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Successive patterns of clonal cell dispersion in relation to neuromeric subdivision in the mouse neuroepithelium

Luc Mathis[1,*], Johan Sieur[1], Octavian Voiculescu[2], Patrick Charnay[2] and Jean-François Nicolas[1,‡]

[1]Unité de Biologie moléculaire du Développement, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France
[2]Unité INSERM 368, Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Paris Cedex 05, France
*Present address: Beckman Institute (139-74), California Institute of Technology, Pasadena, CA, 91125, USA
‡Author for correspondence (e-mail: jfnicola@pasteur.fr)

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SUMMARY

We made use of the *laacz* procedure of single-cell labelling to visualize clones labelled before neuromere formation, in 12.5-day mouse embryos. This allowed us to deduce two successive phases of cell dispersion in the formation of the rhombencephalon: an initial anterior-posterior (AP) cell dispersion, followed by an asymmetrical dorsoventral (DV) cell distribution during which AP cell dispersion occurs in territories smaller than one rhombomere. We conclude that the general arrest of AP cell dispersion precedes the onset of morphological segmentation and is not imposed by the interface between adjacent rhombomeres. This demonstrates a major change in the mode of epithelial growth that precedes or accompanies the formation of neuromeres. We also deduced that the period of DV cell dispersion in the neuroepithelium is followed by a coherent growth phase. These results suggest a cell organization on a Cartesian grid, the coordinates of which correspond to the AP and DV axis of the neural tube. A similar sequence of AP cell dispersion followed by an arrest of AP cell dispersion, a preferential DV cell dispersion and then by a coherent neuroepithelial growth, is also observed in the spinal cord and mesencephalon. This demonstrates that a similar cascade of cell events occurs in these different domains of the CNS. In the prosencephalon, differences in spatial constraints may explain the variability in the orientation of cell clusters. Genetic and clonal patterning in the AP and DV dimensions follow the same spatial sequence. An interesting possibility is that these successive patterns of cell growth facilitate the acquisition of positional information.

Key words: Cell lineage, Central nervous system, Clonal analysis, Compartment, *laacZ*, Mouse embryo, Myelomere, Neuromere, Rhombomere, Segmentation

INTRODUCTION

The central nervous system (CNS) in vertebrates develops from the neural plate that extends along the anteroposterior (AP) and dorsoventral (DV) axes of the embryo to form the neural tube. The neural tube is subdivided into broad AP domains, which contain the precursor cells for the forebrain, midbrain and hindbrain, and posteriorly, for the spinal cord. These domains are subsequently further subdivided into transverse domains, termed neuromeres (Lumsden and Krumlauf, 1996), which eventually become organised along the DV axis (Tanabe and Jessell, 1996). If there is little doubt that most features of genetic patterning of the CNS are conserved between species, it is not evident that this is true for cellular patterning. In particular, cell organization before gastrulation and cell movements during gastrulation, at the time of the delimitation of the neural plate in the disc-shaped cellular blastoderm of the chick and in the egg cylinder of the mouse, may well be different due to different topological constraints. In fact, conservation of the cellular patterning of the CNS between species at a given stage of development would suggest a coordination between genetic and cellular organization of the neuroepithelium. Non-conservation would suggest that such a co-ordination is unnecessary at the stages observed and would illustrate the potential variety of strategies in different organisms. The comparison of cell behaviour between species during development will therefore enrich our understanding of embryogenesis.

Whereas our knowledge of the genetic basis of neuromere formation mainly comes from experiments performed in the mouse, most of cell lineage analyses of neuromere formation have been carried out in the chick embryo and it is unknown whether similar rules underlie neuromere development in mammals. It is not possible to experimentally manipulate the mouse embryo as has been done in the chick and we have therefore used a genetic method of cell labelling and retrospective analysis to examine this problem. The method is based on the generation of a functional *lacZ* reporter gene during normal development of transgenic mice carrying an inactivated *laacz* transgene, through a random intragenic recombination event that re-establishes the open reading frame of the reporter gene (Bonnerot and Nicolas, 1993; Nicolas et al., 1996). The recombined *lacZ* gene is then clonally transmitted to the descendants of the labelled cells. To study

clonal relationships in the central nervous system, the *laacz* gene has been placed under transcriptional control of the neuron-specific enolase (NSE) promoter (Forss-Petter et al., 1990; Mathis et al., 1997). The low frequency of recombination events (Bonnerot and Nicolas, 1993) results in the labelling of individual cells during development, and permits the analysis of clones derived from such labelled cells. These characteristics permit the visualisation, a long time after their birth, of clones labelled prior to the subdivision of the neural tube into neuromeres as well as after this subdivision. NSE-*laacZ* is expressed in most neurons in the embryonic and adult CNS (Forss-Petter et al., 1990; Mathis et al., 1997) and in the cells of the ventricular zone of the embryonic CNS at embryonic day 12.5 (E12.5) (this article), enabling us to study clonal cell behavior during the development of the CNS. These clones have been used to analyze the patterns of cell dispersion in the rhombencephalon, the spinal cord and more anterior domains of the CNS. We have depicted clones at a relatively late developmental stage, at which AP and DV genetic organizations are set up in all CNS domains. We chose to analyze embryos at E12.5 because this stage corresponds to the time at which the production of neurons begins at almost all levels of the CNS, from the prosencephalon to the spinal chord (Altman and Bayer, 1995); at this stage, the basic organization of the neuroepithelium must therefore be in place. In addition, many developmental genes (reviewed in Rubenstein et al., 1998) are already expressed at E12.5 and AP and DV patterning of the CNS are at least partially established.

We show that, as in the chick, there is a constraint acting on longitudinal cell dispersion already evident at the time of neuromere formation. In addition, we find that there is a general arrest of AP cell dispersion that precedes the formation

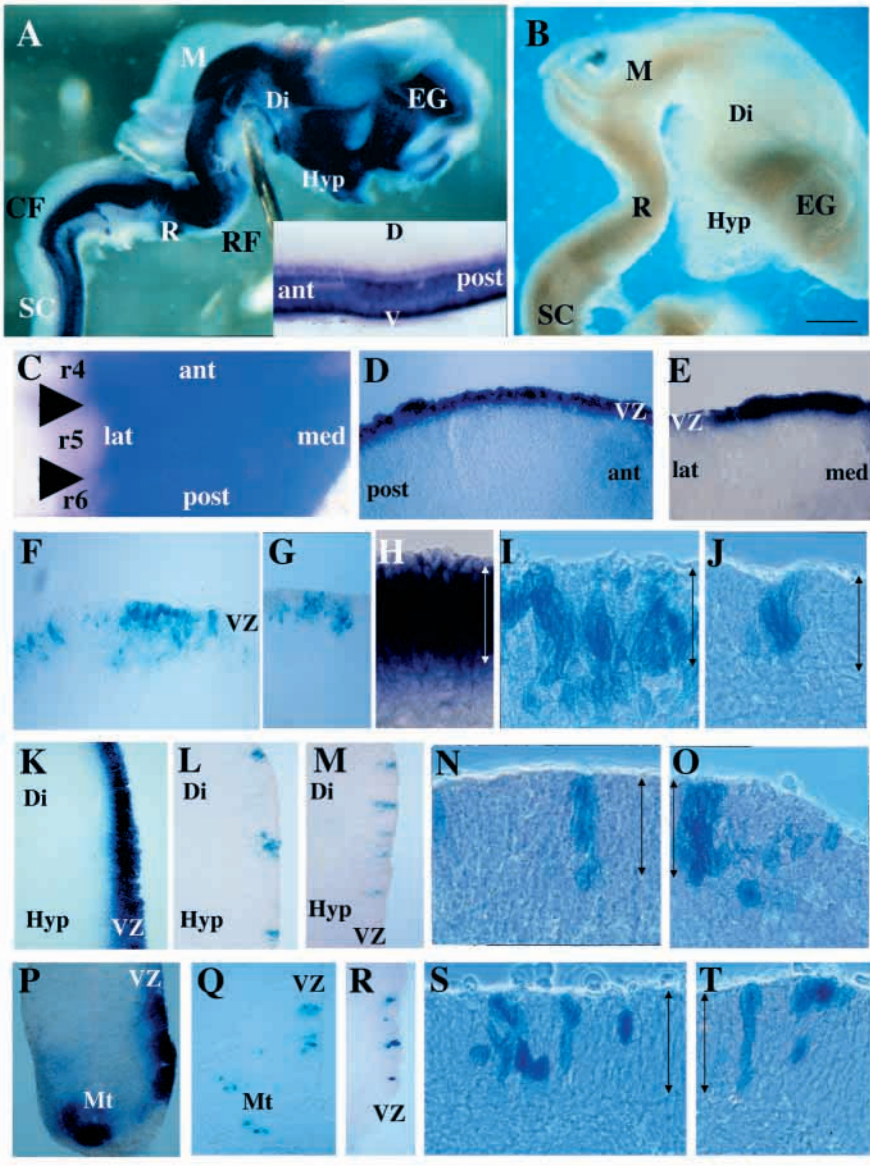

Fig. 1. Expression pattern of *laacZ* in the NSE-1 mouse transgenic line. (A-E) In situ hybridisation with a digoxigenin-labelled *lacZ* RNA probe. (A) Medial view of a whole-mount CNS of a NSE-*laacZ* E12.5 embryo. The surface exposed corresponds to the ventricular zone (VZ). Inset, higher magnification of the spinal cord. (B) Control, a non-transgenic embryo. (C) Dorsal view of the rhombencephalon from r4 to r6 showing the labelling in VZ. The r5 anterior and posterior inter-rhombomeric interfaces are indicated by the arrowheads. (D) Longitudinal section in the rhombencephalon from the cephalic (posterior) to the rhombic (anterior) flexures. Magnification is ×20. (E) Transverse section in the rhombencephalon ×20. (F,G) Transverse sections in the rhombencephalon of clone 95-534, (F) and of clone 94-906, (G) ×20. (H) Transverse section in the rhombencephalon, *laacZ* transgene expression detected by in situ hybridisation, ×100. The double arrow shows the extension of the neuroepithelium. (I,J) Transverse sections in the rhombencephalon of clone 95-534 (I) and 94-906 (J), ×100. Most β-gal+ cells are clustered in the neuroepithelium and a few cells are located in the underlying differentiating zone. (K) Transverse section in the prosencephalon; the *laacZ* transgene expression detected by in situ hybridisation is mainly restricted to the VZ, ×20. (L,M) Transverse sections in the prosencephalon of clone 94-906 (L) and 95-534 (M) ×20. (N,O) Higher magnification of the same clones, ×100. Most β-gal+ cells are in the neuroepithelium. (P) Transverse section in the spinal cord; the expression of the *laacZ* transgene detected by in situ hybridisation is in the VZ and in a ventrolateral position (area of the motor neurons), ×20. (Q,R) Transverse sections in the spinal cord of clone 95-208 (Q) and 95-928 (R) at ×20. (S,T) Higher magnification of the VZ in clone 95-208, ×100. ant, anterior; post, posterior; med, medial; lat, lateral; V, ventral; D, dorsal; CF, cephalic flexure; Di, diencephalon; EG, ganglionic eminencies (telencephalon); Hyp, hypothalamus; M, mesencephalon; Mt, motoneurons; P, prosencephalon; R, rhombencephalon; RF, rhombic flexure; SC, spinal cord; VZ, ventricular zone. Scale bar, A,B, 500 μm; C, 130 μm; D-G, K-M, P-R, 100 μm; H-I, N-O, S,T, 20 μm.

of neuromeres. This initial arrest of AP cell dispersion appears to affect most cells within neuromeres and has been found at all axial levels of the neural tube. Furthermore, our results show that a transition from AP to DV cell dispersion precedes the final coherent growth of neuroepithelial cells. Our results thus reveal a cascade of cellular events and specific arrests of cell dispersion underlying the development of the entire neural tube.

MATERIALS AND METHODS

Whole-mount in situ hybridisation

Whole-mount in situ hybridisations were performed on transgenic NSE-*laacz* and wild-type C57BL/6×DBA/2 E12.5 embryos as described (Mathis and Nicolas, 1998). The probe used for the detection of *laacZ* expression was a digoxigenin-labelled *lacZ* antisense RNA. Before hybridisation, the neural tube was bilateraly sectioned and dissected by removing the skin and the mesenchyme after fixation and methanol dehydration, to allow full access of probe to the tissue. The telencephalic vesicles were removed to avoid the appearance of background activity in this area.

Production and analysis of β-gal$^+$ clones

The NSE-1 NSE-*laacz* transgenic line has been described previously (Mathis et al., 1997). β-gal$^+$ clones were produced from 10 homozygous males. The progeny of the crosses between these males and wild-type C57BL/6×DBA/2 females was analysed at E12.5. The morning of copulation is taken as E0.3. After 30 minutes of fixation in 4% PFA, the E12.5 transgenic embryos were incubated in X-gal for 2 days at 30°C and then for 1 week at 4°C to allow full access of X-gal to the cells, and stronger labelling of individual cells. The embryos were then cleared for 48 hours in 70% glycerol in PBS (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.3) and bilaterally sectioned. The screen for embryos with β-gal$^+$ cells was performed by observation of the embryos at ×60. The description of the distribution and number of β-gal$^+$ cells in the clones was performed after complete dissection of the neural tube from skin and mesenchyme. Parts of the neural tubes to be sectioned were transferred into PBS containing 30% (w/v) sucrose and soaked for 12 hours. 14-30 μm cryostat sections were then cut and mounted in Mowiol The neuroepithelium in unstained sections was visualised at ×100 using phase-contrast optics.

Identification of the neuromeres at E12.5

In the spinal cord, the neuromeres were visualised by the roots of peripheral nerves, which were preserved during the dissection of the neural tubes. Each myelomere has an AP dimension of about 200 μm. Neuromeres in the rhombencephalon were visualised by two methods. In a first procedure, mice containing the Cre recombinase coding sequence inserted by knock-in into the *Krox-20* locus (O. V., S. Schneider-Maunoury and P. C., unpublished data) were crossed with mice bearing a *lacZ* transgene, which can be activated upon excision of a floxed sequence (Akagi et al., 1997). In the second method, a 0.5% (w/v) solution of Toluidine blue in water was injected into the third ventricle of E12.5 mouse neural tube fixed in 4% PFA for 1 hour. From this description, we derived a map of r2 to r6 in relation to the rhombic flexure anteriorly and the cervical flexure posteriorly. In the mesencephalon, two neuromeres have been described whose AP dimension is roughly 600 μm. A rough estimate of the surface of the neuromeres in the prosencephalon (0.2 mm^2 each) was obtained according to the prosomeric model (Puelles and Rubenstein, 1993). The surface of the clusters was estimated using NIH-image software after camera-lucida drawing.

Description of the β-gal$^+$ cells in the clones

We have described the distribution of cells in the ventricular zone (VZ) of the CNS. In the spinal cord, mesencephalon and prosencephalon, the VZ is located in the medial neural tube and is dorsoventrally oriented. In these domains, clones have been examined from the medial surface of bilaterally sectioned neural tubes. In the spinal cord, we did not attempt to distinguish between VZ and motor neurons, and serial sections show a correlation between the AP location of cells in these two domains. The axial position of the β-gal$^+$ cells in relation to myelomeres was recorded by camera-lucida drawing, using the roots of peripheral nerves as morphological landmarks. In the rhombencephalon, the ventricular zone is located dorsally in the neural tube and clones have been described from a dorsal view. A few labelled cells occasionally observed in deeper layers of the neural tube have not been taken into account in our description. The axial position of β-gal$^+$ cells in relation to rhombomere boundaries was obtained using our map of the rhombomeres described above. Independent measurements lead to a maximal variation of 25 μm in the determination of the position of the β-gal$^+$ cells in relation to the rhombomeres. Although this indirect method permitted the β-gal$^+$ cells to be positioned with respect to the rhombomeres, the relationship between rhombomere boundaries and the borders of β-gal$^+$ cell clusters are only estimated here. Given the results presented below, this inaccuracy is not a major problem.

RESULTS

Production of clones in the ventricular zone of the embryonic CNS at E12.5

We have used a transgenic line (NSE-1 *laacZ*) expressing the *laacz* reporter gene under transcriptional control of the NSE promoter (Mathis et al., 1997), which confers pan-neuronal expression in the embryonic and mature CNS (Forss-Petter et al., 1990; Mathis et al., 1997). We have analyzed the expression

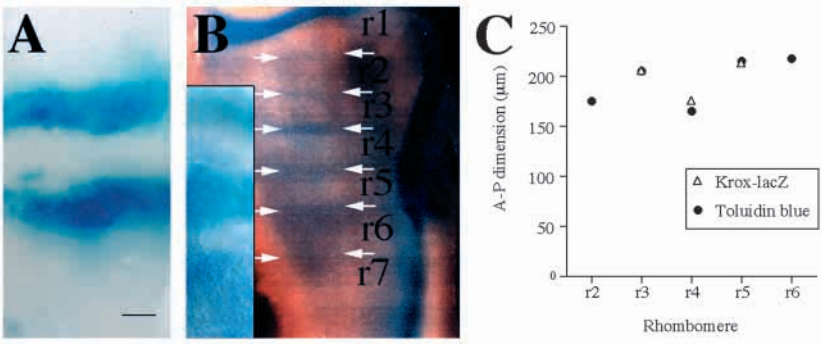

Fig. 2. Identification of the limits of the rhombomeres at E12.5. (A) Dorsal (ventricular) view of *Krox-20-Cre* × *β-actin-lox/lacZ* E12.5 embryos. Labelled cells in r3 and r5 are distributed in two stripes. (B) Identification of five repeated units in the rhombencephalon ventricular zone of E12.5 embryos after injection of Toluidin blue in the neural tube (dorsal view). The boundaries visualised are indicated by white arrows. On the left is shown the result of an injection of Toluidine blue in a *Krox-20 Cre* × *β-actin Lox/LacZ* embryo, demonstrating that the territories of r3, r4 and r5 delimited by both methods superimpose. (C) Size (y axis) of the different rhombomeres (x axis) determined by the two methods. Scale bar, 200 μm.

of *laacZ* in NSE1-*laacZ* mice at E12.5 (Fig. 1), a stage at which the ventricular zone (VZ) and the differentiating zones (DZ) are clearly visible histologically (Altman and Bayer, 1995). Whole-mount in situ hybridizations shows that the *laacz* transgene is strongly expressed along the whole neural tube (Fig. 1). In toto examination of the ventricular surface (Fig. 1A-C) and analysis of histological sections (Fig. 1D,E,H,K,P) show homogeneous expression in the VZ at different axial levels. In particular, transgene expression shows no variation of intensity within and between neuromeres (Fig. 1C,E). The NSE-laacZ transgenic line therefore constitutes an excellent tool with which to observe the development of the neuroepithelium.

The analysis of 3000 NSE-*laacz* transgenic E12.5 embryos yielded 163 embryos with β-galactosidase-positive (β-gal+) cells in the CNS (clones restricted to the telencephalon were not taken into account in this analysis). Histological examination of clones shows labelled cells distributed within the VZ and the DZ (Fig. 1F,G,I,J,L-O,Q-T) as expected from the in situ hybridization.

Identification of the rhombomeres at E12.5

In the mouse, the limits between rhombomeres are only visible from E9 to around E11. To analyze the distribution of β-gal+ cells with respect to rhombomere boundaries at E12.5, we generated a map of the region between the rhombic flexure and the cervical flexure (Fig. 1A). This was obtained using two procedures. Firstly, we made use of a mouse line containing the Cre recombinase gene inserted by knock-in into the *Krox-20* locus (O. V., S. Schneider-Maunoury and P. C., unpublished data). These mice were crossed with transgenic mice bearing *lacZ* under the control of the chicken β-actin promoter, but separated by a floxed transcriptional stop (Akagi et al., 1997). As the *Krox-20* gene is expressed in the rhombomeres 3 and 5 at the onset of segmentation (Wilkinson et al., 1989), the resulting embryos activate *lacZ* in r3 and r5 cells and maintain this expression in their descendants (O. V., unpublished data). At E12.5, the labelled cells were distributed in two transverse stripes separated by a stripe of almost exclusively unlabelled cells (Fig. 2A). This observation demonstrates that very limited cell mixing occurs between adjacent rhombomeres after the onset of *Krox-20* expression at E8. Mouse rhombomeric domains are thus maintained after the disappearance of morphological boundaries, as was previously shown in the chick (Marin and Puelles, 1995; Wingate and Lumsden, 1996). These embryos allowed us to determine that the AP dimension of a rhombomere is about 200 μm at this stage (Fig. 2C). In the second procedure, we identified the territories of the other rhombomeres by Toluidin blue injection into the third ventricle, which revealed six transversal boundaries at the level of the ventricular zone (Fig. 2B). Three of the territories delimited by these boundaries were shown to superimpose with r3, r4 and r5 as defined by the *Krox-20/Cre* line (Fig. 2B).

Clones in the rhombencephalon are organized into DV clusters that identify two main phases of rhombomere formation

In the rhombencephalon, the VZ is located dorsally in the neural tube and appears as a mediolateral sheet. Comparison with the VZ in the rest of the CNS shows that the apparent mediolateral dimension of the rhombencephalic VZ corresponds to its dorsoventral (DV) axis (Kaufman, 1998; Altman and Bayer, 1995). To facilitate comparison with other domains of the CNS, the apparent mediolateral dimension of the VZ has been termed DV.

Table 1. Statistical analysis of clonality

	Clones with one D-V cluster	Clones with several D-V clusters	Expected frequency of large clones following the hypothesis
Frequency	P=19/3000	39/3000	$\frac{P}{1-P} - P$

We have tested whether the number of clones with several labelled D-V clusters (39) equals the expected number of clones with several D-V clusters ($4.04\ 10^{-5}$) following the hypothesis of two or more events of recombination in precursors of D-V clusters in the same embryo. Unilateral test of frequency, z=6.2, $P<10^{-4}$, ns. Therefore, we rejected the hypothesis. When several clusters are observed in the rhombencephalon, they belong to the same clone.

Labelled β-gal$^+$ cells in the rhombencephalon VZ were organized in clusters (Fig. 3). Statistical analysis of the frequency at which positive embryos were obtained demonstrates the clonality of the observed labelling in each embryo (Table 1).

19 clones were obtained comprising a single cluster of cells (Fig. 3D-H). No positive cells outside these clusters were observed in the embryo. This shows that genealogically closely related cells are also geometrically close. These clones presented a preferential dispersion along the DV axis (Figs 3D-H, 4E).

32 clones were observed with a more extensive labelling in the CNS, which were either restricted to the rhombencephalon (13 clones called “restricted, several cluster” containing 26 clusters) or extended into other domains of the CNS (19 clones called “non-restricted” containing 40 clusters). Those clones with different longitudinal extension are likely to be born at different stages, the earliest corresponding to the non-restricted category. Both types of clone, however, were systematically organized into separate DV clusters (Figs 3A-C, 4C,D) demonstrating that the clones are organised into DV clusters independently of their presumed date of birth.

Further analyses of the DV clusters for clones of the three categories (clones with a single cluster, clones with several clusters in the rhombencephalon and clones with extensive labelling in the CNS) indicate that their AP dimension (Fig. 4A) and their cell number (Fig. 4B) are comparable. Therefore, the DV clusters share similar characteristics (DV orientation, AP dimension and cell number) independently of their inferred date of birth, consistent with the notion that DV clusters define subclones within the larger clones (Fig. 5A). In fact, if cells between separate DV clusters were more closely related genealogically than cells within DV clusters, the smallest clones should be longitudinally oriented (Fig. 5B) and this was not observed.

We define the founder of a DV cluster as the first cell in a lineage whose descendants will all populate a single DV cluster. Therefore a clone with two (or more) DV clusters probably derives from genealogically related founder cells that have dispersed longitudinally after the labelling of their common ancestor. In consequence, the clonal arrangement of genealogically closely related cells into separate DV clusters (Fig. 3A-C) suggests two phases in the formation of the

rhombencephalon: an initial phase of longitudinal dispersion and intermingling of the *precursors* of the founders of the DV clusters, followed by a local growth of the *descendants* of the founders to produce the DV clusters. These descendants correspond to neuroepithelial cells arranged in clusters in the VZ and to neurons in the DZ (Fig. 1F-J).

Local growth of the descendants of the founder cells of the DV clusters indicates a general arrest of AP cell dispersion in the VZ before E9

Comparison between the AP and DV extension of the DV clusters shows that intermingling of the descendants of the founder cells is asymmetrical. Whereas the AP dimension of the DV clusters was systematically less than that of one rhombomere (200 μm), their maximal DV dimension was as much as 1 mm, which corresponds to the AP dimension of five rhombomeres. This applies to the three categories of clones already mentioned: those with a large participation in the CNS (Fig. 4C), those restricted to the rhombencephalon (Fig. 4D) and those restricted to a single rhombomere (Fig. 4E), showing that this characteristic is independent of the date of birth of the clones. Therefore, this analysis shows that the dispersion of the descendants of the DV cluster founders is preferentially dorsoventral and is constrained along the AP axis.

This observation raises the question of the origin of the constraint acting on AP cell dispersion. Even though almost nothing is known about cell behavior in the mouse rhombencephalon, we can suppose that the AP cell dispersion may be restricted at the interface between adjacent rhombomeres (as has been described in the chick) or that a general arrest of longitudinal cell dispersion affect all the cells in the rhombencephalon. In this last hypothesis, the arrest of AP dispersion could occur either before or after the formation of rhombomere boundaries.

To address these points, it is important to estimate the date of birth of the clones in relation to the formation of rhombomere boundaries. We have shown that there is a limitation of cell movement between rhombomeres at E8 when *Krox-20* is first expressed (*Krox-20/Cre* experiment and Fig. 2A). To estimate the date of birth of β-gal$^+$ clones in relation to this event, the distribution of the DV clusters in clones with several DV clusters was described in relation to the rhombomeric limits from r2 to r6 (Fig. 2B). Strikingly, most of the DV clusters (87.5%, *n*=32) were separated by at least one rhombomere limit (Fig. 6A). This distribution implies that most of those founders are born before the restriction of cell movements between rhombomeres at the onset of *Krox-20* expression.

This observation does not indicate whether the constraint acting on AP cell dispersion observed for the DV clusters is imposed **only by** the interface between future rhombomeres at E8. If this were the case, it would be expected that clones initiated before interface formation would disperse within and between rhombomeres and should frequently have an AP dimension equal or superior to that of one rhombomere (Fig. 7A). However, we observed that the AP dimension (mean value of 86 μm) of the DV clusters in clones with several DV clusters is systematically lower than the AP dimension of one rhombomere (Figs 4A, 6A). Because, in these clones, most of the DV cluster founders are born before the limitation of cell movements between rhombomeres, these data show that the arrest of AP cell dispersion is not imposed simply by the interfaces between rhombomeres. This shows that the arrest of AP cell dispersion affects most of the cells in the rhombencephalon, regardless of their initial AP position.

Secondly, we have investigated whether the general arrest of AP cell dispersion observed for DV clusters is established after a phase of AP dispersion of their precursors within rhombomeres. In this case, we should systematically observe several DV clusters per rhombomere (Fig. 7B). However, in clones with several DV clusters, we observed a large majority of cases (78%, *n*=32) with rhombomeres containing only one DV cluster (Fig. 6A). This confirms that most of the DV cluster founders are born before the limitation of cell movements between rhombomeres.

In conclusion, our observations suggest that the AP dispersion of the descendants of DV cluster founders is very limited within the rhombencephalon before or at the time of the restriction of cell dispersion between rhombomeres at E8. Thus, a general arrest of AP cell dispersion occurring in the whole rhombencephalon precedes morphological compartmentalization of the rhombencephalon at E9 (Fig. 7C). The AP order of the DV cluster founders and their descendants is therefore already established when cell dispersion between rhombomeres becomes restricted at E8.

Further characteristics of cell dispersion in the rhombencephalon

We have compared the DV position of different clusters in individual clones with several labelled DV clusters (Fig. 6B). DV clusters were generally not dispersed along the entire DV dimension of the rhombencephalon. Interestingly, clonally related DV clusters frequently display a similar DV position (Fig. 6B, for example, clones 95-860 or 95-542.1), and a similar DV extension (see for instance clones 95-860, 95-534, 95-542.1, or 95-714.2 in Fig. 6B) suggesting that precursors maintain their relative position along the DV axis during longitudinal dispersion, and that position along the DV axis may determine the dispersal potential of the cells. These observations suggest that only moderate dorsoventral dispersion occurs during AP dispersion of the precursors of the DV cluster founders and that the dispersion of the founder precursors is mainly longitudinal.

The DV clusters were themselves frequently organized into separate groups of cells aligned along the DV axis (see Fig. 3A, clusters in r4 for instance and Fig. 3B-H). Furthermore, the smallest DV clusters consisted of one or two separate groups of cells similar to those observed in larger DV clusters (compare Fig. 3A-E and G,H). This characteristic suggests that these groups of cells define subclones within the DV clusters. Therefore, this clonal arrangement suggests that there are two phases during the local growth of the descendants of the founder cells of the DV clusters: an initial phase of DV dispersion and intermingling of the precursors of the groups, followed by a phase of relatively coherent growth to produce the small groups of cells. These two phases of dispersion lead to the delimitation of small clonal areas within the neuroepithelium, which suggests that neuroepithelial cells are clonally positioned on a Cartesian grid along the AP and DV axes of the neural tube.

The spinal cord

Clonal organization in the VZ of the spinal cord (SC) presented

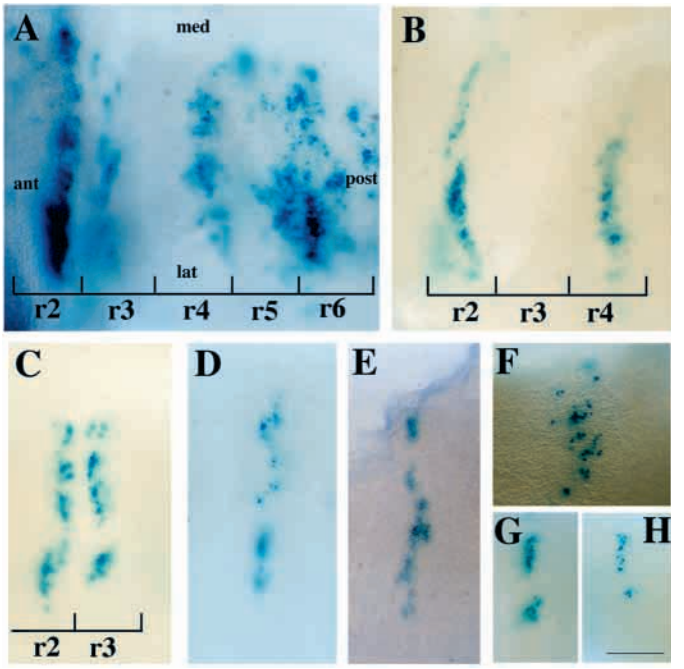

Fig. 3. Clonal arrangement in DV clusters in the rhombencephalon. (A-H) Dorsal (ventricular) views of recombinant *lacZ* clones. The mediolateral dimension correspond to the DV axis (see text). (A) A non-restricted clone, clone 95-534 with several DV clusters in r2 to r6. This clone also presented labelled cells anteriorly in the mesencephalon and posteriorly in the spinal cord. (B,C) Two restricted clones, (B) clone 95-911.2 with two DV clusters of 73 cells in r2 and 38 cells in r4 and (C) clone 95-714.2 with two DV clusters of 64 cells in r2 and 57 cells in r3. This clone presented another labelled DV cluster in r7. (D,H) Several one cluster clones, clone 94-804 presenting a single DV cluster of 55 cells in r3 (one cluster clone). (D) Clone 94-908 presenting a single DV cluster of 45 cells in r2. (E) Clone 95-876, 58 cells in r7. (G) Clone 95-862 presenting two separate groups arranged mediolaterally (and therefore arranged along the DV axis), 48 cells in r4. (H) Clone 95-742, 22 cells in r4. (F) In A-H, note the arrangement of DV clusters into separate cell groups arranged along the DV axis. Scale bar, 200 μm.

striking similarities to that in the rhombencephalon. Cells were also organized into separate clusters with a DV orientation, and the AP dimension of these clusters was less than that of a myelomere (Fig. 8A,B). This clonal organization was observed both in the anterior and in the posterior SC (Fig. 8C). That the DV clusters define subclones within clones with several DV clusters (Fig. 5) was suggested by the similar geometric properties of DV clusters (orientation and AP dimension) in clones with several DV clusters distributed in different neuromeres, and those clones consisting of a single DV cluster (Fig. 8C-E). The DV clusters were themselves organized into separate smaller groups of cells (Figs 1Q-T, 8A,B,F), suggesting that the DV clusters are produced by the intermingling of several neuroepithelial cells. These clonal arrangements show that the successive phases of neuroepithelial clonal development deduced in the rhombencephalon – an initial phase of AP (longitudinal) dispersion of the precursors of the cluster founders, followed by a DV dispersion of the descendants of the cluster founders with moderate AP cell dispersion and finally a relatively coherent growth in the neuroepithelium – also apply to the SC.

The size of the DV clusters in clones of more than one DV cluster was smaller than that of the myelomeres (Fig. 8A,B,E). Therefore, only weak AP clonal dispersion must occur within myelomeres. Together with the distribution of the DV clusters in separate myelomeres (Fig. 8A,B), this observation suggests that, in clones with several DV clusters, most of the DV cluster

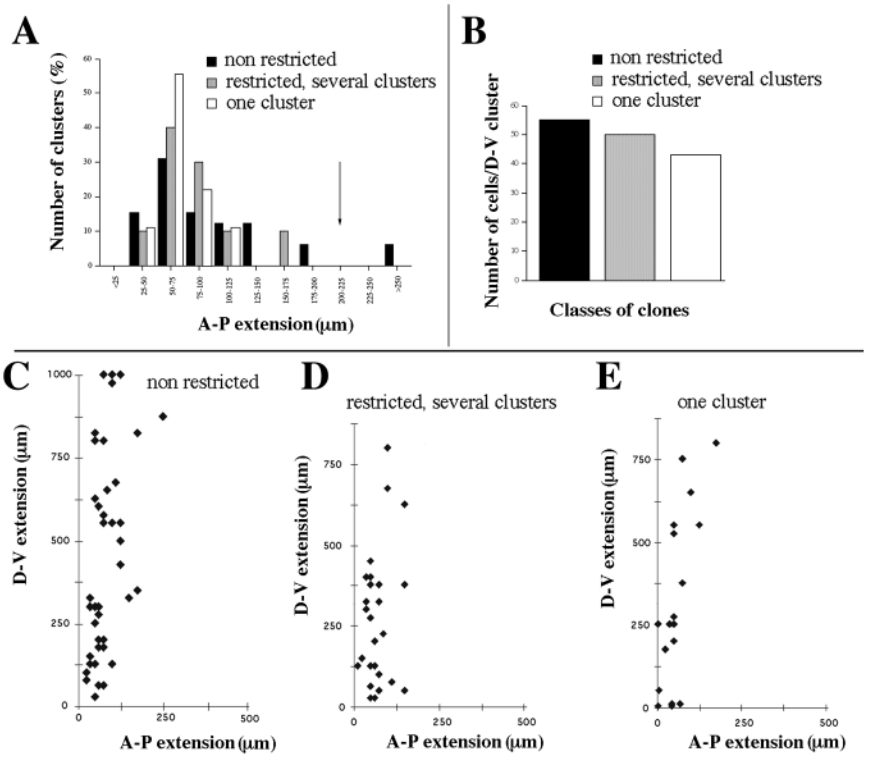

Fig. 4. Characteristics of the DV clusters of the three main categories of clones. (A) Percentage of DV clusters (y axis) with different anterior-posterior extension (x axis) in clones with several DV clusters in the rhombencephalon and labelled cells in other parts of the CNS (non restricted clones, n=32 DV clusters), clones with several DV clusters restricted to the rhombencephalon (restricted clones, n=10 DV clusters) and clones with a single DV cluster (one cluster, n=9). The vertical arrow shows the AP dimension of one rhombomere. We have tested whether the values in the different classes of clones are different (variance analysis, 2 & 48 df, f=2.52, $P<10^{-2}$, ns). (B) Mean cell number of the DV clusters (y axis) for clones of the three different categories. We have tested whether the values in the different classes of clones are different (variance analysis, 2 & 48 df, f=0.4, $P<10^{-2}$, ns). (A,B) Numerical analyses are based on the DV clusters located in the r2-r6 area. (C-E) The mediolateral (DV) dimension of the clusters (y axis) has been compared to their AP dimension (x axis), (C) non-restricted clones, (D) restricted clones, (E) one cluster clones.

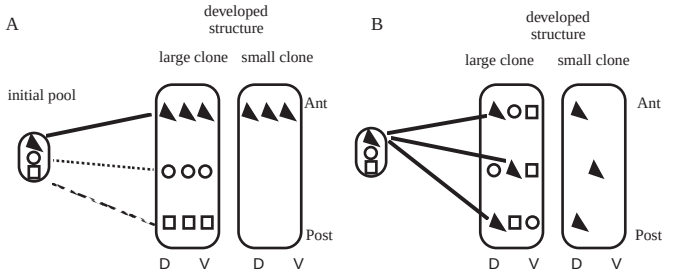

Fig. 5. Clonal organisation in the large clones. Two hypotheses as to relationship between the pool of founder cells and the distribution of their descendants in the DV clusters are presented. Clonally related cells are indicated by identical geometrical symbols. (A) Cells located in a DV cluster are closely related genealogically and the DV clusters define subclones. In this hypothesis, the smaller clones have characteristics of a DV cluster in the large clones. (B) Cells located in separate DV clusters are more closely related genealogically than cells within DV clusters. In this hypothesis, smaller clones are longitudinally oriented and do not correspond to DV clusters.

founders are born before the arrest of dispersion between myelomeres. Therefore, AP cell dispersion becomes very weak within myelomeres before or at the time of the arrest of cell dispersion between myelomeres.

Next, we analysed whether this arrest of AP cell dispersion was due to the presence of fixed boundaries within the myelomeres. This study was simplified by the presence of a morphological landmark, the roots of the peripheral nerves (Fig. 8F). The distribution of β-gal$^+$ cells in relation to this landmark has been described by camera-lucida drawings for a sample of 62 DV clusters randomly chosen from 13 clones with several DV clusters. We subdivided the myelomeres into 5 domains of equal AP extension (Fig. 8F), and the distribution of DV clusters was analyzed in relation to these domains (Fig. 8G). Each of the five arbitrary domains was populated by a similar number of DV clusters of different AP dimensions (Fig. 8H). The most-extensive DV clusters do not stop at any particular level as shown by the fact that each domain was crossed by a similar number of DV clusters (Fig. 8I) and by the fact that DV cluster borders are distributed at random within myelomeres (Fig. 8J). These observations suggest that the arrest of AP cell dispersion does not occur at particular axial levels within myelomeres. This conclusion suggests a general and uniform arrest of AP dispersion affecting most of the cells, which is independent of their initial AP position within myelomeres.

The mesencephalon and the prosencephalon

A clonal arrangement of separate clusters of labelled cells was also systematically observed in the other domains of the CNS (Fig. 9). In the mesencephalon, these clusters presented a dorsoventral orientation (Fig. 9A,B). In the prosencephalon, cluster orientation was more variable (Fig. 9C, D). The transition in the orientation of the cell clusters appears to occur at the level of the thalamic zona limitens (Fig. 9G). Similar geometric properties of the DV clusters (orientation, AP dimension in the mesencephalon, surface of the clusters in the prosencephalon) were observed between the clones with several DV clusters distributed in different neuromeres, and the clones consisting of a single DV cluster (Fig. 9E,F). These data suggest that the DV clusters define subclones within the clones with several DV clusters in the different parts of the CNS (Fig. 5). The DV clusters were themselves organized into separate smaller groups of cells (Figs 1L,O, 9A-D), suggesting that the DV clusters are produced by the intermingling of several neuroepithelial cells. This clonal arrangement shows that the successive phases of neuroepithelial clonal development deduced in the rhombencephalon and in the spinal cord – an initial phase of AP dispersion and intercalation of the precursors of the cluster founders, followed by local growth of the cluster founders with reduced AP neuroepithelial cell dispersion and a final coherent growth phase – also apply to the rostral domains of the CNS.

In the mesencephalon and prosencephalon, the size of the DV clusters was smaller than that of the neuromeres (Fig. 9E,F), showing that clonal dispersion is weak within neuromeres. Together with the distribution of the DV clusters in separate neuromeres (Fig. 9A-D), this observation suggests that, in clones with several DV clusters, most of the DV cluster founders are born before the formation of neuromeres. Cell dispersion is therefore constrained within neuromeres and the arrest of cell dispersion precedes or accompanies neuromere formation.

DISCUSSION

The arrest of clonal cell dispersion during embryogenesis is generally thought to accompany cell fate determination and the acquisition of positional information. In this respect, the relationship between the arrest of clonal dispersion and neuromere development constitutes an important issue which has not been completely resolved in the different domains of the CNS. In the present study, we have analysed the patterns of neuroepithelial cell dispersion underlying the growth of the mouse neural plate and neural tube by looking at clones born before and after the formation of neuromeres. We deduce at least three periods in the clonal history of neural precursors, which are common to the development of the prosencephalon, the mesencephalon, the rhombencephalon and the spinal cord.

A novel method to analyse the development of the mouse neuroepithelium

We have exploited a genetic method of single cell labelling and retrospective analysis of the distribution of genealogically related cells, to analyse the development of the mouse neuroepithelium. In the transgenic line used in this study, the *laacZ* transgene is broadly expressed in the ventricular zone (VZ) of the neural tube at E12.5 in addition to the expression in embryonic neurons (Forss-Petter et al., 1990). The pattern in the VZ was not expected since previous studies reported neuron-specific enolase expression only in differentiated neurons (Marangos et al., 1980; Schmechel et al., 1980). In the cerebellum, analysis of NSE *lacZ* and NSE *laacZ* transgenic mice revealed qualitative as well as quantitative expression of the two reporter genes to be remarkably similar to the expression of the endogenous neuron-specific enolase gene (Forss-Petter et al., 1990; Mathis et al., 1997). However, in addition, we now report that the NSE-driven *laacZ* transgene in line NSE-1 is also expressed in the neuroepithelium using digoxigenin in situ hybridization and β-galactosidase activity.

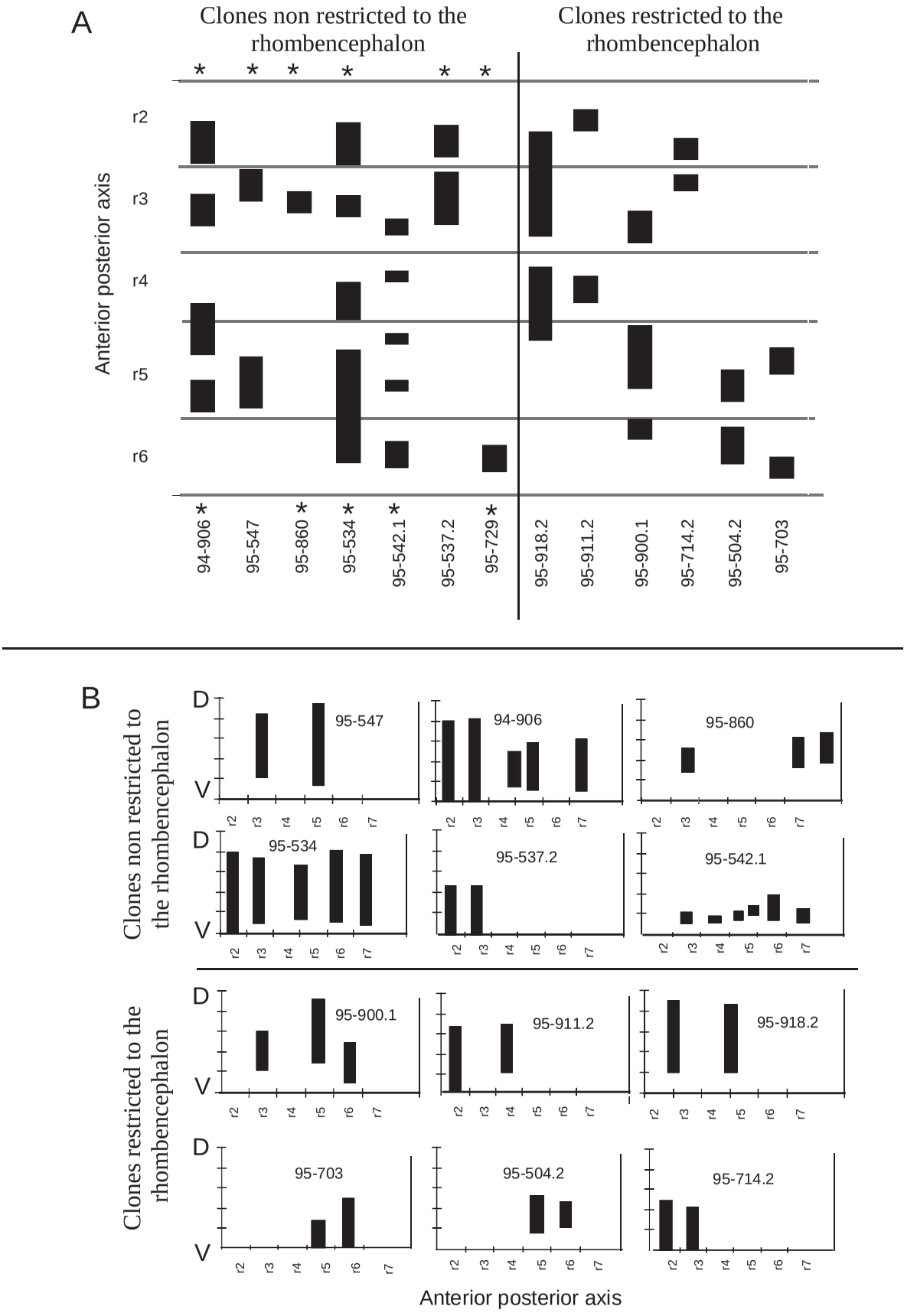
A
Clones non restricted to the rhombencephalon
Clones restricted to the rhombencephalon
Anterior posterior axis
r2
r3
r4
r5
r6
94-906
95-547
95-860
95-534
95-542.1
95-537.2
95-729
95-918.2
95-911.2
95-900.1
95-714.2
95-504.2
95-703
B
Clones non restricted to the rhombencephalon
Clones restricted to the rhombencephalon
D
V
r2 r3 r4 r5 r6 r7
95-547
94-906
95-860
95-534
95-537.2
95-542.1
95-900.1
95-911.2
95-918.2
95-703
95-504.2
95-714.2
Anterior posterior axis

Fig. 6. Distribution of DV clusters in the rhombencephalon in clones with several labelled DV clusters. (A) Distribution of the DV clusters in individual clones in relation to the rhombomeric limits (mapped according to Fig. 2). The stars indicate clonally related labelled cells outside the r2-r6 area, anteriorly and/or posteriorly. Note that clonally related DV clusters are generally separated by at least one rhombomeric boundary. The DV dimension is not to scale. (B) Mediolateral (DV) distribution of the clusters in individual clones. In each case, the entire DV dimension of the rhombencephalon is represented. Note that the dorsoventral position and extent are similar among clonally related DV clusters. The AP dimension is not to scale.

There are several hypotheses that may explain this result. Firstly, it could be due to the high degree of sensitivity of these detection methods. Secondly, the transgene may lack *cis*-acting elements that negatively control expression in these neuronal precursors; alternatively sequences at the integration site may contribute to transgene expression. Whatever the case may be, analysis of the expression pattern by in situ hybridisation and histological examination of β-gal$^+$ clones demonstrated that this transgenic line is useful to describe clonal relationships in the VZ, and thus to study the growth of the neuroepithelium.

The method used here (Bonnerot and Nicolas, 1993; Nicolas et al., 1996; Mathis et al., 1997) presents several advantageous characteristics for the analysis of successive stages of clonal development. Firstly, precursor cells are labelled at a low frequency, which permits the assumption that the majority of labelled embryos contain β-gal$^+$ cells which are derived from a single recombination event. Secondly, clones are initiated at random during development, implying that clones representative of all developmental stages are obtained. In this study, we have assumed that the extent of longitudinal dispersion of clones within the neural tube is related to their date of birth. This hypothesis is supported by fate maps of the mouse neural primordium and is confirmed by our present study, since both suggest a progressive decrease of AP cell dispersion. Thirdly, the long-term nature of the labelling allows clones with different dates of birth to be described at the same developmental stage. This is perhaps the most important characteristic of the method since it permits us to define the successive cellular events underlying neuroepithelium development.

A transition from a dispersive to relatively coherent AP growth pattern in the developing neuroepithelium

The systematic distribution of early labelled clones not restricted to any one region of the CNS in separate DV clusters demonstrates an initial phase of AP cell dispersion of the precursors of the DV clusters. This result therefore identifies an initial phase of low coherent growth during neuroepithelium development. A loss of coherence of epithelial growth has been demonstrated in the mouse epiblast from E6 (Gardner and Cockroft, 1998; Lawson et al., 1991) and the cell intermingling reported here may reflect the continuation of this dispersive growth phase. In sharp contrast, cell dispersion is very reduced in the AP dimension from before the time at which neuromeres form. For instance, in the rhombencephalon, the arrest of AP cell dispersion must occur before E8, that is, prior to the first manifestation of segmental organization revealed by Krox-20 expression (Wilkinson et al., 1989; Chavrier et al., 1990). Subsequently, clones can still disperse in the AP dimension, but this dispersion is much weaker than that in the DV dimension. These results suggest an arrest of AP cell dispersion and a transition from a dispersive to a more coherent AP growth pattern in the neuroepithelium of the rhombencephalon. This demonstrates a major change in the mode of epithelial growth that precedes or accompanies the formation of rhombomeres. An interesting possibility is that the transition from a dispersive to a more coherent AP cell growth pattern facilitates the acquisition of AP positional information in relation to the genetic delimitation of the CNS and of rhombomeres. In agreement with this hypothesis, we show here that AP cell dispersion is restricted prior to the onset of *Krox-20* gene expression, one of the earliest markers of genetic segmentation in the rhombencephalon (Wilkinson, 1995).

We deduced a similar dispersive to coherent transition in different AP domains of the CNS, the spinal cord, the prosencephalon and the mesencephalon. Therefore, most probably, this transition applies to both rostral and caudal regions of CNS, at more or less the same developmental stage. The mechanisms governing the establishment of neuromeric organisation appear to vary in different AP regions of the CNS (Lumsden and Krumlauf, 1996; Sasai and De Robertis, 1997). This suggests that the different genetic strategies involved in AP patterning all require a similar transition in cell behavior from dispersive to more coherent AP growth.

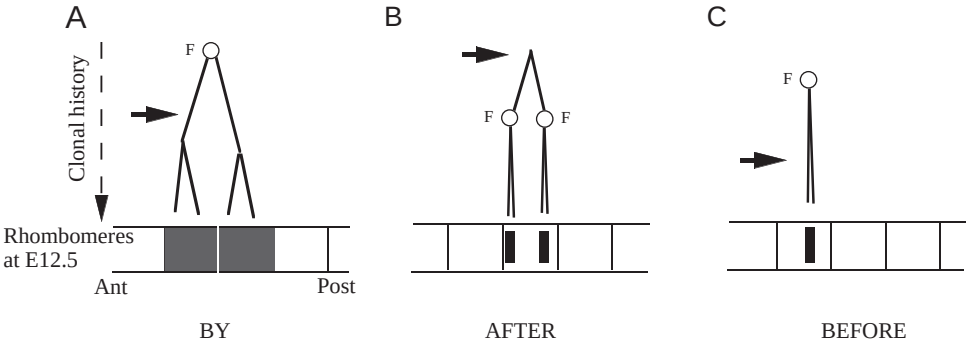

Fig. 7. Expected AP extension and distribution of clones (black boxes) produced following different hypotheses for the mode of arrest of AP cell dispersion with respect to rhombomere formation (horizontal arrows). Founders of the DV clusters (F, indicated by a circle) define the time in the clonal history (dashed vertical arrow) after which clonally related cells show preferential DV cell dispersion. (A) The arrest of AP cell dispersion is imposed **by** the interfaces between rhombomeres. In this hypothesis, clones labelled before the formation of the interfaces between rhombomeres can disperse *within* and *between* rhombomeres. (B) The arrest of AP cell dispersion occurs **after** rhombomere formation. In this hypothesis, DV clusters founders would be produced after a phase of dispersion of the precursors of the DV clusters founders within the rhombomeres. In this hypothesis, more than one DV cluster must frequently be found within one rhombomere. (C) The arrest of AP cell dispersion occurs **before** rhombomere formation. In this hypothesis, clones labelled before rhombomere formation produce only one DV cluster per rhombomere.

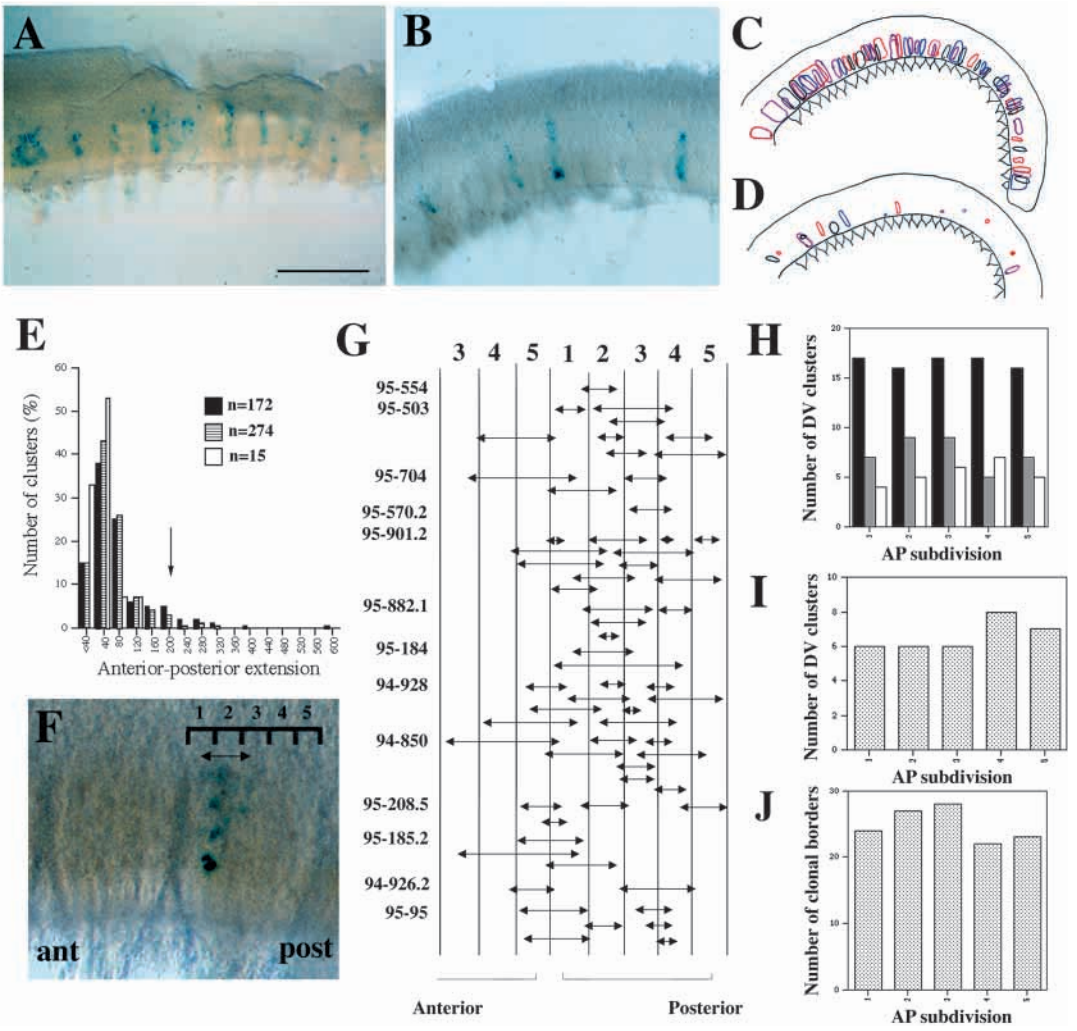

Fig. 8. Clonal arrangement in DV clusters in the spinal cord. (A) Non-restricted clone, clone 94-906, which also presented labelled cells in the mesencephalon and prosencephalon. (B) Restricted clone 95-185.2. Note that, in A and B, the AP extension of the DV clusters is less than that of one myelomere visualised by the root ganglia and that their position within myelomeres is variable. (C,D) Graphic representation of DV clusters of many clones. The shape and AP location of the largest DV clusters has been recorded by camera-lucida in clones with several DV clusters (C) and in clones with a single DV cluster (D). (E) Comparison of the percentage of DV clusters (y axis) with different AP extension (x axis) in clones of different longitudinal extension. The number of clusters analysed in the different classes of clones are indicated. Black, non restricted clones; hatched, restricted clones; white, one cluster clone. (F-J) Analysis of the dispersion of the DV clusters within myelomeres. (F) Method. The myelomere has been subdivided into 5 units of equal AP extension of 40 μm, and the AP dispersion into these units has been represented for each DV cluster by a double arrow. The represented DV cluster (27 cells, belonging to clone 95-901.2) crosses entirely subdivision 2 and its borders are in subdivisions 1 and 3. (G) The dispersion of 62 DV clusters (13 clones) is represented. DV clusters participating to two consecutive myelomeres are represented on the left. (H) Number of DV clusters of different AP dimension that give descendants in the indicated myelomeric subdivisions (1 to 5). Black, DV clusters that give descendants into three or more consecutive subdivisions; grey, DV clusters with an AP dimension of more than 40 μm and that give descendants into 2 consecutive subdivisions; white, DV clusters with an AP dimension of less than 40 μm. (I) Number of clones that cross entirely the different subdivisions. For each subdivision, the value is the number of DV clusters that give descendants in this subdivision as well as in more anterior and posterior subdivision. (J) Number of times a given subdivision is a clonal border of a DV cluster. Scale bar, A,B, 500 μm; F, 140 μm.

A transition from dispersive to relatively coherent dorsoventral growth in the developing neural tube

We deduced that the phase of AP cell dispersion is followed by the acquisition of a mode of asymmetrical DV cell dispersion in the rhombencephalon, the SC and the mesencephalon. The organisation of DV clusters in separate small groups of cells suggests that this second phase of neuroepithelial formation involves the dispersive DV growth of precursor cells. The coherent small groups of cells arranged dorsoventrally suggest a third phase of neuroepithelial development that corresponds to an arrest of both AP and DV dispersion and the acquisition of a coherent mode of growth. The case of the prosencephalon presents a particularity since cell clusters in this region of the CNS have a variable – not a DV – orientation. However, the clusters were still organised into separate coherent groups of cells. This suggests that similar steps of clonal development occur in the prosencephalon, although spatial constraints may be different in the rostral CNS. As discussed above for the arrest of AP cell dispersion, the arrest of DV cell dispersion may facilitate the inductive interactions that lead to the DV organisation of the neural tube and may, for instance, be involved in generating the diversity of neuronal cell types (Echelard et al., 1993).

A similar cascade of cellular events in the mouse and chick embryo

Our results suggest a cascade of cellular events that lead to the static position of cells within the neuroepithelium. We deduce two successive orientations of cell dispersion, an initial, mainly longitudinal, cell dispersion followed by a mainly DV cell

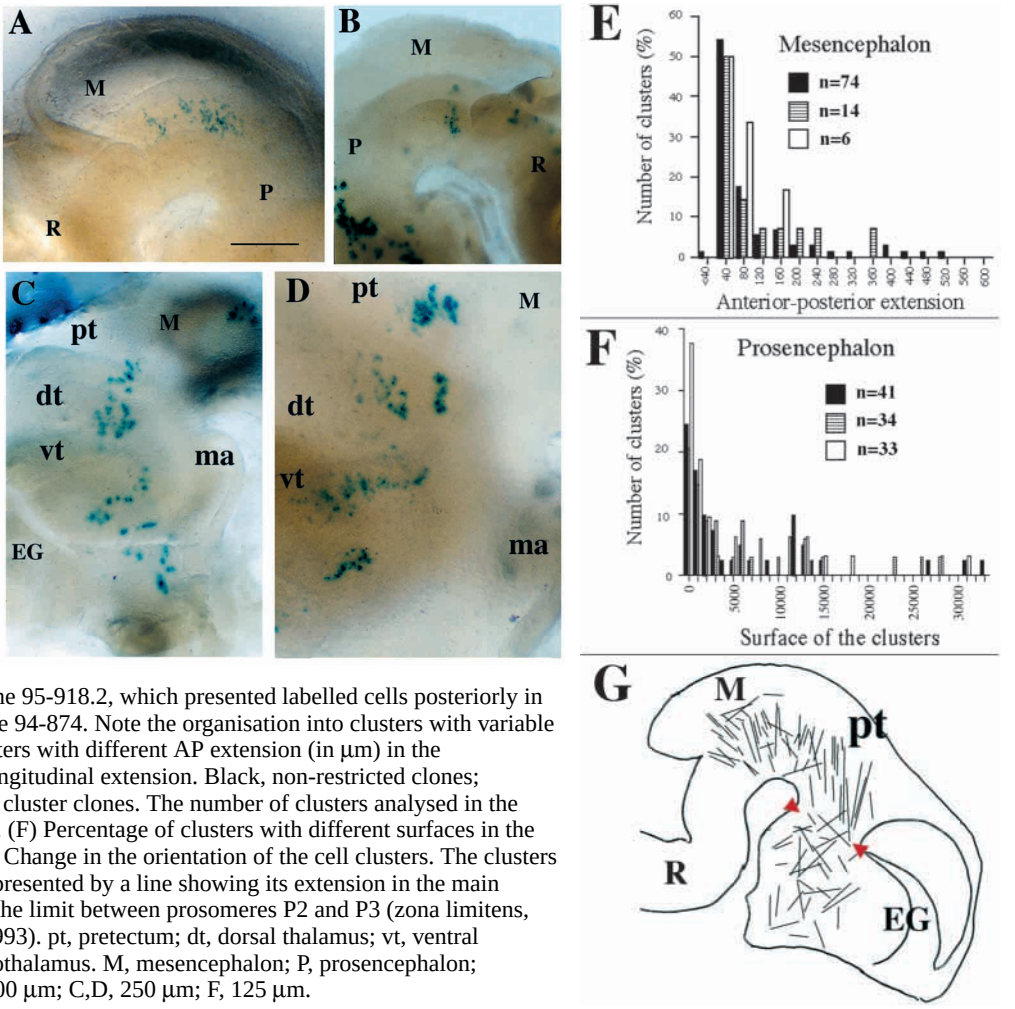

Fig. 9. Clonal arrangement in DV clusters in the anterior domains of the embryonic CNS. (A,B) Clusters in the mesencephalon. (A) Restricted clone 95-192.2. (B) Non-restricted clone 95-860, which presented labelled cells anteriorly in the prosencephalon and posteriorly in the rhombencephalon. Note the DV orientation of the clusters. (C,D) Clusters in the prosencephalon. (C) Non-restricted clone 95-918.2, which presented labelled cells posteriorly in the mesencephalon. (D) Restricted clone 94-874. Note the organisation into clusters with variable orientations. (E) Percentage of DV clusters with different AP extension (in μm) in the mesencephalon in clones of different longitudinal extension. Black, non-restricted clones; hatched, restricted clones; white, single cluster clones. The number of clusters analysed in the different classes of clones are indicated. (F) Percentage of clusters with different surfaces in the prosencephalon. Values are in μm^2. (G) Change in the orientation of the cell clusters. The clusters with a marked orientation have been represented by a line showing its extension in the main direction. The red arrowheads indicate the limit between prosomeres P2 and P3 (zona limitens, according to Rubenstein and Puelles, 1993). pt, pretectum; dt, dorsal thalamus; vt, ventral thalamus; ma, mamillary area; Hy, hypothalamus. M, mesencephalon; P, prosencephalon; R, rhombencephalon. Scale bar, A,B, 500 μm; C,D, 250 μm; F, 125 μm.

dispersion. We also suggest that a transition from dispersive to more coherent growth follows these phases of cell dispersion. This description is in general agreement with results obtained in the chick embryo. In the chick, clones labelled in the epiblast show extensive cell intermingling (Hatada and Stern, 1994). Clones labelled shortly before or after the appearance of neuromeres are never organised into separate clusters in different neuromeres (Fraser et al., 1990; Birgbauer and Fraser, 1994). This is consistent with our own conclusion that, in the mouse embryo, AP neuroepithelial cell dispersion is already arrested at the time of neuromere formation. An asymmetrical DV cell dispersion has also been observed in both clonal and multiclonal analyses of chick rhombomere (Fraser et al., 1990; Guthrie et al., 1993; Wingate and Lumsden, 1996) and spinal cord (Stern et al., 1991) development. Finally, the arrest of DV cell dispersion reported here is compatible with the early allocation of neuroepithelial cells leading to early phenotypic choices in the chick rhombencephalon (Lumsden et al., 1994; Clarke et al., 1998) and to the transition from a dispersive to a coherent growth observed in the chick SC (Leber et al., 1990). Therefore, a similar cascade of cellular events may occur in the chick and in the mouse neuroepithelium.

In addition, analyses performed in the chick embryo revealed a supplementary constraint restricting cell movement at the interface between neuromeres at the time of their morphological appearance (Fraser et al., 1990; Stern et al., 1991; Figdor and Stern, 1993). We have not analysed this restriction in the mouse, since we have mainly analysed clones labelled prior to the formation of neuromeric limits. If a similar constraint is also acting in the mouse neuroepithelium, this would define a supplementary event in the cellular cascade described here, probably involved in the fine definition of neuromeric units.

The cascade of cellular events shares characteristics with the genetic patterning of the CNS

The clonal patterning described here shares similarities with the genetic patterning of the CNS. Genetic patterning follows the same temporal order with the establishment first of AP regionalisation generated at least in part by inductive signals from the organiser (Wilson and Hemmati-Brivanlou, 1997), and second, of DV patterning by signals emanating from the notochord and the dorsal epidermal ectoderm (Tanabe and Jessell, 1996). Furthermore, the strict DV patterning of β-gal$^+$ clones is observed from the SC to the mesencephalon, which are domains under the influence of DV patterning controlled by the notochord, whereas the orientation of cell dispersion changes dramatically in the anterior prosencephalon, whose patterning appears to be controlled by other structures such as

the anterior neural ridge (Shimamura and Rubenstein, 1997). Finally, neuroepithelial growth on a Cartesian grid (two successive cell dispersions in orthogonal directions) is strikingly reminiscent of the way positional information is thought to be delivered to these cells (Lumsden and Krumlauf, 1996). The correlation established by our clonal analysis suggests, therefore, a rather strict coordination between cellular and genetic events during neural tube formation. The cellular cascade described here is compatible with a model involving firstly an arrest of AP cell dispersion related to the establishment of AP patterning, and a subsequent arrest of DV cell dispersion in relation to the establishment of DV patterning. It remains to be established which signals may induce the change in cell dispersion behaviour from AP to DV. Novel expression patterns of cell adhesion molecules, the involvement of morphogens directing cell migration or a change in the orientation of mitosis are all possibly involved.

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