Patterning of chick brain vesicles as revealed by peanut agglutinin and cholinesterases

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Summary
Differentiation of individual rhombomeres of the chicken hindbrain directly follows the emergence of primary brain vesicles. Immediately after the constriction of the prosencephalon at HH9, a series of vesicles of decreasing size is established almost simultaneously between HH9 and HH10, including mesencephalon, four preotic (R2–R5) and one postotic (R6/R7) rhombomeres. Thereby, the cranial neural tube is ventrally embedded in a mesodermal PNA-binding matrix that particularly accumulates underneath vesicular constriction sites, as demonstrated for the segregation of the prosencephalon at HH9 and the cerebellar rhombomere R1 from R2 at HH13. The subsequent period of hindbrain differentiation is analyzed by cholinesterase (AChE, BChE) and peanut lectin histochemistry, by the BrdU and the neurite-specific G4 antibodies. Preotically, differentiation of two pairs of rhombomeres (R4+R5, R2+R3) starts in R4, immediately followed by R2. The caudal rhombomeres of both pairs are delayed (R5, R3). Then the postotic rhombomere is subdivided, whereby R7 differentiates before R6. Thus, the development in the direct vicinity of the otic vesicle is delayed (R5, R6). R7 is the last rhombomere that is demarcated caudally.

Based on these findings, we postulate two processes that may regulate rhombomere formation in the chicken embryo: (a) an early rostrocaudal wave establishing the major brain vesicles, (b) a superimposed pairwise segmentation emanating rostrally and caudally from the otic vesicle. The segregation of the cerebellar rhombomere is a late step.

Abbreviations: AChE, acetylcholinesterase (E.C. 3.1.1.7); BChE, butyrylcholinesterase (E.C. 3.1.1.8); BrdU, bromodesoxyuridine; D, diencephalon; E, eye stalk; G4, neurite-specific antigen; M, mesencephalon; NP, neuropore; OV, otic vesicle; PNA, peanut agglutinin; P, prosencephalon; R1, rhombomere No. 1; R, rhombencephalon; T, telencephalon.

Key words: brain segmentation, cerebellum, cranial nerves, mesoderm, rhombomere, neuromere, otic vesicle.

Introduction
‘... deswegen ich denn auch nur kürzlich meine vieljährig gehegte Überzeugung wiederhole: daß das Oberhaupt des Saugetiers aus sechs Wirbelknochen abzuleiten sei. Drei gelten für das Hinterhaupt, als den Schatz des Gehirns einschließend, und die zarten Lebensenden, fein verzweigt, in und über das Ganze und zugleich nach außen hin versendend; drei hinwieder bilden das Vorderhaupt, gegen die Außenwelt sich aufschließend, sie aufnehmend, ergreifend, erfassend.’

The poet and philosopher Goethe articulated his views on a metameric scheme of vertebrate head formation (Goethe, 1820). This so-called ‘Wirbeltheorie des Schädels’ (‘vertebra theory of the skull’) has had a significant impact on developmental biologists of the last two centuries (von Baer, 1828; Balfour, 1878; Gegenbaur, 1887). Orr was the first to introduce the term ‘neuromere’ to indicate a number of symmetrical dorsoventral constrictions of the hindbrain and the diencephalon of the lizard, thereby replacing Kupffer's German term of ‘Medullarfalten’ (Kupffer, 1885). Later on, the prominent transient bulges of the hindbrain were called ‘rhombomeres’ (Gräper, 1913; Vaage, 1969). The neuromeric theory (Kupffer, 1885; Orr, 1887; Neal, 1918; Palmgren, 1921; Bergquist and Källen, 1954; Vaage, 1969; Puelles et al. 1987; for review Kühlenbeck, 1973) suggests that neuromeres are phylogenetic remnants indicating a metameric brain and head organization. Opponents of the metameric head theory have denied any metameric regulation of vertebrate head formation (Kingsbury, 1926; Romer, 1972). Rhombomeres have been even considered as preparational artefacts (see Neal, 1918), irrespective of the fact that they can directly be visualized in the living embryo with a simple microscope. Whereas the original ‘vertebrae theory of the skull’ certainly represented a simplified, idealistic view of the development of the vertebrate head, ‘metameric theories’ have been seriously discussed ever since (see textbooks, e.g. Starck, 1979).

The discovery of somitomeres indicated some degree
of cranial mesoderm segmentation (Meier, 1979; Meier, 1981) and renewed interest in the concept of head segmentation and the question whether rhombomeres represent a manifestation of neural tube segmentation. Interestingly, the spinal cords of lower vertebrates are segmented (amphioxus, Bone, 1960; fish, Hanneman et al. 1988) and those of higher vertebrates are not (see Lumsden and Keynes, 1989). Nevertheless, in all vertebrates, the establishment of segmented motor units is controlled by the segmentation of mesodermal somites (review Keynes and Stern, 1985). However, somitomes in the head seem to be inconsequential for the patterning process (Noden, 1983; Noden, 1984). Instead it has been suggested that the neural crest derived from prepatterned rhombomeres could be responsible for the segmental differentiation of skeletal and muscular components of the hindbrain periphery (Lumsden and Keynes, 1989). They proposed a two-segment repeat scheme of hindbrain organization in which pairs of rhombomeres cooperate to generate the repeating sequence of cranial nerves. Similarities with the segmentation process in invertebrates were pointed out. Reports on the coincidence of expression patterns of homeobox genes with rhombomeric boundaries underline the possible significance of a neuromeric developmental scheme (review see Lewis, 1989).

Therefore, the very first formation of rhombomeric swellings of the early neural tube must be assigned a most significant role in the entire patterning of the future vertebrate head. Using a series of developmental markers, we here describe the development of rhombomeres in the chick embryo between stages HH9 and 22. BrdU injection allows the detection of cells in S-phase with the BrdU antibody. The enzyme butyrylcholinesterase (BChE) has a transient maximum around the last mitotic cycle of neuroblasts (Layer, 1983; Layer and Sporns, 1987; Layer et al. 1987). It precedes the expression of AChE, which accumulates within young postmitotic neuroblasts when they reach the basal surface of the neuroepithelium about 15 h after their final mitotic cycle (Miki and Mizoguti, 1982; Mizoguti and Miki, 1985; Layer and Sporns, 1987). Therefore, AChE has been exploited as a very reliable marker of earliest biochemical differentiation by various authors in different vertebrates (Layer et al. 1987; 1988a; Puelles et al. 1987; Hanneman et al. 1988; Moody and Stein, 1988). Moreover, a technique combining AChE histochemistry with neurite-contrasting with the G4 antibody (Layer et al. 1988b; Rathjen et al. 1987), allows direct correlation of neuritic with biochemical differentiation (Weikert et al. 1990).

In this study, we use PNA to trace sensitively the primary formation of brain vesicles and of rhombomeres. Beginning with the prosencephalon, a rostrocaudal series of brain vesicles is established rapidly. An inductive mechanism from a mesodermal PNA-binding matrix onto the neural tube is discussed. By applying all our markers during periods of maximal rhombomere expression (HH17–HH19), we are able to resolve a complex pattern of differentiation within the hindbrain of the chicken embryo, which follows the primary establishment of vesicles. Rhombomeres R2–R7 can be subdivided into two preotic and a postotic pair, whereby differentiation of R4/R5 slightly precedes that of R2/R3 and the postotic pair R6/R7. Rhombomere R1 contributing to the cerebellum has a delayed development. Therefore we postulate that the second phase of rhombomere development must originate at the rostrocaudal level of the otic vesicle, assigning it a regulatory role in hindbrain differentiation.

### Materials and methods

#### (1) Preparation of frozen sections

White Leghorn chicken embryos were washed and then fixed in 4% PBS–formalin for 16 h at 4°C. They were staged according to Hamburger and Hamilton (1951). Embryos were soaked in 25% sucrose-PBS for 16 h at 4°C. Frozen sections were cut at 10–15 μm thickness in frontal (horizontal) and sagittal directions. The following reactions have been performed on frozen sections according to previously described procedures.

#### (2) Cholinesterase staining

Cholinesterase histochemistry was performed using a modified Karnovsky–Roots technique (Karnovsky and Roots, 1964; Layer, 1983; Layer and Sporns, 1987). Selective staining of AChE was performed by incubation of sections for 3 h at 37°C in the presence of 2.56 mM acetylthiocholine iodide (ATC) as substrate, plus 10−7 M iso-OMPA to inhibit BChE. Alternatively, to stain for BChE activity, incubation was performed 6 h at 37°C plus 10–15 h at room temperature in the presence of 3.1 mM butyrylthiocholine (BTC), plus 5×10−5 M BW 284C51 to inhibit AChE. These concentrations of iso-OMPA and BW 284C51 have been shown to fully and selectively inhibit BChE and AChE, respectively (Layer, 1983). In order to diminish the diffusion of the reaction precipitate, some sections were stained in a reaction solution that had been thoroughly mixed with a 30% solution of polyvinylalcohol (Polyviol G04/140, Wacker Chemie, Burghausen, FRG). The final substrate concentration was 3 mM both for ATC and for BTC; otherwise procedure was according to Kugler (Kugler, 1987). The original Karnovsky/Roots protocol is called ‘K&R’, the latter protocol is called ‘polyviol’.

#### (3) Staining with PNA-lectin

PNA histochemistry was essentially performed as described (Liu et al. 1983; Layer et al. 1988b). Frozen sections were washed overnight in PBS at 4°C. Then they were incubated in 50 μg/ml−1 HRP-coupled peanut lectin (Sigma) in a moist chamber at room temperature. The HRP was detected by the chloronaphthol reaction (50 ml TBS, 10 ml chloronaphthol of a 3 mg ml−1 methanol-solution, 20 μl 30% H2O2, reaction time 5–10 min at room temperature; according to Hawkes et al. 1982). This procedure is called ‘sucrose/chloro’. Control experiments were performed by preincubating parallel sections with 10 mM, with 50 mM (final concentration) D-(+)-galactose (Merck), and with 10 mM 1-O-methyl-D-galactopyranoside (Sigma) for 15 min, respectively, before incubating them in a mixture of the hapten sugar plus HRP-PNA lectin at the indicated final concentrations at room temperature. All sections were washed threefold in PBS and once more in TBS (5 min each), before the chloronaphthol reaction followed as described above. PNA binding was fully blocked under all
these control conditions, as shown for 10 mM D-galactose in
Fig. 7 (lower).
Following an alternative procedure (called 'acryl/poly'), we
have embedded the fixed specimens in a polyacrylamide gel as
described previously (Layer and Kotz, 1983). Frozen sections
were prepared as described, washed in PBS for 15 min, and
incubated in HRP-PNA as described above. In order to
decrease the background staining during longer incubation
periods, the chloronaphthol reaction was performed by over-
laying the sections with a mixture of 4.5 ml 30 % Polyviol
GO4/140 (Wacker Chemie, Burghausen, FRG) in 0.1 M Hepes
buffer, pH 7.0, plus 1 ml chloronaphthol as above, plus 20 µl
3 % H2O2. Reaction time was 4 h at room temperature
(Figs 1-4, 9).
(4) Staining with G4 antibodies
To detect neurite growth in the tissues, a separate staining
procedure with G4 antibodies, and a combined procedure of
AChE histochemistry and G4 immunohistochemistry has
been described previously (Layer et al. 1988; Weikert et al.
1990). G4-staining was performed as follows. Sections were
washed in PBS plus 0.1 % BSA for 10 min, followed by
incubation with 10-30 µg ml⁻¹ of either monoclonal or poly-
clonal antibodies to G4 (Rathjen et al. 1987) for 1 h in a moist
chamber. After appropriate washes in PBS/BSA, binding of
antibodies was monitored with an HRP-coupled rabbit anti-
mouse IgG or with goat anti-rabbit (both Dianova) and
detection of the HRP-group as described above. Alterna-
tively, an RITC-coupled second antibody was used.
(5) BrdU-injection
250 µl of a 10 mg ml⁻¹ solution of BrdU (5-bromo-desoxyuri-
dine, SERVA) in N2-medium was injected onto the choioal-
lantoic membrane of an embryo of the appropriate stage.
After 45-90 min, the embryos were isolated, washed in F12-
medium and fixed overnight in 4 % formalin at 4°C. After
three washes in PBS, the embryos were soaked overnight in
25 % sucrose and frozen sections were cut at 14-16 µm
thickness.
(6) Staining with BrdU antibody
The sections were washed 10 min in PBS and then treated for
30 min with 2 N HCl at 37°C, followed by two washes (5 min
each) with 0.1 M sodium borate (pH 8.5), two 5 min washes in
PBS, 10 min in PBS plus 5 % rabbit serum. Then the BrdU
monoclonal antibody (Bio-Science Products AG) at a 1:10
dilution in PBS was applied for 50 min at 37°C. The sections
were washed three times (5 min each) in PBS and 5 min in
PBS/rabbit serum, followed by a 30 min incubation with a
rabbit anti-mouse-RITC second antibody at a 1:30 dilution in
PBS. After three washes in PBS (5 min each), the sections
were mounted in glycerol/gelatine. Trypsinization before acid
treatment was omitted, because the preservation of the
sections was much superior and detection of BrdU was still
satisfactory.
(7) Microscopy and microphotography
Stained sections were observed under a ZEISS 'Axiophot' microsco-
pe using DIC-Nomarski, light-field, or fluorescence
optics. Kodak Ektachrome DX160 color, Ilford HP5 and Agfa
PanF films were used.

Results

1. Primary rostrocaudal formation of brain vesicles
   and mesodermal PNA matrix
Shortly after the invagination of the neuroectoderm,
the neural tube starts to form brain vesicles including rhombomeres of the hindbrain. The period from the first onset of brain vesicle formation until the time of maximal expression of rhombomeres within the hindbrain spans the time from stage HH9 to stage HH18 (1–3.5 days; 5–34 somites). Since the borders of rhombomeres at later stages are clearly demarcated by the binding of the PNA-lectin (see Figs 5–7), we have attempted to correlate the earliest morphological formation of brain vesicles with the expression of PNA-binding proteins within the neuroectoderm and its mesodermal environment.

Stage HH9; the first brain vesicle emerges
The first sign of a bulging of the neural tube is detectable around stage HH9. Thus at a stage when the neural tube at its frontal end is still open (NP, neuropore, Fig. 1), a rostral constriction of the neuroepithelium indicates the formation of the prosencephalon (P). In Fig. 1, a series of parallel longitudinal frozen sections of an HH9 embryo has been stained by an HRP–PNA conjugate. As well as strong staining to the internal and external surfaces of the neural tube, PNA labels a mesenchymal cellular matrix that accumulates preferentially underneath the neural tube. The label decreases dorsally and more caudally. In the most dorsal section (Fig. 1D), PNA binding in the mesoderm is restricted to a perpendicular strip coinciding with the primary prosencephalic constriction site (arrows). Thus, the newly forming prosencephalon is supported by a mesodermal wedge-shaped ridge of PNA-binding matrix.

Stage HH10; rapid emergence of the major vesicles
Immediately following the segregation of the telencephalon, most preotic brain vesicles form in a rapid sequence. At stage HH11 (Fig. 2), a clear distinction can be made between prosencephalic (P; including the eye stalk, E), mesencephalic (M) and hindbrain anlage. The otic vesicle (O) marks the caudal end of the brain vesicles. At this stage, the hindbrain consists of four preotic rhombomeres, which we term R2–R5. This numbering system (Kupffer, 1885) allows us to retain the same rhombomere numbers throughout development of the hindbrain; accordingly, the cerebellar rhombomere forming later will be called R1 (see below). Since the formation of brain vesicles starts with the prosencephalon (Fig. 1) and the vesicle size de-

Fig. 2. Rapid establishment of major brain vesicles. PNA binding to longitudinal sections (A–C, ventral to dorsal) of a HH11 head is shown. A clear distinction between prosencephalic (P), mesencephalic (M) and hindbrain anlage is depicted. The hindbrain consists of four preotic rhombomeres (2–5). Note decreasing size of brain vesicles. O, position of otic vesicle; E, eye stalk. Note patchy accumulation of PNA binding along the mesenchymal surface of the neural tube, with an increase to more ventral positions; at some positions, patchy PNA binding extends into the nervous tissue (e.g. diencephalon in A). Materials and methods as in Fig. 1. Bar=250 µm.
creases sequentially from mesencephalon to the 5th rhombomere (Fig. 2C), the early formation of vesicles may be governed by a rostrocaudal gradient. However, we have failed so far to resolve unequivocally the exact sequence of establishment of the vesicles caudally to the prosencephalon and rostrally to the ear vesicle. By HH11, PNA-binding material still embeds the ventral surface of the neural tube. However, the pattern within the neural tube, the mesoderm and the ectoderm has become more structured, e.g. the lens placode and the base of the otic vesicle are heavily labelled (Fig. 2A), the ventral diencephalon is packed with PNA matrix, and patches of radially oriented staining invade this part of the neuroepithelium. Nonlabelled mesodermal spaces are detectable in all three sections (Fig. 2A–C). Within these spaces, cranial nerve bundles can be seen (see Fig. 9).

Stage HH11–13; indentation of ear vesicle
By stage HH13, the ear vesicle has significantly increased (Figs 3, 5). Its deep invagination into the embryo leads to a direct contact with the neural tube. Now a sixth rhombomere follows right behind the ear. The ear anlage under its ventral and behind its caudal aspects is embedded in PNA-binding material. The four preotic rhombomeres have grown, in particular R3 and R5 have diminished. Note strong PNA binding underneath and caudally to the ear vesicle, as well as to specific mesenchymal and ectodermal locations (near border R2/R3). D, diencephalon; M, mesencephalon. Materials and methods as in Fig. 1.
R5 have increased relatively in size. The constriction of separate rhombomeres is most advanced near the ventral floor part (Fig. 3, left). Dorsally, the hindbrain widens and the borders between rhombomeres fade away (Fig. 3, right). Mesodermal spaces that are unlabelled by PNA coincide with areas of developing facial-acusticus and trigeminal ganglia complexes (Fig. 3, middle, on the left side of R2–R4; see also Fig. 9 for details; Layer and Kaulich, in preparation).

Stage HH13 onwards; PNA-binding foreshadows cerebellar development

Since the degree of vesicular constriction is highest near the ventral floor which is supported by a bed of mesenchymal PNA-binding material, an inducing effect of the mesodermal PNA matrix on the formation of brain vesicles seems possible. This suggestion is supported by events during the segregation of the prosencephalon (see Fig. 1) and of the future cerebellum. The first signs of cerebellar segregation emerge from the ventral surface of the hindbrain (Fig. 4). PNA-staining of a parasagittal section of a HH13+ embryo reveals a bulky mass of PNA binding underneath all brain vesicles. Patchy PNA binding along the ventral neuroepithelium extends from the external surface into the inside of the neural tube (arrow). The morphological constriction of this rhombomere follows later. We could not decipher cerebellar development earlier with any marker other than PNA.

II. Subsequent rhombomeric differentiation as revealed by AChE, BChE, BrdU and PNA labelling

Following the rapid establishment of the major brain vesicles in a rostrocaudal series, further differentiation processes within the hindbrain are complex. As has been analyzed by others (Lumsden and Keynes, 1989), pairs of rhombomeres (R2/R3 and R4/R5) cooperate to contribute to the trigeminal and the facialis-acusticus complex, respectively. The first postmitotic cells as indicated by AChE staining reach the rhombomeric surface first in R4, shortly followed by AChE-positive cells in R2 (Fig. 5; see also Discussion).

The structure of rhombomeres is most prominently expressed in the chicken embryo around stages HH17–19. At this time, the rate of cell production is still significant in all rhombomeres (Figs 6, 7, BrdU). After the short BrdU pulse applied, differences between the different rhombomeres cannot be demonstrated.

By comparing the expression of AChE and of BChE in parallel sections of a HH17 embryo (Fig. 6, AChE, BChE), we can deduce which rhombomeres at this stage are just about to differentiate (BChE) compared with those that already contain a significant number of postmitotic cells (AChE). These data show that at stage HH17 the cerebellar and the trigeminal rhombomeres (R1–R3) are in an actively differentiating state with many cells just leaving their final cycles (high BChE), but they also include many postmitotic cells (AChE-positive cells). R3 is clearly delayed compared with R2.

The highest density of postmitotic cells is found in R4, but production of further postmitotic cells will be very limited, as indicated by the low content of BChE within the facialis-acusticus rhombomeres R4/R5. Both rhombomeres neighboring the ear anlage (R5 and R6) are almost void of postmitotic cells. Noticeably the only AChE-positive cells in R5 and R6 are located near the rhombomeric borders, distant from the otic vesicle. However, the high BChE activity in R6 indicates that their production has just started there. In R7, production of postmitotic cells already has approached its end, shown by high AChE and low BChE. PNA binding in R1 (cerebellum), R3 and in R5 is enhanced (Fig. 6, PNA), correlating with their delayed state of differentiation.

III. Transience of rhombomeres

From HH21 onwards, the longitudinal subdivisions of the hindbrain (rhombomeres) slowly start to disappear.
Fig. 5. Preotic differentiation starts in R4. AChE-stained longitudinal section of a stage HH13 embryo depicts the area from R2–R6. At this stage, several AChE-positive postmitotic neurons have reached the surface of R4. The strong diffuse staining in R2 indicates that R2 will be the next rhombomere to differentiate. Note a few AChE-positive cells at the R5/R6 border (arrows, pointing also to the position of the otic vesicle), otherwise the neuroepithelial vicinity of the otic vesicle is void of postmitotic cells. Procedure 'K&R', see Materials and methods. Bar=100 μm.

Fig. 6. Comparative patterns of rhombomere differentiation. Parallel sections of a HH17 embryo have been stained for AChE-detecting postmitotic neurones, for BChE-detecting neurones during their last mitotic cycle, with the BrdU-antibody detecting cells in S-phase, and with the PNA lectin demarcating neuroepithelial radial extensions including rhombomeric borders. AChE is negative in R5/R6 and low in R3, which indicates their retarded development. BChE staining is high in R1–R3 and R6, indicating that many cells are about to differentiate. Based on a high AChE/BChE ratio, R7 is further advanced than R6. PNA binding is strong at rhombomeric borders. Note that in R1 (cerebellum), R3 and in R5 the strength of PNA-binding fibers is enhanced, correlating with their delayed differentiation. BrdU uptake into S-phase cells is achieved into semicircular units of cell bodies, which reveal deep furrows of cell bodies at the rhombomeric borders (compare Nom. with BrdU). Procedures: PNA, sucrose/chloro, AChE, BChE, polyviol, further details see Materials and methods. Bar=200 μm. R, rostral; C, caudal.
Fig. 7. Structure of rhombomeres. Postmitotic, AChE-producing cells are located on the surface of rhombomeres, reaching far inside the sulci (upper left; DIC-optics). G4-positive neurites originate from these AChE-positive cells (same section of a HH17 embryo is shown in RITC-G4 fluorescence lower left; note two prominent dark AChE cell bodies in fluorescence. HRP-PNA lectin (upper right) binds to a network of radial extensions within the neuroepithelium including their apical and basal sides. PNA labelling at rhombomere borders is particularly strong (upper right, see text). BrdU antibody demonstrates the semicircular arrangement of S-phase cell bodies (lower right). PNA and BrdU are from a HH19 embryo. Note that PNA binding is fully blocked in the presence of 10 mM d-galactose (right of two lower frames, parallel sections of a HH17 hindbrain are shown). Procedures: AChE, K&R, PNA, sucrose/chloro, for further details see Materials and methods. Bar=50 μm.

(Fig. 8). Their pronounced circular shape has vanished and concomitantly the transversal PNA borders have become weaker. Noticeably, a new longitudinal (laminar) band has evolved, which divides the neural tube into a marginal and an internal zone (see Liu and Layer, 1984). Similarly, the distinctive rhombomeric pattern as revealed at earlier stages by the BChE activity has faded.

Discussion

Spatial subdivisions by PNA matrices
In this study, we have detected PNA matrices that delineate spatial subdivisions of the neural tube in both its longitudinal and transverse dimension and the surrounding mesodermal space (Figs 1–4, 6–9). The peanut lectin (PNA) has been shown to bind specifically to
Fig. 8. Transience of rhombomeres. PNA binding to semisagittal sections of a stage HH21 rhombencephalon (upper). Note that the pronounced circular shape of rhombomeres has vanished, transversal PNA borders have become weaker, but a new longitudinal (laminar) band has evolved caudally to Rl. Lower pictures demonstrate the decrease of BChE activity along the hindbrain at stage 21. Dorsal side is oriented upwards. Procedures: PNA, sucrose/chloro, BChE, polyviol, further details see Materials and methods. Bar=200 μm.

Functions for PNA-binding glycoproteins?
During emergence of PNA binding to brain vesicles and rhombomeres, four findings deserve further attention: (a) before any vesicles form, the neural tube is embedded ventrally in a massive PNA-binding matrix of mesodermal origin, (b) PNA borders are more diffusely distributed before forming concentrated radial alignments (see cerebellum, Fig. 4), (c) during formation of borders, PNA binding is more concentrated along the outside surface of the neural tube. Often, there exist direct connections with the PNA-labelled matrix of the neighboring mesoderm (Figs 2, 3; see also Liu and Layer, 1984); further experiments have to show whether the mesodermal PNA-binding matrix exerts an inductive effect for the early subdivision of the neural tube, and thus offer a molecular basis for the interaction between mesoderm and folding brain vesicles, which has been reported before (Jacobson and Tam, 1982); and finally (d) along with further differentiation, the exit and the mesenchymal course of cranial nerves remain void of PNA matrix. In contrast, ventral areas of the tube without cranial nerve connections are embedded in PNA matrix (at R1, R3, R5 and R6 of Fig. 9). Given the patterns of expression of PNA-binding molecules in the head (this study) and the trunk (Stern et al. 1986; Layer et al. 1988b; Oakley and Tosney, 1989), their functional involvement in morphogenesis seems likely (as proposed in Liu and Layer, 1984; 1988). Interestingly, a number of PNA-binding glycoproteins have been partially characterized that show an inhibitory action on neurite growth (Liu and Layer, 1988; J. Raper and F. Bonhoeffer, personal communications; Davies et al. 1989).

Scheme of brain vesicle and rhombomere formation
The semicircular organisation of rhombomeres as separate proliferating and differentiating cellular compounds ('proliferation centers'; Källen, 1956) is revealed by patterns of AChE-positive neuroblasts, their corresponding G4-positive axons, and cell bodies of BrdU-labelled S-phase cells that all reach deep into the gyri of the rhombomeres, whereas the borders between rhombomeres are particularly well stained by PNA (Figs 6, 7). Studies at the electron-microscopic level will further clarify the exact course of the PNA-labelled processes at the interrhombomeric borders. Apically, PNA binding is restricted to the base area of the rhombomere, from which the radial processes emanate. As has been suggested for mammals (Tuckett and Morris-Kay, 1985), blocks of microtubules between sulci and microfilament-rich luminal borders could contribute to the bulging process.

Using this series of differentiation markers, we were able to resolve the spatiotemporal emergence of brain vesicles and the sequence of rhombomere formation. All numbers from five to eight rhombomeres have been reported for birds (Gräper, 1913), with an exceptional 13 rhombomeres described for the seagull (Meck, 1907). Based on PNA, AChE, BChE and BrdU labelling, we could detect seven rhombomeres for the chick, including R1 representing the cerebellar precursor,
Fig. 9. PNA subdivisions inside and outside of the hindbrain. PNA binding to a parasagittal section of a HH19 hindbrain is shown. Borders of rhombomeres are clearly demarcated by radial PNA binding. The ventral exit and the mesenchymal course (arrows) of cranial nerves are void of PNA binding. V, trigeminal nerve; VII, VIII, acustico-facialis complex, IX, glossopharyngeal nerve. Procedure: PNA, acryl/poly, further details see Materials and methods. Bar=250 μm.

R2–R5 as further preotic and R6 and R7 as postotic rhombomeres. The last border detectable by PNA is that caudally to R7 (Fig. 6). Our scheme confirms that of Kupffer (1885, 1906). Vaage defines eight rhombomeres, which may be due to the fact that he conceives the mesencephalic–rhombencephalic isthmus area as a separate rhombomere (Vaage, 1969). Recently, the rostral limit of the cerebellum has been shown to be located within the caudal mesencephalon (Martinez and Alvarado-Mallart, 1989). On the basis of PNA binding (Fig. 4), we could detect the caudal limit of the cerebellum earlier than by any other means.

The very early formation of vesicles has turned out to be such a rapid process that we have not been able to unequivocally resolve the establishment of the first four preotic rhombomeres (R2–R5). We could not observe a caudorostral gradient as suggested in some of the early literature (reviewed in Graper, 1913; see also Vaage, 1969; Tuckett et al. 1985). Rather, based on the decreasing size of R2 through R5, we postulate a primary rostrocaudal wave of brain vesicle formation. We have analyzed the following steps of rhombomeric differentiation using cholinesterase histochemistry. Thus, the pairwise differentiation of trigeminal (R2/R3) and the facio–acusticus rhombomeres (R4/R5), as demonstrated before (Lumsden and Keynes, 1989; see also Zimmermann, 1891) has been further resolved. Both pairs start to differentiate almost simultaneously. Based on Fig. 5 and on computer reconstructions, we were able to determine the very first cell group expressing AChE to be located in R4, shortly followed by cells in R2 (Layer et al. 1988a, Weikert et al. 1990). Therefore, R4/R5 (facio–acusticus complex) develops before R2/R3. Although R6 is morphologically discernible around stage HH12 (Figs 3, 5) and produces motor cell bodies of the IXth nerve before HH16 (Lumsden and Keynes, 1989; note also a few individual AChE-positive cells in R6 of Fig. 5), the late expression of AChE and BChE in R6 (Figs 5, 6) demonstrates that its major period of differentiation is delayed, following that of R7. Thus, both otic rhombomeres are very much retarded.

In summary, we have described the expression of a PNA-binding matrix along the ventral surface of the hindbrain, accompanying the spatial subdivision of the cranial neural tube. These observations now must lead to experiments showing, whether a prepattern of mesodermal PNA matrix is functionally involved in the formation of rhombomeres. In order to account for the complex sequence of rhombomere differentiation as revealed in here, we postulate that it may be governed by (a) a rostrocaudal wave of vesicle formation and by (b) a superimposed pairwise segmentation process emanating rostrally and caudally from the otic vesicle. The segregation of the cerebellar rhombomere R1 from the more caudal parts of the hindbrain is a late process.

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References


PNA binding, cholinesterases and chick brain vesicles


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