from basal plate forebrain levels, i.e., the posterior tuberculum (PT), into alar plate forebrain levels (Fig. 1C through G). A typical teleostean condition is that, already at that stage, the hypothalamic inferior lobe – although representing the basal part of the anterior forebrain – has considerably extended posteriorly and lies below the basal posterior forebrain (compare Fig. 1C and B). The hypothalamic and tegmental tissues can be seen to be slightly superimposed at their point of contact (Fig. 1B). This artifact occurred during sectioning since the neural tube is evidently not continuous between hypothalamus and mesencephalic tegmentum.

Day 2

In contrast to day 1, in the zebrafish brain at 48 h, proliferative activity becomes restricted to cells in a ventricular position judged from the PCNA material and many clearly unstained postmitotic cells are present more peripherally, constituting a mantle zone almost everywhere in the brain. This mantle zone is adjoined more peripherally in turn by a neuropilar marginal zone. The pallial proliferation zone is restricted to a few cell rows near the ventricular lining of the pallium including its medial non-everted and lateral everted portions and the postmitotic telencephalic cells can now be distinguished easily from the proliferative ones (Fig. 2A). However, there is as yet no distinct gap to the subpallial proliferation that emerges more ventrocaudally. Also, at this stage there is no gap between the subpallial and the caudally adjacent preoptic proliferation; they appear contiguous dorsal to the anterior commissure. In contrast, ventrally, a strip of proliferative subpallial cells extending caudally below the anterior commissure is interrupted by a gap of non-proliferative cells well before the preoptic proliferative cells emerge more caudally.

Virtually all cells of the habenula and of the most anterior dorsal and ventral thalamus as well as of the pretectum (i.e., the whole alar plate of the posterior forebrain) are proliferative and can, thus, not easily be distinguished by means of their separate proliferation zones. More caudally, however, the PCNA reactivity is increasingly restricted to the ventricular lining. There, the dorsal thalamic proliferation (DT*) at the ventricular side of its (postmitotic) cellular protuberance can be clearly delineated from the proliferation of the ventral posterior tuberculum (PTv). A corresponding smaller, dorsal posterior tubercular proliferation (PTd), emerges also in between these two proliferation centers, but is hard to be delineated at times from the ventral posterior tubercular proliferation. The latter can be followed caudally into the dorsal aspect of the inferior lobe that, thus, already at that stage includes a portion of the posterior tuberculum in addition to its hypothalamic main cellular masses. At 48 h, the posterior tubercular proliferation (PTv) is essentially contiguous with the more posteriorly emerging caudal proliferative zone of the hypothalamus (Hc*), since at least most medially there are no apparent postmitotic cells between these two proliferation zones. However, the two zones are separated by the commissure of the posterior tuberculum (Fig. 2A). As with respect to the rest of the hypothalamus, the rostral and intermediate proliferations are massive and it is to be

Fig. 2 Microphotographs (Nomarski optics) of PCNA-stained zebrafish brain parasagittal sections at **A** 48, **B** 72 h, **C** 5 days. Note the progressive increase of unstained postmitotic cells and the distribution of particular proliferation centers in these first five days of development. The alar plate ventral thalamus (*VT*) is not seen on sagittal sections as a separate entity, but it can unambiguously be differentiated from the dorsal thalamus in horizontal sections (Wullimann and Puelles 1999). *Bars* 50 µm. ▶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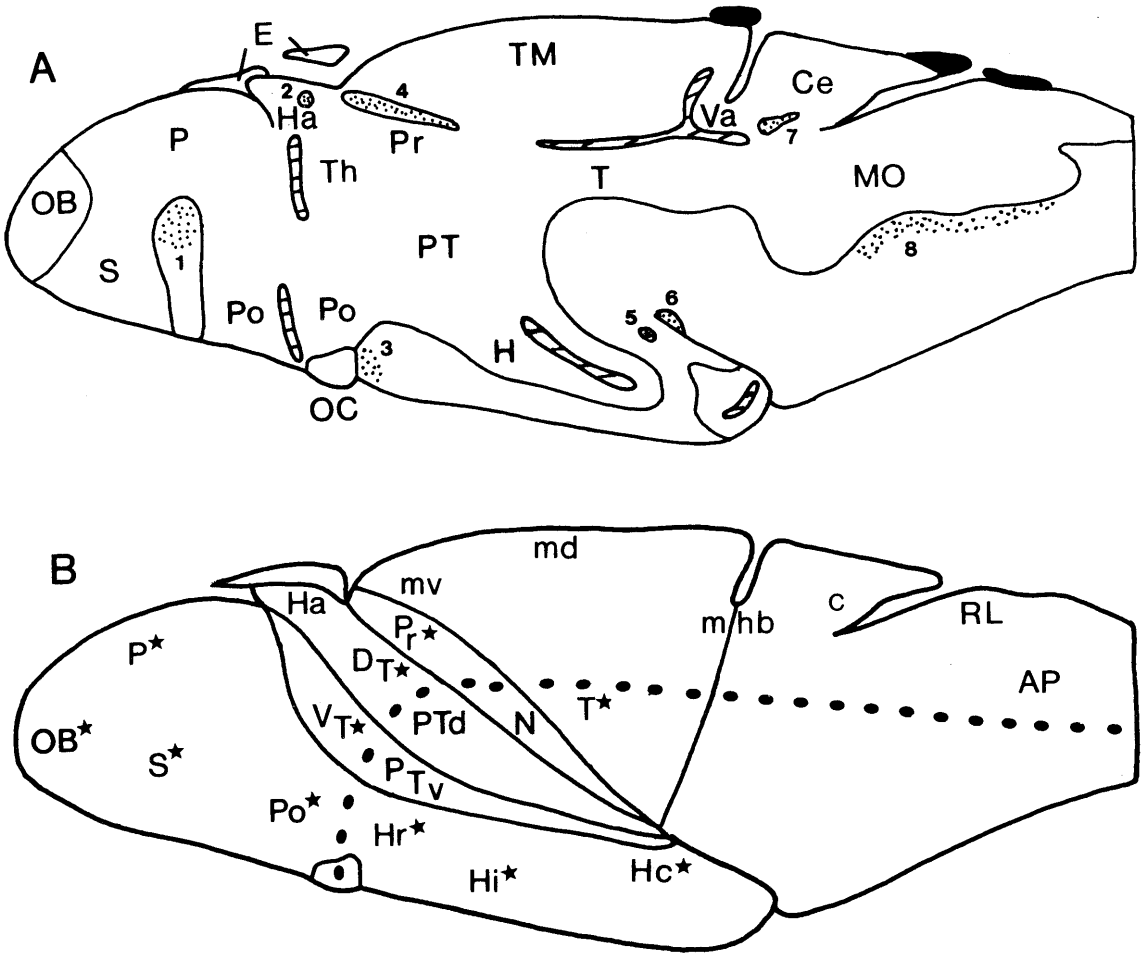


Fig. 3 **A** Drawing of a Bodian-stained parasagittal section of a 5 day zebrafish brain. The boundary between white and gray matter is indicated by a thin black line. *Letters* designate major brain subdivisions in the center of their respective gray matter. *Black areas* represent pigment in the meninges. Commissures are indicated with *stippling* (*1* anterior commissure, *2* habenular commissure, *3* postoptic commisssure, *4* posterior commissure, *5* commissure of posterior tuberculum, *6* ansulate commissure, *7* cerebellar commissure, *8* ventral rhombencephalic commissure). Ventricles are indicated by *hatching*. **B** Outline of same drawing includes all proliferative zones at 5 days schematically (except the lateral tectal proliferation not present in a sagittal section and the patchy proliferation of the medullary basal plate). *Letters* are positioned centrally in the respective proliferation zone they designate. *Line of black dots* indicates the longitudinal brain axis according to the model of Puelles and Rubenstein (1993). *Black lines* perpendicular to this axis indicate segmental boundaries supported by the proliferative pattern at 5 days. *Bar* 100 µm.

noted that the most lateral extent of the intermediate one (Hi*) is deflected dorsally within the inferior lobe. It is thus feasible that the isolated spot of PCNA reactivity seen in the dorsocaudal aspect of the inferior lobe from 72 h onward is derived from the intermediate hypothalamic proliferation. In contrast to the two rather well-separable proliferation zones of the posterior tuberculum, distinct proliferation zones of the basal synencephalon and mesencephalic tegmentum can not be recognized at 48 h, although there is some proliferative activity along the ventricle in this general region.

Day 3

At 72 h, the proportion of postmitotic brain cells has again drastically increased compared with 48 h. A gap of non-proliferative cells has now developed between the pallial and the subpallial proliferations, but not between the dorsal and ventral subpallial divisions. Also the preoptic proliferation may be delineated well from the adjacent ones. The posterior forebrain clearly shows a prosomeric alignment of proliferative stripes (Fig. 2B) reminiscent of the situation described at 5 days. Regarding the diencephalic alar plate, a thin pretectal stripe is followed basally (actually rostrally, respective to the longitudinal forebrain axis; compare with Fig. 3B) by a thick dorsal thalamic stripe. In addition, a separate proliferation of the habenula is present more dorsally. The ventral thalamic proliferation of the alar plate is more restricted in the rostrocaudal axis than the dorsal thalamic one (Fig. 2B). Turning to the basal plate, proliferation in the general region of the nucleus of the medial longitudinal fascicle (N) is represented by a small cell cluster and is replaced caudally by the stronger mesencephalic

tegmental proliferation (see below). The basal plate portion of the dorsal thalamic prosomere exhibits a relatively thin proliferative stripe (i,e., the dorsal posterior tubercular proliferation) underlying its alar plate complement, but clearly separated from it by non-proliferative cells (Fig. 2B). Even more basal is the much thicker proliferative stripe belonging to the ventral thalamic prosomere represented by the ventral posterior tubercular proliferation (PTv). It lies immediately above (actually: posterior to) the hypothalamic proliferation centers (Hr*, Hi*, Hc*). It is to be noted that all these proliferative stripes lie perpendicular to the longitudinal axis of the vertebrate neural tube (compare with Fig. 3B) as proposed by Puelles and Rubenstein (1993). In the hypothalamus, the anterior and intermediate proliferations are well distinguished from the caudal one (Hc*). Small bilateral isolated spots of proliferative activity are seen within the laterodorsal inferior lobes at 72 h.

Days 4–5

Day 4 is not further considered here, because all forebrain proliferative zones described in detail for day 5 in earlier reports (Wullimann and Puelles 1999; Wullimann et al. 1999) are qualitatively present. For completeness, the situation at 5 days will be summarized shortly here (compare with Figs. 2C, 3). Briefly, an uninterrupted sheet of pallial proliferation (P*) lines the ventricular surface of the relatively small, non-inverted medial and the larger, everted lateral portion of the pallial telencephalon. Basal to the pallial proliferation lies the subpallial proliferation, (S*) which has dorsal and ventral divisions. Posterior to the anterior commissure, the preoptic proliferation emerges, also displaying dorsal and ventral divisions. Posterior to the optic chiasma and postoptic commissure, the hypothalamus is seen to proliferate along its entire ventricular lining. A rostral hypothalamic proliferation (Hr*) is continuous with the intermediate hypothalamic proliferation (Hi*), the latter including the proliferation of the lateral recess protruding into the inferior lobes (Figs. 2C, 4, 5). At the very posterior pole of the inferior lobe, yet another, caudal hypothalamic proliferation zone (Hc*) emerges on top of the intermediate one (Figs. 2C, 5C, D). However, this caudal proliferative zone is well-separated by a gap – consisting of white matter (that includes the commissure of the posterior tuberculum; Figs. 2C, 4B, C) and of proliferation-free gray matter (Fig. 2C, 5A, B) – from the ventral posterior tubercular proliferation. Again separately, a spot of isolated lateral proliferation within the dorsal inferior lobe is seen at that age (Figs. 4B, D, 5A).

Regarding the diencephalon proper (pretectum, dorsal and ventral thalamus), the proliferation zones may be grouped according to their prosomeric domains (compare with Fig. 3) as well as according to their longitudinal column domains (i.e., alar and basal plates) along the longitudinal brain axis newly defined by Puelles and Rubenstein (1993; see Fig. 3). There are separate pretectal (Pr*), dorsal thalamic (Ha, DT*), and ventral thalamic (VT*) alar plate proliferations corresponding to the first through third prosomeres and these are complemented by respective basal plate proliferations, i.e., a proliferation in the area of the nucleus of the medial longitudinal fascicle (N), a dorsal posterior tubercular proliferation (PTd), as well as a ventral posterior tubercular proliferation (PTv). The basal plate proliferation of the synencephalon (N) is replaced more caudally by a mesencephalic tegmental proliferation zone (T*) (Fig. 4A). The cell-poor area below the tegmental proliferation zone is indicative for the boundary between mesencephalon and diencephalon (Wullimann and Puelles 1999). Typically at this cross-section level, the most caudal extent of the diencephalic posterior tuberculum – represented by the ventral posterior tubercular proliferation (PTv) – and, more ventrally, the intermediate hypothalamic proliferation (Hi*) zones are still present. It is important to note that the most ventral part of the posterior tuberculum extends and becomes enclosed into the inferior lobe. Thus, this brain part includes a portion of the posterior tuberculum (i.e., part of prosomere 3) in addition to its hypothalamic domains (suspected basal parts of prosomeres 4 to 6; compare with Fig. 3B).

Changes in mid- and hindbrain proliferation patterns between the 1st and 5th day

Day 1

As in the forebrain, major neural tube subdivisions have formed already in the zebrafish mid- and hindbrain at 24 h (Fig. 1). The basally located mesencephalic tegmentum (Fig. 1B) may be followed through successively more dorsal horizontal sections into its corresponding alar plate complements of that piece of the neural tube, i.e., the tectum mesencephali (Fig. 1B–G). A posterior protuberance of the future torus semicircularis may already be delimited from the anteroposteriorly more extended optic tectum proper, together comprising the tectum mesencephali. Dorsoposteriorly to the torus semicircularis, the tectum forms a fold, refered to here as the posterior tectal membrane (ptm), that deeply invaginates towards the midline (especially nicely to be seen in Fig. 1C; compare also with Figs. 6 and 7). Here, in the midline, both sides of the alar plate neural tube wall run caudally for some distance, closely adjoined to each other (Fig. 1C, 7). This piece of neural tissue may be called the rostral cerebellar thickening (rct), since the neural tube wall is increasing its diameter here compared to the posterior tectal membrane. The rostral cerebellar thickening is also seen more dorsally to extend as a wedge-shaped structure into the mesencephalic tectum. There is little doubt that once the developmental roles of the rostral cerebellar thickening are fulfilled, it will give rise to the adult valvula cerebelli, a teleostean-specific portion of the cerebellum. These two pieces of tissue (ptm and rct) represent the alar plate midbrain-hindbrain boundary (mhb); (best

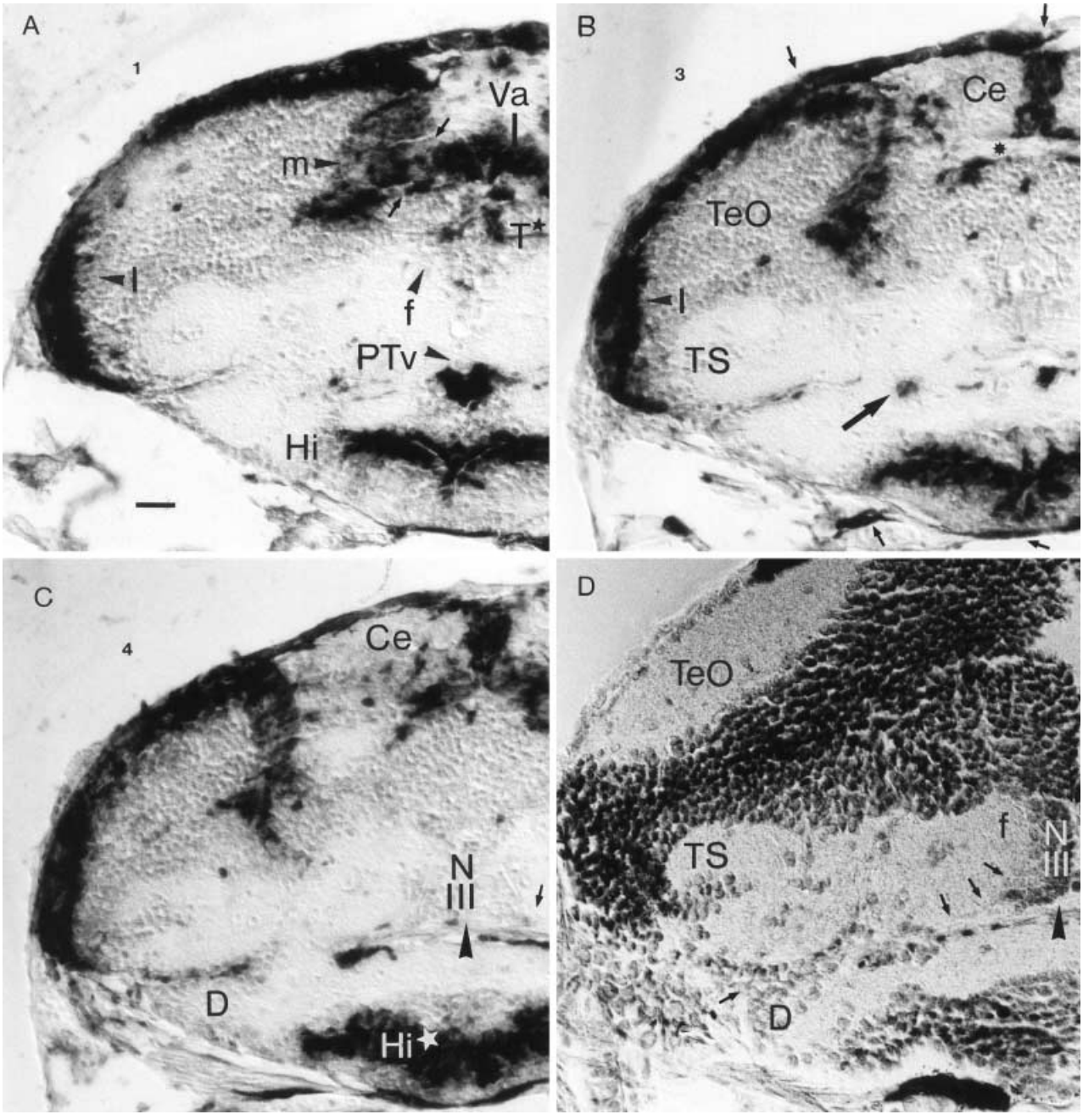
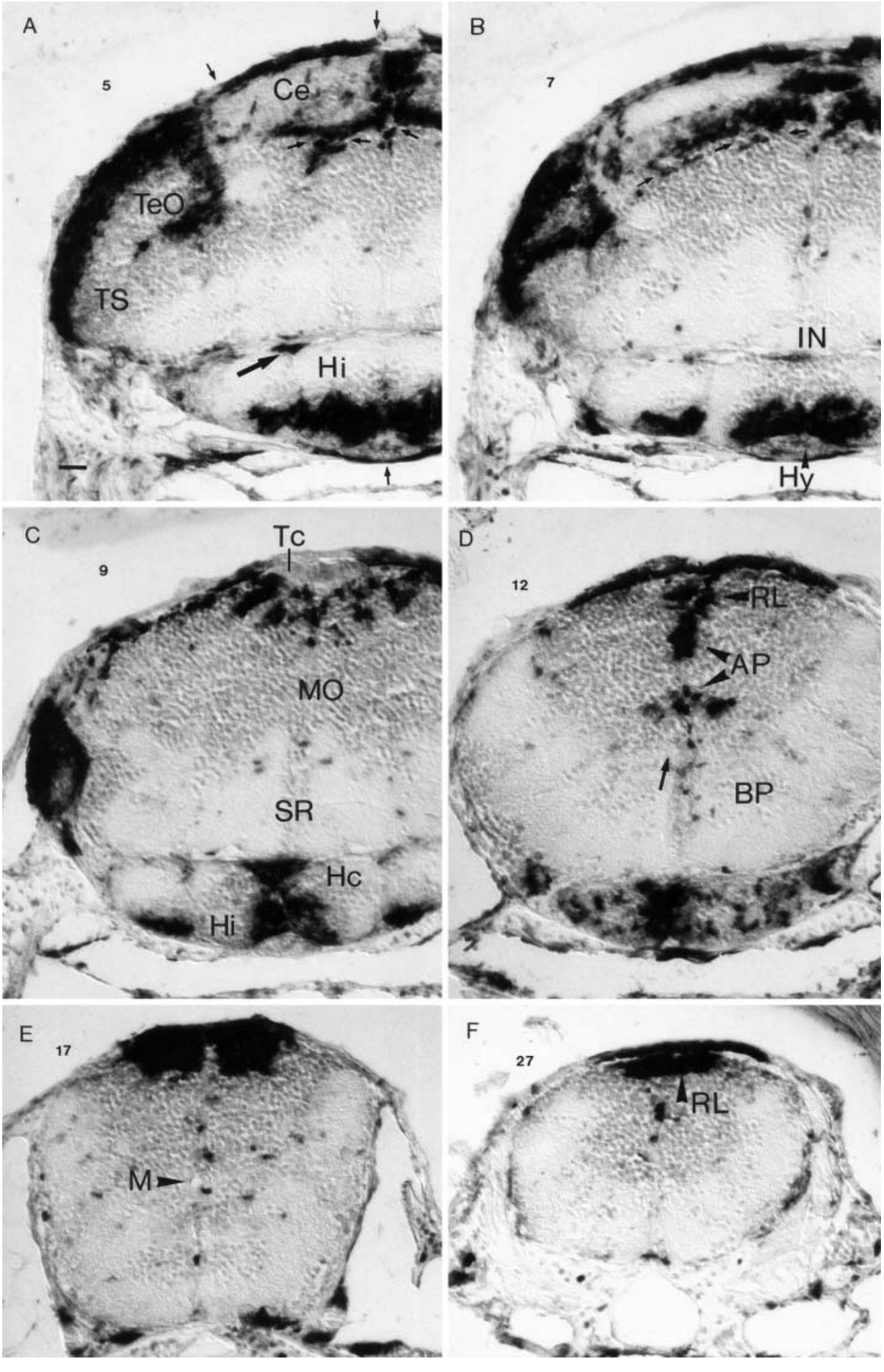


Fig. 4 Microphotographs (Nomarski optics) of PCNA-stained **A–C** or Bodian silver-stained (**D**) zebrafish brain cross sections (5 days) starting at most rostral cerebellar levels. In **A**, some proliferation of the mesencephalic tegmentum is visible (*T**) below the strong proliferative activity of the prospective valvula of the cerebellum. *Arrows* in **A** delineate cerebellum from tectum. Note heavy midline proliferation in the future corpus cerebelli in **B** and **C**. *Small arrows* in **B** meningeal pigment, *asterisk* cerebellar commissure, *large arrow* lateral hypothalamic proliferative spot. *Large arrowhead* in **C** and **D** ansulate commissure; *small arrows along the NIII* oculomotor fibers. *Small arrow at the left lateral brain side* sulcus between hypothalamus (inferior lobe) and mesencephalon. Due to a slight difference in section level between **C** and **D**, the cerebellum is not yet visible on **D** as is the case in **C**. Arabic numbering on PCNA material indicates relative distance between sections. *Bar* in **A** 10 µm

to be seen in Fig. 1C), which is known to have an organizer-like inductive influence on the surrounding tissue (Marin and Puelles 1994; Picker et al. 1999). Many of the developmentally early, spatiotemporally complex regulatory gene expression patterns and interactions of the respective gene products in the mhb have been elucidated

Fig. 5 Microphotographs (Nomarksi optics) of PCNA-stained zebrafish brain cross section (5 days) from posterior tectal/cerebellar **A** to obex levels **F**. *Small arrows outside the brain* in **A** pigment dorsal to cerebellum and ventral to hypothalamus; *small arrows in the brain* boundary between cerebellum and medulla oblongata. *Large arrow* in **A** lateral hypothalamic proliferative spot. *Arrow* in **D** ventral rhombencephalic commissure. Arabic numbering on PCNA material indicates relative distance between sections. *Bar* 10 µm ▶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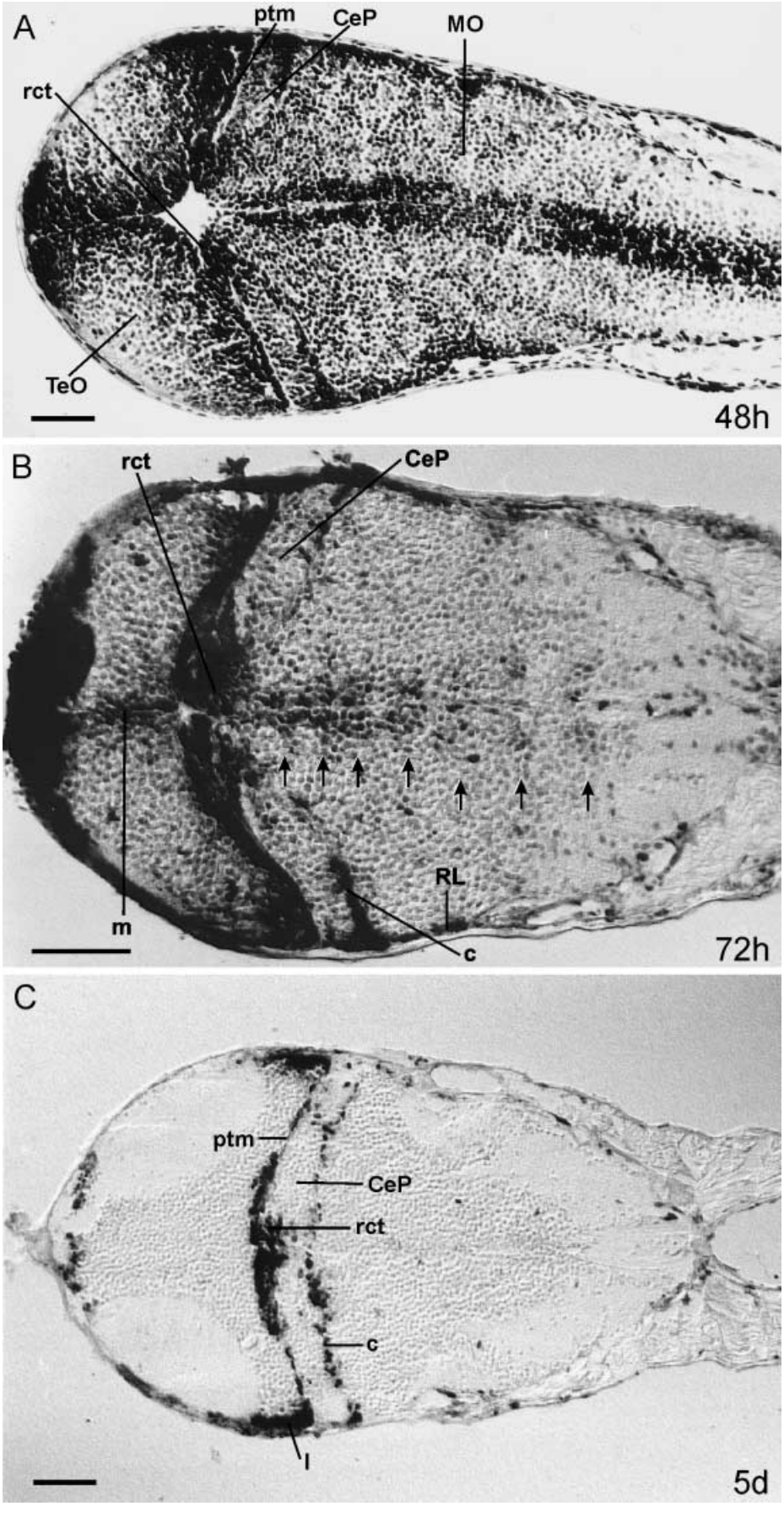

Fig. 6 Microphotographs (Nomarski optics) of PCNA-stained zebrafish brain horizontal sections at **A** 48 h, **B** 72 h, **C** 5 days. Note the persistence of proliferation in the mhb (i.e., the posterior tectal membrane and the rostral cerebellar thickening) during these first 5 days of development. Also, the emerging rhombomeric organization of the alar plate proliferation (indicated by *arrows*) is evident between days 2 and 3 but disappears at day 5. Most of the black stain at the rostral optic tectum is pigment outside the brain. *Bars* 50 µm

already (Brand 1998; Lun and Brand 1998; Reifers et al. 1998). Caudal to this area of close apposition of the alar plates of both brain sides, the neural tube walls suddenly arch out laterally again to form the paired cerebellar plates, thus, complementing the bilateral neural tube fold that is composed of a midbrain and a hindbrain (cerebellar) component. At this point of the neural tube – between the cerebellar plate and the rostral cerebellar thickening – the cerebellar commissure will be located at later stages (see below). In accord with our results, previous data (incl. TEM material) provided by Wilson et al. (1990) showed no cerebellar commissure at day 1.

In the rhombencephalon and mesencephalon, a strong PCNA labeling of most cells is visible at day one (Fig. 1A) as everywhere in the brain. However, segmentally organized postmitotic hindbrain neurons are present at this stage in the zebrafish (Kimmel 1993) and in amniotes (Wilkinson and Krumlauf 1990). This discrepancy is likely due to the fact that early postmitotic cells retain enough PCNA to be immunoreactive for 24 h after their last division (see Discussion). However, in the surrounding mesoderm, a sizable number of unstained cells are visible (Fig. 1A). Also, the lining of the otic vesicle is PCNA-reactive throughout. A rhombomeric organization – although it was clearly established with various other approaches for this developmental stage (Hannemann et al. 1988; Trevarrow et al. 1990; Macdonald et al. 1994) – is not apparent in the distribution of PCNA reactive cells at day 1.

Day 2

As in the forebrain, many cells in the mid- and hindbrain turn into postmitotic cells and proliferation becomes increasingly restricted to cells located near the ventricles at 48 h. Proliferation of the mesencephalic tegmentum is as yet neither clearly separable from that of the rostrally located basal synencephalon (basal plate of P1), nor from that of the caudally adjacent rhombencephalon. However, in sagittal sections, one might suspect the separation between rhombencephalic and mesencephalic proliferation (Fig. 2A), especially in comparison with older stages. Although tectal proliferation is still prominent along most of the ventricle, a tendency towards receding to mediodorsal and lateral edges is visible (nicely demonstrated in Fig. 6A). Interestingly, the posterior tectal membrane has essentially thinned out to a unicellular layer at 48 h, but all of its cells are still proliferative, as are the cells of the rostral cerebellar thickening (Fig. 6A). However, the cerebellar plate is only proliferative at its basal and medial aspects; most of the dorsal cells are unstained postmitotic cells (Fig. 6A). Most cells near the ventricle of the rhombencephalic alar plate – and fewer of the basal plate – are still proliferative (Fig. 2A). The beginning rhombomeric organization of the alar plate proliferation is especially clearly visualized in ideally levelled horizontal sections (Fig. 6A). Dorsal to this rhombomeric alar plate proliferation, there is equally strong proliferation throughout the dorsal medulla oblongata extending far laterally. This extensive dorsal medullary proliferation will later become the more restricted rhombic lip proliferation.

Day 3

In the 72 h zebrafish, mid- and hindbrain proliferation has greatly diminished. The tectal proliferation has receded even more towards the lateral and medial edges enclosing the many more central postmitotic cells. However, all cells of the posterior tectal membrane are still proliferative as are those of the rostral cerebellar thickening. The situation in the cerebellar plate is similar to 48 h in that only the basal and midline cells are proliferative (Figs. 2B, 6B). The cerebellar commissure is now clearly visible as a landmark at the boundary region between cerebellar plate and rostral cerebellar thickening (Fig. 2B).

Although the proliferation of the mesencephalic tegmentum is not well separated from the rostrally located one of the basal synencephalon, it is better to be delineated from the rhombencephalic proliferation (Fig. 2B). The latter displays now a proliferation of the rhombic lip. Its lateral extent is now restricted to rostral medullary levels immediately behind the attachment of the cerebellar plate (Fig. 6). Also, the more caudal proliferation of the rhombic lip is already restricted to a medial position (Fig. 2B). A more ventral alar plate proliferation becomes increasingly separate from that of the rhombic lip more caudally (Fig. 2B). The segmental (rhombomeric) organization of this rhombencephalic alar plate proliferation is now overtly evident in the PCNA preparations, since the proliferative cell clusters in the center of each rhombomere segregate by virtue of postmitotic cells lying in between them (Figs. 2B; 6B). A smaller alar plate proliferation in the rostral medulla oblongata may also be seen more ventrally, in addition to scattered proliferation in the basal plate.

Days 4–5

Between day 4 and 5, the relative extent of proliferative zones is further restricted, especially with regard to the segmental proliferative clusters. In the following, the situation at 5 days will be described. The exact caudal boundary of the mesencephalic tegmentum towards the rhombencephalon, i.e., the isthmic region, is hard to discern in the PCNA material. However, the distinct mesencephalic tegmental proliferation center (T*) (Fig. 4A) is replaced by a more patchy, single-cell PCNA-reactivity in the adjacent caudal region situated below the cerebellar commissure. We interpret this area (Fig. 4B) as the rhombencephalic isthmic region. Caudal to the cell-poor diencephalo-mesencephalic boundary zone mentioned above (seen below the tegmental proliferation in Fig. 4A), the (non-proliferative) cells of the mesence-

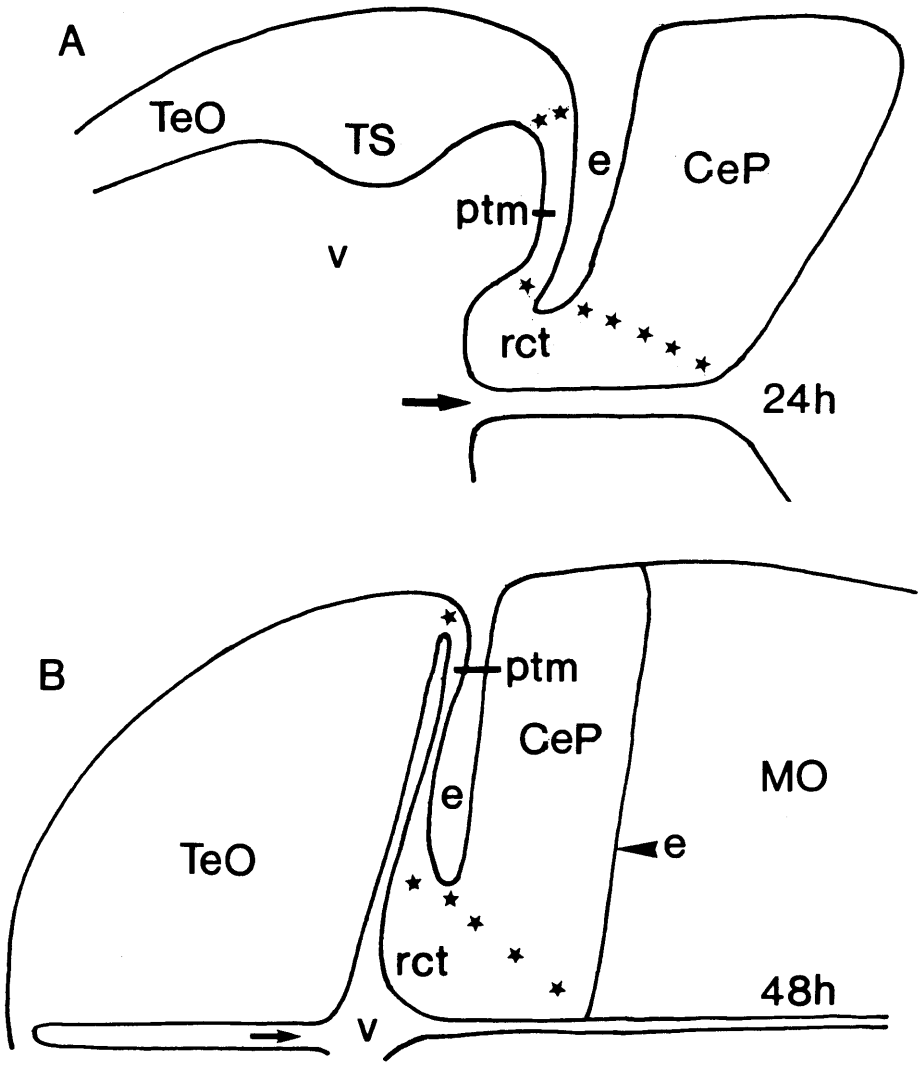

Fig. 7 Semischematic horizontal sections at **A** 24 h and **B** 48 h show development of midbrain-hindbrain boundary. While proliferation in the cerebellar plate proper (future corpus cerebelli) recedes to a ventral and medial position already at 48 h **A**, the rostral cerebellar thickening (*rct*, possibly the future valvula cerebelli) and the posterior tectal membrane (*ptm*) remain proliferative throughout. Together, ptm and rct (delineated by *asterisks*) form the midbrain-hindbrain boundary. Note that the posterior tectal membrane is getting thinner with progessive age, while the rostral cerebellar thickening remains a massive structure. *Arrows* indicate midline

phalic tegmentum eventually extend to the base of the brain, forming the oculomotor nucleus (NIII); (Fig. 4B, C, D). This nucleus is diagnostic for the early postmitotic basal plate mesencephalon and can be identified unambiguously by virtue of the oculomotor axons coursing lateroventrally away from their motor somata (arrows in Fig. 4D) and exiting the brain basally. Equally indicative for the location of the oculomotor nucleus is the presence of the mesencephalic ansulate commissure immediately ventral to this nucleus (arrowhead in Fig. 4C,D). The alar plate portion of the mesencephalon is represented by the mesencephalic tectum, comprising the optic tectum (superior colliculus homologue of mammals) and the torus semicirularis (inferior colliculus homologue of mammals). The latter is only present at posterior mesencephalic levels and – together with the optic tectum – fades out laterally to the medulla oblongata. Proliferation in the mesencephalic tectum (i.e., tectum opticum and torus semicircularis) at 5 days has receded to the lateroventral and mediodorsal edges and includes only few scattered proliferative cells along most of the tectal ventricle. In a horizontal section, the tectal ventricle is cut longitudinally in the tectal midline and transversely at the posterior end of the mesencephalic tectum (compare with Fig. 7B). In the horizontal plane, one can see that the lateroventral tectal proliferation extends from this transverse part of the ventricle to the peripheral neuropil (Fig. 6C). Similarly, the mediodorsal proliferation extends from the medial tectal ventricle to peripheral tectal neuropil. This situation becomes even more clear in comparison with earlier stages (Figs. 6B; 7B). Lateral and medial proliferation zones of the optic tectum merge at caudal levels and form a continuous cap of tectal (alar) proliferation, also including that of the torus semicircularis (Fig. 5).

The strong proliferative activity of the cerebellum includes a midline streak present throughout the anteroposterior cerebellar axis, except in the most rostral aspect (Fig. 4A), as well as a paired ventral proliferative zone, which is particulary well-developed in the anterior cerebellum, i.e., the future valvula cerebelli. This rostral cerebellar area is separated laterally from the medial tectal proliferation by a small non-proliferative gap. This gap actually represents a thin sulcus between mesencephalon

and cerebellum separating the valvular from the medial tectal proliferation, as can be nicely seen in earlier stages (compare with Figs. 1C, 7). Towards the pial surface of the cerebellar plate (future corpus cerebelli), a thinner superficial proliferation is seen that may correspond to an external granular layer. This confirms the findings of Pouwels (1978) on the development of the rainbow trout cerebellum based on Nissl material: following a first phase characterized by proliferation at the ventricular matrix layer, a second developmental phase emerges where a proliferative superficial secondary matrix in the corpus cerebelli is formed, which gives rise to inwardly migrating neuroblasts that develop into granular cells, very much like the situation in amniotes. A proliferative external granular layer remains to be present in the corpus cerebelli of teleosts into later stages (Pouwels 1978) and into adult life (Zupanc and Horschke 1995). In the early postembryonic zebrafish, the ventral and superficial proliferative cells are separated by a sizable, cell-poor white matter, especially in the posterior cerebellum (i.e., the future corpus cerebelli) indicating that a molecular layer has already developed at 5 days.

In general, the cerebellum is still rather small at 5 days compared with its adult configuration and compared with the extent of other major brain parts such as the optic tectum. The rostral cerebellum (valvula or rostral cerebellar thickening; see below) has not yet grown anteriorly to the degree to be located substantially within the tectal ventricle (compare with Fig. 3A) as is the case in the adult zebrafish (Wullimann et al. 1996) and is strongly immunoreactive for PCNA (Fig. 4A). The cerebellar commissure is very prominent by day 5 (asterisk in Fig. 4B).

As the cerebellum fades out caudally and laterally on top of the medulla oblongata (Fig. 5B,C), the midline tela chorioidea covers the fourth ventricle (Fig. 5C). Below it, proliferative cells of the most dorsal medulla oblongata consolidate medially to form a new compact proliferation center slightly more caudally that is maintained down to the obex (Fig. 5D-F). Proliferative cells in the most dorsal medulla oblongata extend very far laterally only at the level where the cerebellar plates are attached (Fig. 5B,C). Caudal to this point, dorsal medullary proliferation recedes to a medial position. These PNCA-reactive cells in the most dorsal medulla oblongata may be considered the rhombic lip proliferation. Ventral to it – but restricted to the most rostral rhombencephalic level – two additional proliferations are present (Fig. 5D). They likely both belong to the alar plate, since (dorsal) sensory and (ventral) motor areas of rostral medullary levels are rather well delimited by the two descending giant axons of the Mauthner cells (Fig. 5E) and the underlying ventral rhombencephalic commissure (arrow in Fig. 5D) in the adult zebrafish brain (Wullimann et al. 1996). In contrast, the basal plate proliferation is patchy throughout rhombencephalic levels at day 5, as is the more caudal alar plate proliferation. An obvious rhombomeric organization is neither evident in the arrangement of alar nor of basal plate proliferation zones at this age (compare also with Figs. 2C, 6C).

Discussion

PCNA as a marker for proliferation

The proliferating cell nuclear antigen (PCNA) is an auxiliary protein of the DNA-polymerase-δ (pol δ; Mathews et al. 1984) and functions as a sliding clamp in concert with pol δ and replication factor C (RFC) in the elongation process of eukaryotic DNA replication, probably of both leading and lagging DNA strands (Tsurimoto 1998). Absence of PCNA greatly limits the potential of pol δ to synthesize long DNA strands (Tsurimoto 1998). PCNA represents a reliable marker for cycling cells versus long-term quiescent (i.e., postmitotic) cells (Waseem and Lane 1990). A summary of the history of the recognition and description of this protein, its functional role and the production of antibodies against PCNA has been given in an earlier paper (Wullimann and Puelles 1999) and will not be further addressed here. Also, questions of specificity and of how the results gained with the PCNA-antibody compare with studies using other proliferation markers has been dealt with in the said paper rather extensively (Wullimann and Puelles 1999) and these elaborations pertain to the data presented here equally. The specificity of the monoclonal PCNA-antibody used here has been described by Waseem and Lane (1990). The antibody has been applied to detect PCNA in several mammalian species in addition to our previous studies in the zebrafish, *Danio rerio*. Our controls consisted of parallel runs – one using the antibody, the other one using PBS for incubation instead of the primary antibody with the zebrafish tissue – and were intended to reveal reactivity unrelated to the PCNA-antibody. Control sections showed absolutely no reactivity within the central nervous system. Naturally, the intrinsic color of the eye pigment layer and of additional pigmented structures within the meninges (e.g. on top and at the base of the brain) is visible in the control sections. Such pigment is indicated by arrows in some of the photographs (Figs. 4B; 5A) showing the PCNA results. The degree of reliability of PCNA as a marker for proliferation has been discussed extensively elsewhere (Wullimann and Puelles 1999). Meanwhile, our own results with BrdU as a mitotic marker in the early zebrafish brain confirm furthermore qualitatively the proliferation pattern revealed by the PCNA data, since a highly similar proliferation pattern is seen in the BrdU preparations (Th. Müller, M.F. Wullimann, unpublished results).

However, we may also have labelled recently postmitotic cells, since in cells leaving the mitotic cell cycle, PCNA levels decrease to about 30% only within 24 h (Bravo and Mcdonald-Bravo 1987). Indeed, some postmitotic cells must exist at that age since postmitotic cell clusters forming the early axonal scaffold of tracts have been described in the zebrafish brain at day 1 and earlier (Mendelson 1986a,b; Hannemann and Westerfield 1989; Chitnis and Kuwada 1990; Wilson et al. 1990; Ross et al. 1992). These early postmitotic cell clusters were demonstrated by way of acetylecholinesterase-reactivity and neuronal tracing in the telencephalon, epiphysis and ven-

tral diencephalon (future hypothalamus), in addition to such postmitotic clusters in the dorsal (often called: nucleus of the posterior commissure) and ventral (often called: nucleus of the medial longitudinal fascicle) forebrain-midbrain boundary zone, and in addition to various early postmitotic cell clusters in the hindbrain.

The heuristic value of the neuromeric model

A correct interpretation of the location of proliferative zones critically depends on the concept of the basic vertebrate CNS bauplan one uses. Following the segmental model of Puelles and Rubenstein (1993), it is assumed here that central nervous longitudinal zones (floor, basal, alar and roof plates) extend into the general region of the optic chiasma and, thus, that the anteroposterior axis of the neural tube is considerably deflected in its rostral part in the zebrafish (compare Fig. 3B; see also discussion of related evidence in Wullimann and Puelles 1999). Equally important in the segmental model is that segmental units (neuromeres) are present perpendicular to the anteroposterior axis and that these neuromeres exist not only in the rhombencephalon (rhombomeres) but also in the prosencephalon (prosomeres). A variable number of prosomeres has been suggested both historically and more recently, i.e., between 4 (Figdor and Stern 1993) and 6 (Puelles and Rubenstein 1993; Rubenstein et al. 1994). Irrespective of the correct number of such segmental units, it is of prime importance to take the longitudinal axis proposed in the neuromeric model into consideration in order to interpret adequately the topological transformation of a theoretically straight neural tube during development. For example, the hypothalamus and posterior tuberculum/basal synencephalon are recognized to represent basal (and floor) plate portions of the forebrain neural tube only when this axis is respected. The corresponding forebrain alar and roof plate complements would be represented by the telencephalon/preoptic region and the alar plate portions of the posterior forebrain (i.e., ventral and dorsal thalamus, pretectum), respectively. In contrast, a widely accepted textbook opinion holds that the diencephalon (i,e., hypothalamus, posterior tuberculum, thalamus, epithalamus, pretectum) is a transverse piece of the neural tube (i.e., the diencephalic vesicle) in which the hypothalamus represents the most ventral (i.e., basal) part, and the ventral thalamus, dorsal thalamus and epithalamus are successively more dorsal parts of the diencephalic neural tube in that model. In contrast, in the segmental model, the hypothalamus is considered to represent the basal (ventral) part of the neural tube associated with the dorsally located telencephalon (together representing the anterior forebrain or secondary prosencephalon; i.e., the most rostral piece of the neural tube). Accordingly, the posterior tuberculum/basal synencephalon are considered the basal (ventral) forebrain portions associated with the alar plate portions (i.e., ventral thalamus, dorsal thalamus, pretectum) of the caudally adjoining piece of the neural tube (together forming the posterior forebrain, i.e., anterior parencephalon, posterior parencephalon, and synencephalon). This example shows that it is far from trivial to disclose which CNS bauplan or model one uses when interpreting particular facts, such as the distribution of proliferation centers, since the interpretation of their correct topological position within the neural tube may differ using different models.

How does the forebrain proliferation pattern emerge during the first five days of development?

In the forebrain, the best delineation of proliferation centers in terms of their segmental (prosomeric) domains is seen around day 5 (Wullimann and Puelles 1999). This is in contrast to the situation in the hindbrain, where the distribution of PCNA immunoreactive cells in the more dorsal part of the alar plate proliferation is increasingly becoming arranged into a rhombomeric pattern already between 48 and 72 h, but this pattern is lost between 4 and 5 days. So, there is about a 48 h delay with regard to the appearance of the segmental organization of the neural tube in PCNA preparations between hindbrain and forebrain. Thus, we were able to show with the present report that the well-documented segmental organization of the hindbrain indeed becomes apparent using PCNA as a proliferation marker, although at an earlier stage of development compared to the situation in the forebrain (Wullimann and Puelles 1999). However, it has to be kept in mind that these observed segmental patterns are not causal to a segmental organization of the neural tube, but rather represent a late consequence of it. It is clear, for example, that the rhombomeres as physical entities and developmental units exist before 24 h (Lumsden 1990; Kimmel 1993; Lumsden and Krumlauf 1996) and a similar situation may be present in the forebrain. However, Guthrie (1995) has suggested that the function of the apparent segmentation in the diencephalon might be different from that of the rhombencephalon in that the former might underlie later processes of regionalization and not be related to the early subdivision of the neural plate/neural tube as in the rhombencephalon. The later appearance of a segmental pattern of PCNA-proliferation zones in the posterior forebrain would be consistent with such a hypothesis.

How do mid- and hindbrain proliferation patterns spatiotemporally develop during the first 5 days and what are the major proliferation zones in the zebrafish hindbrain – including midbrain-hindbrain-boundary and cerebellum?

Apart from the rhombomeric proliferative clusters mentioned above, the major proliferation zones in the zebrafish hindbrain include the two composing the alar plate midbrain-hindbrain boundary, i.e., the posterior tectal membrane (ptm) and the rostral cerebellar thickening (rct), which remain active until 5 days and beyond. In addition, a midline and ventral proliferation remains present into postembryonic stages in the cerebellar plate in addi-

tion to a few pially migrated proliferative cells (comparable to an external granular layer). An additional postembryonic alar plate proliferation that also does not appear to be organized segmentally is visible at rostral medullary levels more ventrally between 72 h and 5 days. Also, an anteroposteriorly extended proliferation of the rhombic lip is seen more dorsally beginning with 72 h. The rhombencephalic basal plate never assumes an apparent rhombomeric pattern in the PCNA preparations and proliferation is becoming patchy around 72 h already, which is in line with the earlier exhaustion of its proliferative powers.

It may be of considerable interest for future research that the spatiotemporal changes of proliferation patterns during the early postembryonic development are not uniform throughout the neural tube forming the zebrafish brain, as is evident from the foregoing. The midbrain-hindbrain boundary (mhb) is a case in point, where proliferative activity is maintained long after surrounding areas have become postmitotic. There is a wealth of research going on regarding the inductive potential of this region and its roles as a distinct, singular boundary region, which is currently intensively investigated in molecular genetic terms (Brand 1998; Lun and Brand 1998; Reifers et al. 1998). An outstanding comparative neurobiological problem is related to this topic. While the mhb and its associated mechanisms of proliferation and differentiation are a developmental necessity general to the vertebrate brain in order to establish the difference between the mesencephalic and cerebellar/rhombencephalic brain domains, the piece of neural tissue forming its alar plate portion (ptm, rct) appears to be exhausted with these developmental functions in amniotes. In teleosts (and to lesser degree in other ray-finned fishes) there is a large adult structure (i.e., the valvula cerebelli) in front of the cerebellar commissure (i.e., in the position of the early rostral cerebellar thickening) that lies within the tectal ventricle but, phenotypically, has a cerebellar histology. It is very probable that this structure develops from the rostral cerebellar thickening and its gigantic size in some teleosts (e.g., the African mormyrids: Nieuwenhuys and Nicholson 1969; Meek and Nieuwenhuys 1998) and distinctly different connectivity (and, thus, function) compared with other parts of the cerebellum (Wullimann and Northcutt 1989) is likely to depend developmentally on the long-lasting proliferative activity typical for this region for other ontogenetic reasons. It may be no coincidence that a BrdU study in an adult teleost (the gymnotiform *Apteronotus leptorhynchus*) revealed 75% of mitotic cells to be located in the cerebellum (Zupanc and Horschke 1995). Thus, in dissecting the developmental functions of the mhb in the zebrafish, one has to keep in mind that those functions common to all vertebrates may be mixed with those that lead to a specific teleostean structure that reportedly is an evolutionary novelty (Wullimann and Northcutt 1989, Wullimann 1997).

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft (grant number: Wu 211/1-3). Thanks are due to the remaining members of our workgroup, Katja Ahrens, Elke Rink and Thomas Müller, for providing a fruitful scientific environment during the preparation of this paper. Carolin Pfau is thanked for producing some of the PCNA and Bodian material. Prof. Dr. Dr. Gerhard Roth has generously allowed this study to be carried out in his laboratories. PD Dr. Michael Brand and Prof. Dr. Luis Puelles are thanked for enlightening discussions on the midbrain-hindbrain boundary and more and for critically reading the manuscript. Also, two reviewers are thanked for constructive criticism.

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