

Retrospective clonal analysis of the cerebellum using genetic *laacZ/lacZ* mouse mosaics

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SUMMARY

Analysis of *lacZ* neuronal clones in the mouse cerebellum demonstrates genealogical independence of the primary and secondary germinal epithelia (PGE and SGE) from early development. PGE precursors and their neuronal descendants are organised into two polyclonal groups of similar sizes that exhibit parasagittal patterning and generally respect the midline. The relationship between these two groups cannot be traced back in time to less than 80 independent cells, which were probably recruited following a period of non-coherent growth that distributes unrelated cells into distinct territories of the neural tube. A lateromedial clonal organisation is observed in the mature cerebellum, suggesting the existence of many small

parasagittal domains of clonal restriction and/or of cell dispersion in the rostrocaudal but not in the mediolateral dimension. The organisation is orthogonal with respect to the cellular organisation in the neural tube as is the genetic organisation. Cellular and genetic patterning of the cerebellum therefore share similarities. A possible hypothesis is that distinct cell behaviours create the different clonal domains observed in this study and that the cellular and genetic organisation of the cerebellum are coordinated.

Key words : cell lineage, cerebellum, clonal analysis, clonal growth, compartment, founder cell, *LaacZ*, mouse, Purkinje

INTRODUCTION

Among all the structures of the central nervous system (CNS), the cerebellum is the one whose development is best understood (Sotelo and Wassef, 1991, Hatten and Heintz, 1995). In the mouse, cerebellum morphogenesis begins at embryonic day 7.5 (E7.5) as the anterior portion of the neural tube becomes subdivided into three prospective vesicles: the prosencephalon, the mesencephalon and the rhombencephalon. Subsequently, at E9, a bending of the neural tube creates the pontine flexure (Theiler, 1989). At E10–E11, in the roof of the metencephalon (a subdivision of the rhombencephalon), the cerebellar anlage establishes itself as two bilateral thickenings called the cerebellar plates (Miale and Sidman, 1961). These thickenings increase considerably during development up to E15. This feature has been interpreted as resulting from either progressive fusion of the cerebellar plates at the midline or differential growth of the medial part of the cerebellar primordium. Neurons of the deep nuclei, the Purkinje cell layer (PCL) and Golgi cells are sequentially produced from the primary germinal epithelium of the cerebellum anlage between E10 and E15 (Altman and Bayer, 1985a–c). Between E12 and E15, cells originating from the rhombic lip (the germinative trigone) invade rostrally the cerebellum anlage to produce a second germinative layer, the external granular layer (EGL) (Miale and

Sidman, 1961). Until postnatal day 15 (P15), the EGL produces the granule cells of the internal granular layer (IGL), which reach this deep position after migration along the Bergmann glial fiber processes (Miale and Sidman, 1961, Rakic, 1971, Hatten and Liem, 1981). The adult cerebellum has three cortical layers: the molecular layer (ML), the Purkinje cell layer (PCL) and the IGL.

The precise origin and clonal relationships between cells of the cerebellum anlage have not yet been clearly defined in rodents. Transplantation experiments of portions of the mesencephalic and metencephalic vesicles in the quail/chick system (LeDouarin, 1969) have been used to localise the presumptive territory of the cerebellum at the 10- to 12-somite stage (stage 10 according to Hamburger and Hamilton, HH10) (Martinez and Alvarado-Mallart, 1989, Hallonet et al., 1990, Alvarez-Otero et al., 1993, Hallonet and LeDouarin, 1993). These studies have shown that the cerebellum anlage originates from both the mesencephalon and metencephalon. The metencephalon contributes to the lateral and caudal portions of the cerebellum, and the mesencephalon contributes to a V-shaped mediodorsal portion (Martinez and Alvarado-Mallart, 1989, Hallonet et al., 1990, Alvarez-Otero et al., 1993). In contrast, at HH10, cells that contribute to the second germinative layer, the EGL, are localised only in the metencephalon; whereas, the midbrain tectum and related tegmental nuclei are derived from rostral

regions of the mesencephalon. These partial cerebellar grafts therefore suggest that, as early as HH10, the mesencephalon and the metencephalon have already subdivided along the anterior-posterior axis into regions that will produce different structures. These experiments have also shown that the ventricular epithelium generates not only Purkinje and Golgi cells, but also at least a fraction of the interneurons of the ML (Hallonet et al., 1990, Alvarez-Otero et al., 1993). This observation appears paradoxical since, in the mouse, these interneurons are produced at a time when the ventricular epithelium has no mitotic activity (Miale and Sidman, 1961). It has therefore been postulated that, in rodents, the ML interneurons originate from the EGL (Altman, 1972a-c; Jacobson, 1991).

In comparison, the processes underlying cerebellum development in the mouse are still incomplete. However, tetraparental chimeras (Mintz, 1965) and X inactivation mosaics have been used to analyse another aspect of the origin of cerebellum: the number of cells that are selected as Purkinje cell progenitors during development (Wetts and Herrup, 1982a, Vogel and Herrup, 1993). In the study presented here, a founder cell is one that produces a cohort of Purkinje cells, also called a lineage group. It reasons that a cohort of Purkinje cells contains all cells that can be grouped by common descent from a single founder cell, and that founder cells are selected by a process that excludes other potential founders. This definition of founder cells suggests that their selection occurs at a stage of development when mixing between different cell lineages (whose descendants will form part of another tissue or cell lineage) becomes minimal (McLaren, 1972). Wetts and Herrup (1982) have estimated that cerebellar Purkinje cells descend from 8 founder cells per hemiserebellum; whereas, Baader et al. (1996) estimated 65 founder cells. Whatever the exact number of founders, these findings support the idea of an early allocation of a small number of progenitor cells to the Purkinje cell lineage, possibly during segmentation of the neural tube. However, controversy about the interpretation of these results challenges these important conclusions (Jennings, 1988).

Genetic analysis of hindbrain and midbrain formation has shown that vertebrate homologues of *Drosophila* segment-polarity genes are required very early during development for hindbrain and midbrain formation (Bally-Cuif and Wassef, 1995, Joyner, 1996). *Pax-2*, *engrailed-1* (*En-1*), *Pax-5* and *En-2* are expressed between E8 and E12.5 in domains which overlap with the hindbrain-midbrain junction. The targeted deletion of *En-1* or *Wnt-1* resulted in homozygous mutant mice that lacked a cerebellum as well as other midbrain structures (McMahon et al., 1992, Wurst et al., 1994). The loss of these structures was apparent as early as E9.5. It has been hypothesised that *Wnt-1* is necessary for the maintenance of the hindbrain-midbrain border (Bally-Cuif and Wassef, 1995, Joyner, 1996) and this period of development coincides with the thickening of the cerebellum anlage and significant levels of cell proliferation. Later during development, between E15 and E17.5, *En-2*, *En-1* and *Pax-2* have been shown to exhibit transient and spatially restricted patterns of expression that define 11 sagittal domains in the cerebellum anlage. It has been proposed (Millen et al., 1995) that these genes are involved in the regionalisation of sagittal and transverse domains within the cerebellum (Wassef et al., 1992, Oberdick et al., 1993).

The role of these segment polarity genes with respect to morphogenetic cell behaviour, however, remains obscure. For instance, it is not known whether these genes act independently of cell proliferation or whether they function throughout the various phases of cell growth. This incomplete knowledge stems from contradictory results reported in the literature and the difficulty of estimating the critical parameters of cell organisation and patterning during the formation of the cerebellar primordium, particularly in the mouse where direct experimentation has previously not been possible.

To analyse the clonal behaviour during morphogenesis of the cerebellum, we have developed a novel method of cell labelling to visualise neuronal clones in the central nervous system of mice. It is based on the generation of a functional *lacZ* reporter gene during development of transgenic mice carrying an inactivated *laacZ* transgene, through a random intragenic recombination event that re-establishes the open reading frame for the reporter gene (Bonnerot and Nicolas, 1993b, Nicolas et al., 1996). This results in the labelling of single cells during development. Descendants of these cells are visualised by the means of the neuron-specific enolase promoter (Forss-Petter et al., 1990), which drives expression of the *laacZ* transgene. We describe here the observed patterns of randomly generated neuronal clones in the postnatal cerebellum.

MATERIALS AND METHODS

Production and analysis of β -gal⁺ clones

Transgenic mice bearing a NSE-*nlslacZ* construct were generated as described previously (Bonnerot and Nicolas, 1993b, Forss-Petter et al., 1990). Stocks of transgenic animals were maintained by intercross breeding. Progeny were obtained from crosses between transgenic and non-transgenic C57BL/6xDBA/2 animals, and mice homozygous for the transgene were identified by PCR analysis with the appropriate oligonucleotides for *lacZ* (Bonnerot and Nicolas, 1993). The β -gal⁺ clones were obtained from 10 males of one transgenic NSE-*nlslacZ* line (NSE-1). For the production of clones, homozygous transgenic males were crossed with non-transgenic females. Brains from postnatal day 12 to 20 progeny were dissected. In toto X-gal staining (Sanes et al., 1986, Bonnerot and Nicolas, 1993, Bonnerot and Nicolas, 1993) was performed as follows: dissected brains were fixed for 30 minutes in cold 4% paraformaldehyde in PBS at 4°C, washed several times in cold PBS, incubated for 48 hours in X-gal at 30°C, stored at 4°C in X-gal for several days and cleared in 70% glycerol for 48 hours to increase the detection of the clones. The long incubation period at 30°C and the long storage in X-gal at 4°C increase the efficiency of staining deep neurons. That this protocol is appropriate to detect β -gal⁺ neurons deep in the IGL is illustrated on several examples in Fig. 1. Finally, the brains were carefully examined for the presence of β -gal⁺ cells. The enumeration of the cortical β -gal⁺ cells was done by direct observation using a stereomicroscope and strong illumination. The distribution of labelled cells was recorded in toto by drawings using a camera lucida. From the drawings, a schematic representation of the clones on a flattened cerebellum was established.

In situ hybridisation

In situ hybridisation was performed on 15 μ m-thick cryostat brain sections from P15 animals, using an antisense LacZ digoxigenin-labelled RNA probe (plasmid kindly provided by Sharaghim Tajbakhsh) as described (Groves et al., 1995). Control sections were taken from nontransgenic P15 C57BL/6xDBA/2 brains.

Histological analysis

Cerebella containing β -gal⁺ cells were transferred into PBS (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.3) containing 30% (w/v) sucrose and soaked for 48 hours. 30 μ m cryostat sections were then cut and about 120 sections were obtained for each cerebellum. Two series of slides were produced: one was counterstained with neutral red (1% w/v in 50 mM sodium acetate pH 3.3) and the other was not counterstained. Then the sections were briefly dehydrated in successive dilutions of ethanol (70%, 90%, 99% and 100%), cleaned in toluene and mounted in Eukitt (Kindler GmbH). Histological analysis was performed on both counterstained and unstained slides. General topography of the cerebellum was best determined on neutral-red-stained slides; whereas, the size and the number of β -gal⁺ cells was best obtained on unstained slides. The neuronal classes have been defined using the following criteria, including their position and the size of their nuclei (Fig. 6). Medium and large cells in the ML have been classified as molecular layer cells. Most of them have been clearly identified as basket and stellate neurons on the basis of their typical morphology observed using 100 \times magnification. Other cells were classified as ML neurons when located above the PCL; their sizes were around 5.6 μ m. Finally, large (9 μ m) or medium (6 μ m) cells located below the PCL were classified as Golgi neurons. Typical examples of these various cases are illustrated in Fig. 11-L. Medium and large cell types in the IGL have all been classified as Golgi cells. Small cells in the IGL have been unambiguously identified as granule neurons. Large (10 μ m) β -gal⁺ cells in the PCL were classified as Purkinje cells, based on the size of their nuclei (Fig. 6) and cell morphology.

RESULTS

Production of brains exhibiting β -gal⁺ neuronal clones

A line of transgenic mice harbouring the *NSE-lacZ* (β -gal⁻) reporter gene has been constructed as previously described (Forss-Petter et al., 1990, Bonnerot and Nicolas, 1993b). Recombinant *lacZ* (β -gal⁺) clones in the CNS were obtained from crosses of transgenic *lacZ/lacZ* homozygous males with non-transgenic females. The β -gal⁺/ β -gal⁻ mosaicism in somatic cells is initiated by a random homologous recombination event between the duplicated sequences in *lacZ* and restoration of a functional *lacZ* (Bonnerot and Nicolas, 1993b). The neuron-specific enolase (NSE) promoter (Forss-Petter et al., 1990), which drives expression of *lacZ* transgene, allows visualisation of clonally related β -gal⁺ neurons. Forss-Petter et al. (1990) have shown that NSE *lacZ* lines exhibit neuron-specific, pan-neuronal β -galactosidase activity. Particularly in the adult cerebellum, Purkinje cells, granule cells and neurons of the deep cerebellar nuclei, but not glial cells, all express *lacZ*. In the NSE-1 transgenic line used for this study, RNA in situ hybridisation for the *lacZ* gene revealed a distribution of transcripts throughout the cerebellum, as is expected for a gene under control of the NSE promoter. Clones were obtained that contained Purkinje cells, Golgi cells and neurons in the ML and deep nuclei (Fig. 11-P); and there were also some clones that contained granule cells of the IGL (Fig. 1C). These data confirm that the NSE-1-driven *lacZ* gene is expressed in all classes of neurons and not in glial cells.

Description of the β -gal⁺ clones

The results presented below correspond to the analysis of cerebellum containing β -gal⁺ cell types that were previously shown to derive from the cerebellar plates, namely Purkinje and Golgi cells. Clones that contained inner granule cells will not be discussed here. The examination of 1200 brains of P12-P20 NSE-*lacZ* transgenic mice led to the detection of 128 such recombinant *lacZ* clones. It is likely that we detected all large and medium clones and a majority of small clones. However, clones with only a few cells are in principle difficult to detect when they have no contribution to the visible layers of the cerebellum. We estimate that the visible layers of the cerebellum in glycerol-cleared brains is about 50%. The relatively low frequency of cerebella exhibiting β -gal⁺ cells derived from the cerebellar plates (about one out of 10 brains) suggests that most of the positive cerebella contained β -gal⁺ cells that derived from a single recombinant clone. Because β -gal⁺ cells were always clustered even in small and medium clones, double recombination events would almost always produce two distant clusters of labelled cells. Clonal identity of β -gal⁺ cells for the various groups of clones described in this article has been statistically determined. The results of these tests will be presented at the appropriate place in the article and have been summarised in Table 1A-D.

Clones derived from the cerebellar plates were classified into two main categories depending on their size and longitudinal extent. There were 115 small clones, containing from 1 to about 80 visible cells, populating only one or two consecutive lobes (examples in Fig. 1E-H), and there were 13 large clones, containing up to 2141 visible cells, populating many consecutive lobes (examples Fig. 1Q,R).

Large clones

Cells present in the large clones were uniformly dispersed throughout the longitudinal dimension of the cerebellum (Fig. 3). These large clusters of β -gal⁺ cells are very unlikely to result from multiple recombination events in distinct precursors that have the potential to contribute to medium or small clones (Table 1A). They originate, rather, from a single recombination event in precursors having the potential to populate a **large area** of the cerebellum. Most large clones contributed to both rostral and caudal lobes and six had marked cells in all ten lobes. To give a clearer picture of the organisation of these large clones, they have been represented schematically on a flattened cerebellum (Fig. 4). The visible surface of each lobe was measured and represented by a horizontal rectangle. Folia 4 and 5 were considered together and folia 6 and 7 were split into 6a and b and 7a and b because these latter two displayed a deep secondary foliation. Each large clone corresponded to a unique longitudinal stripe along the cerebellum, and these large clones could be further subdivided into two classes. Five clones (95-258, 94-429, 94-256, 95-83 and 94-826) had a medial border that coincides with the midline; these are called median clones (M) and are allocated mainly to the vermis. Their lateral border is located either in the vermis (95-258, 94-429) or overlaps slightly with the adjacent hemisphere (94-256, 95-83 and 94-826). Eight clones had their two longitudinal borders in the cerebellar hemisphere and these are referred to as lateral clones (L). This longitudinal organisation of the large clones demonstrates that clonally related cells in the cerebel-

lum are widely distributed in the rostro-caudal dimension. Depending on the clone, the borders of the longitudinal stripe were located at different medio-lateral levels (Fig. 4); however, the clonal distributions shared a similar orientation in both the lateral and medial parts of the cerebellum. Therefore, the large clones clearly define a lateromedial level of cellular organisation in the cerebellum.

Clones not restricted to the cerebellum

Further analysis of the brains of 13 animals containing large clones showed that 6 participate in structures outside the cerebellum (Fig. 4). Three clones contributed to ipsilateral hindbrain-midbrain structures (abbreviated HM), of which two were median (clones M-HM 94-256 and 95-83) and the other lateral (L-HM 94-857). The other three clones contributed to many structures in the central nervous system (abbreviated CNS, clones M-CNS 95-258, 94-429 and L-CNS 94-452).

The frequency of large clones in the cerebellum is 13 per 1200 and we found that the frequency of clones contributing to many structures in the CNS is 6:1200. Therefore, the frequency of a double event is only 5.4×10^{-5} (Table 1B). Consequently, it is very unlikely that the two labellings observed in clones 95-258, 94-429 and 94-452 are independent and we consider that they result from a single recombination event. The same conclusion was reached for the HM clones (Table 1C). These observations led to a classification of the 13 clones into three groups based on the extent of their participation outside the cerebellum (Figs 4, 5). The three clones corresponding to precursors capable of participating in numerous structures of the central nervous system correspond to the colonization of either the whole vermis (2 clones) or the whole cerebellar hemisphere (1 clone) (Fig. 4). Altogether these clones colonize the entire surface of the cerebellum with little overlap (Fig. 5A). The clones corresponding to precursors capable of participating in only ipsilateral hindbrain and midbrain, or restricted to the cerebellum correspond to (1) clones with a smaller mediolateral extension but still a very large longitudinal extension (see for instance, 95-498, 94-499, 94-747, Fig. 4); they define smaller longitudinal

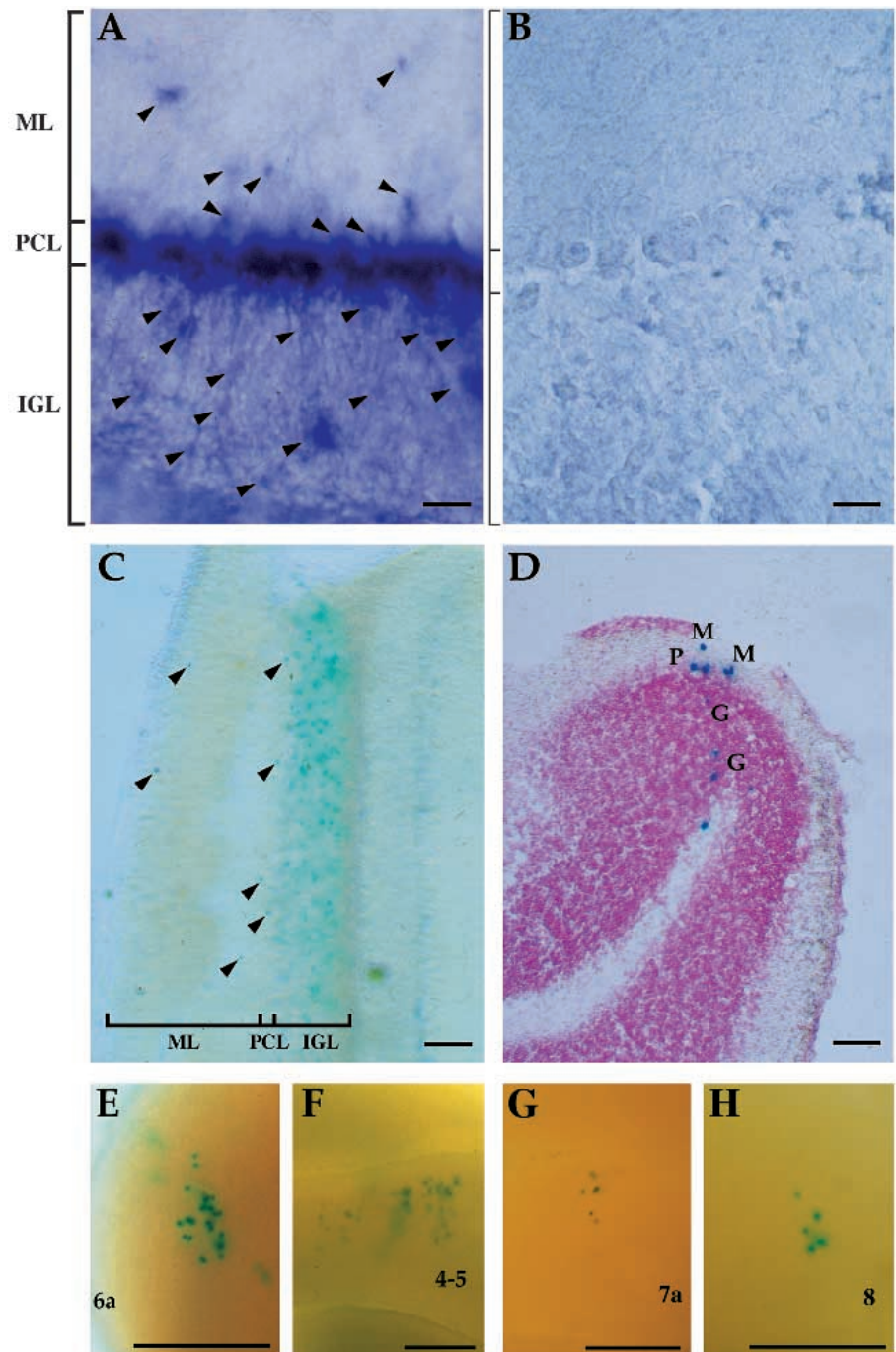
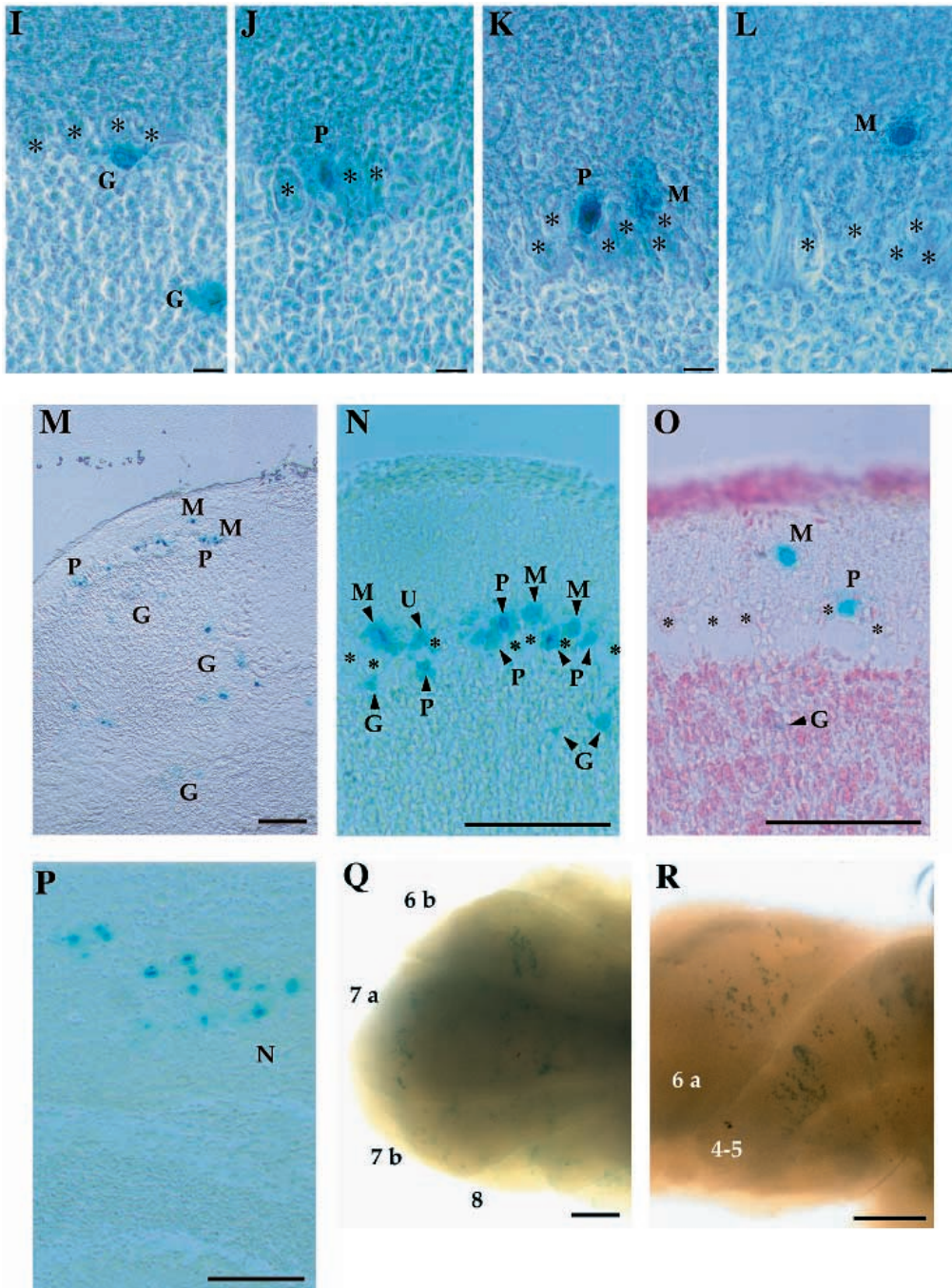


Fig. 1. Histological composition of the clones. (A) In situ hybridisation of a 10 µm cryostat section of a P15 *NSE LacZ* cerebellum with a digoxigenin-LacZ probe. Arrowheads: strongly positive cells in the molecular layer (ML) and internal granular layer (IGL). (B) Nontransgenic control P15 cerebellum, same *lacZ* probe. (C-H) Labelled cells in small clones. (I-L) Labelled cells in large clones. (C) Clone 95-34. Labelled granule cells in the IGL in lobe 7a. A few dispersed cells of the same type are also labelled in the ML and Purkinje cell layer (arrowheads). (D) Clone 95-143. Labelled cells in the ML, Purkinje cell layer and IGL (Golgi cells). (E) Clone 95-143, in toto. 53 visible cells are clustered in lobe 6a. (F) Clone 94-844, in toto. 82 visible cells are clustered in lobes 4-5. (G) Clone 94-566, in toto. 10 visible cells are clustered in lobe 7a. (H) Clone 94-656, in toto. Seven visible cells are clustered in lobe 8. In all cases, note the general orientation of the clone perpendicularly to the main axis of the lobes. (I-L) Identification of the neuron types at $\times 1000$ in clone 95-83. Asterisks: β -galactosidase negative Purkinje cells. (I) Golgi neurons in the IGL. The Golgi neuron just below the PCL can be



distinguished from Purkinje cells by its morphology. (J) A labelled Purkinje cell. (K) A labelled Purkinje cell and a labelled basket cell just above the PCL. (L) A labelled cell (probably a stellate cell) in the ML. (M) Clone 94-452. Labelled cells in ML, PCL and IGL (Golgi cells). (N) Higher magnification of clone 94-452. The three labelled cells in the ML are probably basket cells; the three labelled cells in the IGL are Golgi cells; five Purkinje cells are also labelled. (O) Clone 95-83. The cell labelled in the ML is probably a stellate cell; a Purkinje and a Golgi cell are also labelled. The tearing at the level of the PCL is due to our fixation protocol for the brain. (P) Clone 94-452. Labelled interneurons in a deep nuclei. (Q) In toto dorsal view of clone 94-452 in which there are 2141 visible cells in the hemispheric cerebellum. (R) In toto rostral view of clone 95-83, in which there are 2007 visible cells in the vermis. In Q and R, note the tendency of the clusters of labelled cells to be oriented perpendicularly to the lobes. ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granular layer; M, molecular layer neuron; P, Purkinje cell; G, Golgi cell; N, deep nuclei neurons. U, non-identified labelled cell. Bars are: A,B, 20 μ m; C,D, 50 μ m; E-H, 250 μ m; I-L, 10 mm; M-P, 60 μ m; Q,R, 500 μ m.

stripes and were observed only in the lateral cerebellum (Fig. 5C,D); or (2) clones with a larger mediolateral extension and a maximal longitudinal extension across all cerebellar lobes; they were observed only in the median cerebellum (94-256, 95-83, 94-826) (Figs 4, 5B,C).

Altogether this classification suggests two distinct modes by which progenitors contribute to the lateral and medial cerebellum. The lateral domain would be constructed from clones exhibiting a progressive restriction in their lateromedial extent (Fig. 5A-D); whereas, the median domain would be constructed from clones with no progressive restriction within the domain (Fig. 5A-C) and only later would be restricted into small rostrocaudal domains (small median clones, Fig. 2). Although this last conclusion necessitates the examination of more median clones before being completely accepted, the main difference between the lateral and the median domains would be that precursors of the median domain would be capable of contributing to the whole domain, whereas the corresponding precursors in the lateral domain would exhibit progressive mediolateral restriction (compare lateral and median HM clones). Importantly, the data also suggest that the earliest detectable precursors of the cerebellum – represented by the clones contributing to structures outside the cerebellum – do not constitute a single polyclone but two distinct polyclones, a lateral one and a median one.

Small clones

Cells in the 115 small clones are always clustered (Fig. 1E-H) and there is no systematic organisation into two or more separate groups either rostrocaudally or lateromedially. Therefore, genealogically

Table 1. Statistical tests

(A) Can large clones occur as a result of multiple events of recombination in precursors of medium clones?			
	Medium clones with 50 to 100 cells	Large clones with 1000 cells or more	Expected frequency of large clones following the hypothesis
Frequency	4/1200	8/1200	[4/1200] ¹⁰
We tested whether the observed number of large clones (8) equals the expected number of large clones following the hypothesis of several events of recombination (2×10 ⁻²²). $\chi^2 = 4.10^{26}$ (1 degree of freedom (df)) $P < 10^{-4}$. Therefore, we rejected the hypothesis.			
(B) In large cerebellar clones, is the contribution to the CNS due to a second independent event of recombination?			
	Large CNS clones*	Large cerebellar clones	Large CNS + large cerebellar clones (L and M - CNS)
			Expected (if 2 recombinations)
Number of clones	6	13	0
Frequency	6/1200	13/1200	6/1200×13/1200 = 5.4×10 ⁻⁵
			Observed 3 3/1200 = 2.5×10 ⁻³
*With or without contribution in the cerebellum. We tested whether the observed number of large cerebellar clones with a contribution to the CNS (3) equals the expected number (6.5×10 ⁻²) following the hypothesis. $\chi^2 = 132$ (1 df) $P < 10^{-4}$. Therefore, we rejected the hypothesis.			
(C) In large cerebellar clones is the contribution to the Met-Mes due to a second independent event of recombination?			
	Medium Mes-Met clones*	Large cerebellar clones	Medium Mes-Met + large cerebellar clones (L and M - HM)
			Expected (if 2 recombinations)
Number of clones	9	13	0
Frequency	9/1200	13/1200	9/1200×13/1200 = 8.1×10 ⁻⁵
			Observed 3 3/1200 = 2.5×10 ⁻³
*With or without contribution in the cerebellum. We tested whether the observed number of large cerebellar clones with contribution to the hindbrain-midbrain (3) equals the expected number following the hypothesis (9.7×10 ⁻²). $\chi^2 = 86$ (1 df) $P < 10^{-4}$. Therefore, we rejected the hypothesis.			
(D) Frequency of large clones contaminated by a small clone?			
	Small clones	Large clones	Expected small clones contaminating large clones*
Frequency of clones	115/1200	13/1200	1/1000 = 1.2/1200
*The contaminating clone would be in three cases out of four outside the area delimited by the large clone.			
(E) Are small clones with to distant clusters due to 2 independent events of recombination?			
	Small clones	Expected number of 2 events	Observed number of 2 clusters
Number of clones	115	1200/100 = 12	8
Frequency	1/10	(1/10) ² = 1×10 ⁻²	8/1200 = 0.7×10 ⁻²
We tested whether the observed number of small clones with two distant clusters (8) equals the expected number following the hypothesis (12). $\chi^2 = 1.3$ (1 df) $P = 0.25$. The difference is not significant.			

related cells remain geometrically grouped, although there is a frequent intercalation of β -gal cells between β -gal⁺ neurons. The general topography of small clones is preferentially oriented rostrocaudally (orthogonal to the main axis of the lobes). These three characteristics, clustering, intercalation with β -gal cells and rostrocaudal orientation, are also frequently observed in the patches of the large clones (Fig. 1Q,R).

100 out of the 115 small clones were obtained in animals devoid of any labelling, either in the hindbrain-midbrain or in

the rest of the CNS (Table 2). They reflect the existence of a pool of cells strictly allocated to the primitive germinal epithelium of the cerebellum. In addition, it is possible that the extracerebellar β -gal⁺ cells observed in various structures in the other 15 animals originated from recombination events in independent precursors (Table 2). However, because the strict clonal identity of these 15 small clones is difficult to definitively establish, it remains possible that a small percentage of cerebellar precursors are capable of contributing not only to small clones, located at various positions in the cerebellum

Table 2. Participation of the clones in structures outside the cerebellum

Clones	R	HM	CNS	Total
Large				
Median	1	2	2	5
Lateral	4	1 (2)	1	8
Small				
Median	47	1 (2)	3 (2)	55
Lateral	52	2 (2)	2 (2)	60

Clones have been classified in relation to their participation in structures adjacent to the cerebellum (hindbrain-midbrain structures, HM) and to structures dispersed in the central nervous system (CNS). Other clones are strictly restricted to the cerebellum (R). The numbers in parenthesis refer to the number of clones in which the labelling outside the cerebellum is contralateral.

(Fig. 2), but also to heterogeneous structures in the CNS or HM (not strictly allocated precursors).

Histological composition of the large clones

We have examined the typological composition of the large clones on histological sections and the β -gal⁺ cells have been classified by criteria that compare their relative position (Fig. 1D,I-P), the size of their nuclei (Fig. 6) and their morphology (Fig. 1I-L). It was found that the nuclei of cells within clones obviously restricted to inner granule cells (Fig. 1C) had a mean size of 3.4 μ m (Fig. 6A). In contrast, the nuclei of cells in the IGL of large clones, which also contained β -gal⁺ cells in the PCL and ML, had a size of 5.9 μ m or 9.0 μ m (Fig. 6B). These latter cell types are therefore larger than inner granule cells and must correspond to small and large Golgi neurons. A few of them are located below the PCL. The nuclei of cells in the PCL and ML from large clones have a bimodal size range of 5.6 μ m and 9.7 μ m, respectively (Fig. 6D). We measured the size of individual nuclei in Purkinje neurons from control preparations by staining for Calbindin, and determined a mean size of 9.7 μ m (Fig. 6E). Therefore β -gal⁺ cells with a nuclei of 9.7 μ m in the PCL are probably Purkinje neurons (Fig. 6D), and those cells with a smaller nuclei (5.6 μ m), usually positioned in the ML or just above the PCL, probably correspond to either basket or stellate neurons (ML neurons).

Clones usually contain β -gal⁺ cells in the IGL and/or in the deep nuclei, indicating that the deep regions of the cerebellum do not escape the histochemical labelling in our experimental conditions. All large clones are composed of Purkinje cells, Golgi cells and neurons of the ML (Table 3 and 4). Furthermore, most of them (11/13) also contain neurons in the deep nuclei (Table 3). These results indicate a common clonal precursor for all these neuron classes. The two clones (94-747 and 94-852) that do not contain neurons in the deep nuclei are among the smaller clones of this group and, although it is possible that β -gal⁺ cells in these deep layers escaped detection, another possibility is that the precursors of these clones have a restricted potentiality. The analysis of more clones of this

Table 3. Histological composition of the large clones

Clone	Cerebellar neurons				
	Deep nuclei	G/IGL	PCL	ML	IGL
Median					
95-258	+	+	+	+	—
94-429	+	+	+	+	—
94-826	+	+	+	+	1 group
95-256	+	+	+	+	—
95-83	+	+	+	+	—
Lateral					
94-452	+	+	+	+	—
94-499	+	+	+	+	—
94-857	+	+	+	+	—
94-451	+	+	+	+	—
95-498	+	+	+	+	—
94-747	+	+	+	+	1 group
94-852	—	+	+	+	—
94-698a	+	+	+	+	—

The identification of the neurons was as detailed in Materials and Methods and is illustrated in Figs 1 and 6. G/IGL, Golgi in the internal granular layer; PCL, Purkinje cell layer; ML, molecular layer; IGL, granule neuron in the internal granular layer.

category will help to distinguish between these two hypotheses. Finally, the large clones are devoid of β -gal⁺ granule cells, except for a small group of such cells in clones 94-826 and 94-747 (Fig. 6C). These exceptional large clones likely represent a double recombination event in two distinct precursors. Because β -gal⁺ granule cells can be detected by our labelling protocol, the absence of labelled granule cells in the majority of large clones indicates that the precursors of granule cells have a distinct clonal origin.

Rostrocaudal dispersion of the neuronal classes

The various neuronal classes observed in the rostrocaudal

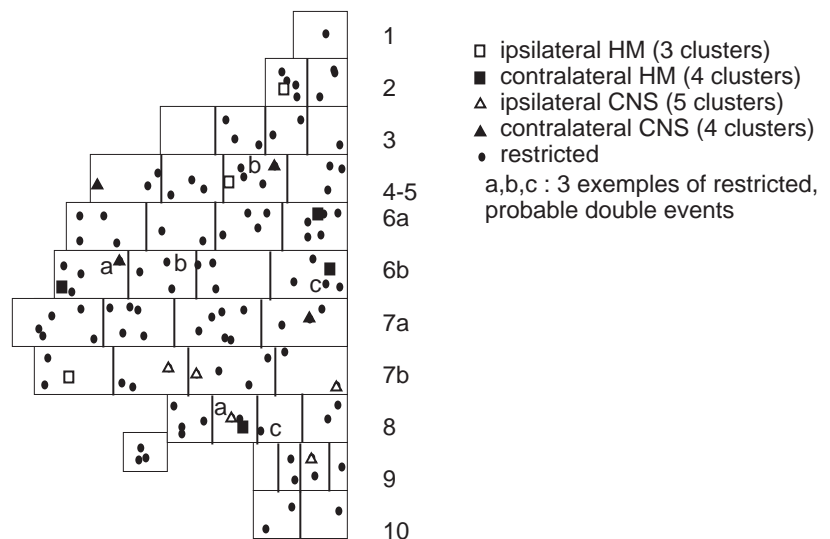


Fig. 2. Distribution of the small clones on the cerebellar surface. The 115 small clones are described on a flattened hemiserebellum. In this representation, the surface of each lobe was measured in the cerebellum and the distribution of small clones is represented by a symbol. The significance of the symbols is indicated on the left. HM, CNS are defined in Fig. 4. Numeric identification of the folia is indicated on the right. Folia 4 and 5 were considered together as a single lobe. Folia 6 and 7 were each considered separately because both displayed a deep secondary foliation.

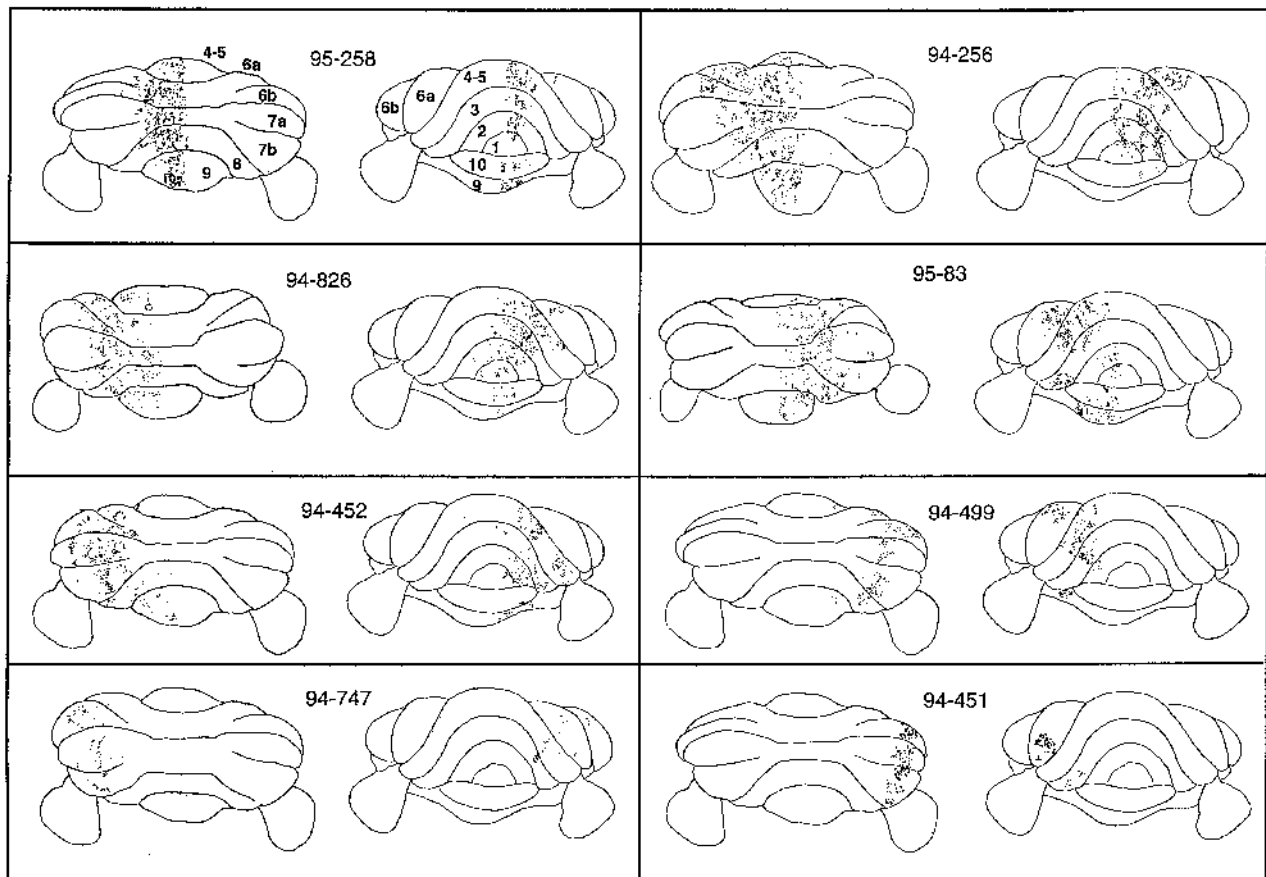


Fig. 3. Distribution of β -gal⁺ cells in the large clones. Camera-lucida drawings of representative large cerebellar clones, showing dorsal and ventral sides and the location of visible β -gal⁺ cells. The number on the top corresponds to the identification of the clones. Numeric identification of folia is also indicated.

dimension of the cerebellum have been analysed by comparing cell distributions along this axis. Examples of two median clones and one lateral clone are detailed in Fig. 7. A similar dispersion profile was observed for Purkinje cells, Golgi neurons and neurons in the ML. What is particularly evident is that all cells were distributed along the whole rostrocaudal dimension of the cerebellum. A similar dispersion pattern was observed in the clustering of β -gal⁺ cells in small clones (Figs 1E-H, 2) and demonstrates a moderate mediolateral and rostrocaudal dispersion of clonally related cells.

Estimation of the number of precursors of Purkinje neurons

The proportions of the various cortical neurons (Golgi cells, Purkinje cells and neurons in the ML) were determined for the five largest clones, which represent the progeny of an early precursor cell labelled before strict allocation to the cerebellum (Table 4). Neurons of each class have been identified and scored for 8% of the histological sections examined, which comprised all regions along the rostrocaudal axis of the cerebellum. From these data, the total number of cells in each clone has been estimated by extrapolation to the whole cerebellum. The comparison of these data with the *in toto* enumeration of β -gal⁺ cells (Fig. 4) shows that twice as many β -gal⁺ cells are scored on histological sections. This result is not

unexpected since only about one third of the neuronal layer occupies a superficial position in the mature cerebellum. Since a ratio close to two is obtained for all five clones, *in toto* counts and counts on histological sections are mutually consistent.

This latter analysis permitted an estimation of the total number of β -gal⁺ Purkinje cells in each clone (value A, Table 4). Obviously this value differed from one clone to another with a maximum difference of 2-fold (clone 95-258, A = 564; clone 95-83, A = 1357), suggesting that the number of Purkinje cells can vary in those clones whose precursors are founded before the period of strict allocation of cells to the cerebellum. These differences between clones are not due to variations of the relative composition of different neuronal classes, since all exhibit the same relative composition (Table 4, column %). The precursor cells of the clones therefore have similar histogenic potentials.

In contrast, a good correlation between the surface occupied by the clones and their Purkinje cell number was observed (Fig. 4, Table 4). The variable potential of clones to occupy a certain area of the cerebellum may therefore explain the variation in cell number. Significantly, clones with the earliest origins (clones M-CNS) are no bigger than later originating clones (clones M-MH). To account for this fact, an estimation of the mean number of Purkinje neuronal precursors was calculated from the mean size of the Purkinje cohorts for these clones. If our sample of large clones is representative of the diversity of

Purkinje progenitor cells and if we approximate the total number of Purkinje cells in the medial and lateral territories to 40800 (which equals the 81,600 total Purkinje neurons in one hemocerebellum (Vogel and Herrup, 1993, Baader et al., 1996)), then the size of the polyclonal group of founders is $(40800 \times A^{-1})$ cells; this number correlates to 45 cells for the medial territory and 34 cells for the lateral territory.

DISCUSSION

The method

In this study, we have examined neuronal clones that constitute at least some of the cells that populate the cerebellum. The targeting of transgene expression to all neurons independently of their topographic domain allows the classification of these clones in relation to their contribution to structures outside the cerebellum. The random nature of the cell labelling technique made it possible to compare the contribution to the cerebellum from clones whose progenitors have different birthdates. Moreover, the targeting of all neurons, independently of their specific cell type, made it possible to define a common progenitor for some classes of cerebellar neurons.

Our analysis differs from other retrospective analyses, in which chimerism is produced very early during embryogenesis, before the allocation of cells from the inner cell mass to the embryo proper (Wetts and Herrup, 1982a), or where mosaicism based on X-inactivation is exploited (Baader et al., 1996), because, in both these approaches, cell labelling is restricted to only one short period of development. Our method also differs from prospective methods using the quail/chick chimeric system (LeDouarin, 1969) since, in our strategy, there is neither temporal nor spatial specification of the group of cells labelled. Furthermore, we follow the fate of descendants from individual cells in unperturbed embryos, rather than a population of transplanted cells whose previous clonal relationships may be masked after relocation in the host embryo. Thus, our analysis brings novel insights into the development of the cerebellum in mice and the data presented here may help to resolve certain contradic-

tions and uncertainties in the literature, in addition to revealing the previously undescribed sagittal clonal patterning of the cerebellum and the subdivision of the presumptive territory of each hemocerebellum into two polyclonal domains.

Progenitors common to the neurons of the deep nuclei, Golgi and Purkinje cells and interneurons of the molecular layer

The traditional view of cerebellar development in mammals is that cerebellar components derive from two sources: a primary

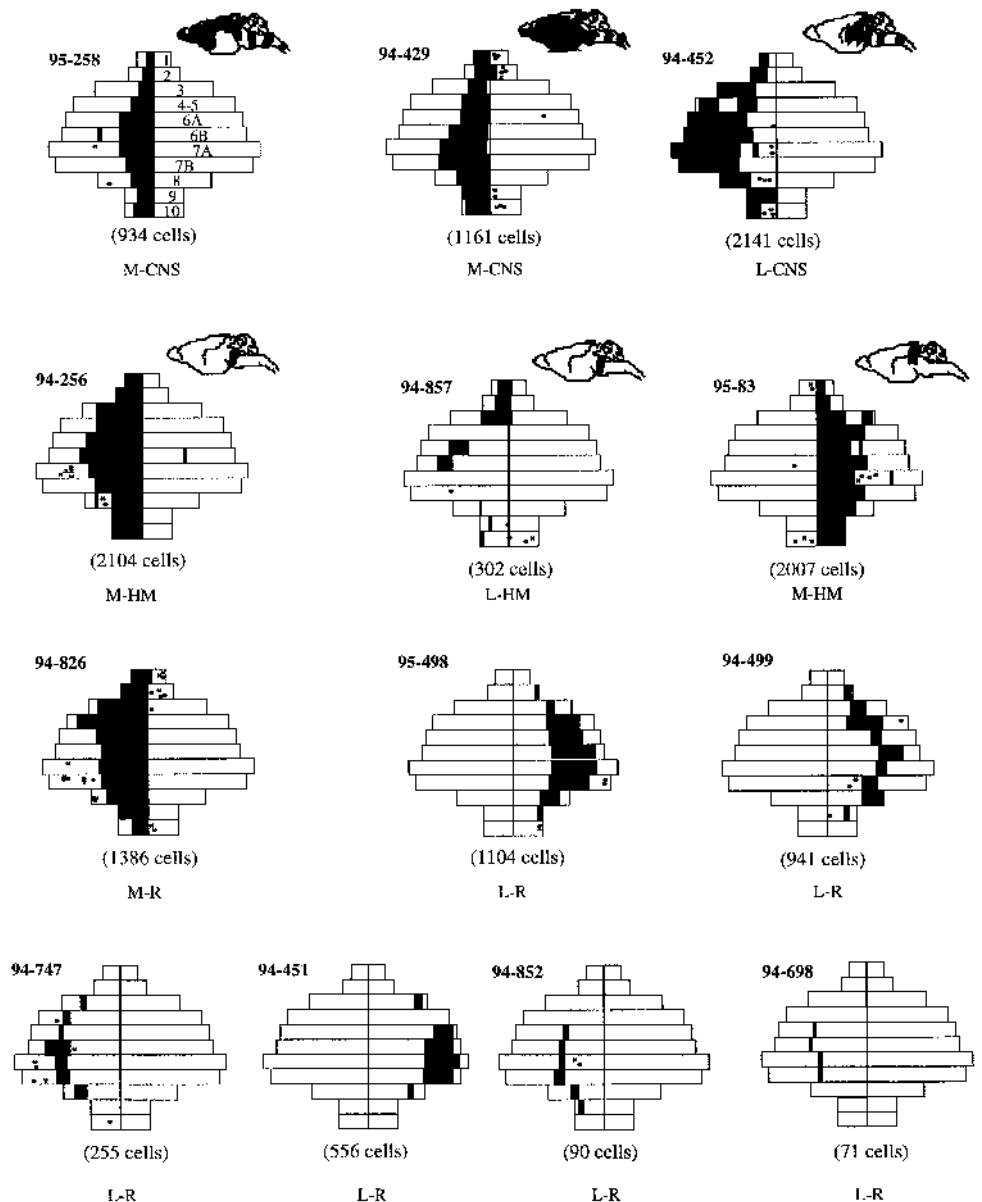


Fig. 4. Schematic representation of the 13 large clones. The clonal distributions of the large clones are depicted on a flattened cerebellum (see Fig. 2). Numeric identification of the folia is indicated on cerebellum 95-258. Numbers in parenthesis are the total number of β -gal+ cells counted per clone (i.e. in toto). Individual cells are represented by asterisks. The presence and location of ipsilateral β -gal+ cells in the brain stem are schematically represented in black dashes. M-CNS and L-CNS, median or lateral clones with extracerebellar participation in numerous structures of the central nervous system. M-HM or L-HM, median or lateral clone with extracerebellar participation in hindbrain and midbrain structures only.

Table 4. Estimation of the number of cells in Purkinje cohorts

Clone	Type	Purkinje % (a)	Golgi % (b)	ML % (c)	T	A	F
95-258	M-CNS	27.8 (47)	58.6 (99)	13.6 (23)	2028	564	72
94-429	M-CNS	29.5 (56)	58.9 (112)	11.6 (22)	2280	673	60
95-83	M-HM	35.9 (113)	56.8 (179)	7.3 (23)	3780	1357	30
94-256	M-HM	34.0 (86)	54.9 (139)	11.1 (28)	3036	1032	39
94-452	L-CNS	29.0 (98)	59.2 (200)	11.8 (40)	4056	1176	34

For each clone, Purkinje (a), Golgi (b) and β -gal⁺ neurons in the ML (c), were identified and counted in 1/12 (that is 10) transverse sections selected randomly from rostral to caudal. Number in parenthesis corresponds to the number of cells and is preceded by the % of neurons of the class within the clone. Then the total cell number in each clone, T, [T = (a+b+c)×12] and the total number of Purkinje cells, A, [A = a×12] were deduced. The A value has been used to calculate a theoretical number of founder cells, F, of the lineage of Purkinje cell, assuming an equal participation of the founders to the lineage (see text). It corresponds to the ratio between the total Purkinje cell number (40800) in either the median or the lateral territory occupied by the clone and the Purkinje cell number in the clone (F = 40800×A⁻¹). M-CNS : median clone contributing to structures in the CNS.

M-HM : median clone contributing to structures in the midbrain. L-CNS : lateral clone contributing to structures in the CNS. ML: Molecular layer cells.

ventricular germinal source produces Purkinje cells and Golgi interneurons, and a second germinal source at the rhombic lip produces the EGL from which granule cells derive. Basket and stellate interneurons have been thought to derive also from the EGL because they are born at a time when no mitotic activity is observed in the ependymal layer. In contrast, transplantation studies in the chick have indicated that at least some basket and stellate interneurons derive from the ventricular epithelium (Hallonet et al., 1990, Alvarez-Otero et al., 1993). This has led to the hypothesis that basket and stellate cells either have a different origin in birds and mammals or that the last mitoses of basket and stellate progenitors occur in a place other than the primary ventricular epithelium (Alvarez-Otero et al., 1993). Zhang and Goldman (Zhang and Goldman, 1996) have used retroviral labelling to mark several cells in the EGL or postnatal cerebellar white matter of the rat, and have suggested that basket and stellate cells are derived from progenitor cells located in the white matter.

The typological analysis of the lateral and medial large *lacZ* clones demonstrate unambiguously that there are common progenitors for interneurons (basket and stellate cells) of the ML and Purkinje and Golgi cells (Table 3, Fig. 1). This finding suggests that, in mammals as in the chick, all these neuron classes derive from the ventricular epithelium and that the mitoses producing interneurons of the ML occur probably in the white matter. Our analysis clearly indicates that neurons in the deep nuclei and the cortex also share common precursors (Table 3, Fig. 1P).

These neuronal classes are known to be produced sequentially from E11 to P15 starting with the neurons of the deep nuclei (E11) and later the Golgi (E12 to 15) and Purkinje cells (E12) (Miale and Sidman, 1961); whereas, basket and stellate cells are produced postnatally up to P15. The common clonal progenitors identified here therefore have the properties of self-renewing stem cells, which give rise to these different neuronal classes asymmetrically and sequentially through cell division. A detailed histological analysis of the 115 small clones has been done in order to study the evolution of the potentialities of these self-renewing stem cells; however, for the sake of simplicity, these data will be presented elsewhere.

Finally, the histological composition of large clones confirms an independent origin for the interneurons of the IGL (Altman and Bayer, 1978), since none of the large clones contained this class of neurons. This same observation was

made for both the median and lateral clones (Table 3), therefore our results demonstrate, for the first time, the clonal independence of the primary and secondary germinal epithelia at the earliest stages of cerebellar formation.

Limited dispersion of cortical cerebellar neurons

The study of Alvarez-Otero et al. (1993) on the fate of transplants in subregions of the presumptive territory of the chick cerebellum has given a general picture of the movement and migration of cerebellar cells. In our experiments, only neurons and not glial cells are detected and this simplified the analysis of neuronal dispersion within the clones. When considering the behaviour of individual cells, there is obviously some rostro-caudal and lateromedial dispersion resulting from intercalation of clonally unrelated cells for all types of neurons and this was, in fact, observed for both large and small clones.

To assess the dispersion of clones at the primitive stage of the germinative ventricular epithelium, we considered, separately, the areas colonised by Golgi, Purkinje cells and neurons in the ML of large clones. The area occupied by Purkinje cells is not distinct from the area occupied by Golgi cells (Fig. 7), and neither can be distinguished from the total area occupied by the clones, which is reflected in the results of the in toto analysis. In addition, the systematic clustering of cells in the small clones (Figs 1, 2) shows that both latero-medial and rostrocaudal dispersions are limited. Therefore, the area of the clones delimited in toto likely represents their primary extension in the germinative epithelium. Limited dispersion and general respect of the midline are observed at the clonal level for Purkinje and Golgi cells, as well as for their precursors. These features may be related to the overall radial organisation of glial processes and to their eventual guidance of cells migrating into the cortex. However, there are obviously instances of scattered cells on the contralateral side of median clones. Therefore, in the adult cerebellum, the midline cannot be considered as an absolute boundary.

The presumptive territory of the cerebellum is organised into distinct polyclonal pools of precursors

It has been shown that at HH10 (10- to 12-somite stage, closed neural tube), the presumptive territory of the chick cerebellum does not respect the mesencephalic-metencephalic constriction of the neural tube (Martinez and Alvarado-Mallart, 1989,

Hallonet et al., 1990, Alvarez-Otero et al., 1993). This fact has posed, but not resolved, the question of the di- or even multi-neuromeric origin of the cerebellum (Marin and Puelles, 1995). This problem is complicated by the recent demonstration of unexpected rostrocaudal morphogenetic movements taking place between stages HH10 and HH16, which change the position of the midbrain-hindbrain boundary (Millet et al., 1996).

Our results indicate that the presumptive territory of the cerebellum is organised into polyclonal pools of precursors and suggests that, in the adult, there are two main intra-cerebellar domains (Fig. 5). Thus, the five large median clones and four of the lateral clones described here are best explained by postulating the existence of two distinct pools of precursors, one contributing to a median territory and the other to a lateral territory.

The clones that labelled very early, which are not restricted to the cerebellum, contribute to either of these territories but not to both. They completely fill them. These two postulated territories share a parasagittal border which consists of an overlapping region. The territories are perpendicular to the main axis of the lobes of the cerebellum and are topographically complementary. Such a complementarity between the two corresponding pools of precursors would be sufficient to explain the formation of the whole primary germinal epithelium of the cerebellum. Before the morphogenetic 'rotation' of the two hemispheres (see next paragraph), the median pool may occupy a rostral ('mesencephalic') position, and the lateral pool a caudal ('metencephalic') position, as suggested by fate mapping in the chick. Again, our data also indicate that at least one, clonally independent pool is at the origin of the EGL. However, our analysis does not provide any information on the location of the parasagittal overlapping region between the two pools of precursors in relation to the midbrain-hindbrain boundary or to the gene expression domains of *En-1*, *Wnt-1*, *Fgf-8* or *Otx-2* (McMahon et al., 1992, Crossley and Martin, 1995, Millet et al., 1996).

These observations also raise questions about the segmental origin of the cerebellum. The early embryonic hindbrain is initially characterised by a segmental organisation (Lumsden and Keynes, 1989) into eight rhombomeres, each of which represents an independent polyclonal unit (Fraser et al., 1990) whose transverse boundaries delimit domains of developmental gene expression (Wilkinson et al., 1989, Sundin and Eichele, 1990, Wilkinson, 1993), which are themselves cell autonomous (Guthrie et al., 1992, Kuratani and Eichele, 1993). This segmental rhombomeric organisation has also been observed in the adult (Marin and Puelles, 1995, Wingate and Lumsden, 1996). However, although the adult hindbrain is composed of a segmental array of lineages derived from individual rhombomeres, the boundaries between these polyclonal units are not sharp (Marin and Puelles, 1995, Wingate and Lumsden, 1996).

In this study, we have examined the fate of individual clones in adult cerebellum. We show that it is formed from at least two polyclonal pools of precursors that are defined early in development. They share with the adult hindbrain the characteristic that their common border is not sharp. Furthermore, the clonal organisation observed in the lateral territory is reminiscent of the cellular organisation in rhombomeres 2 to 6 (Wingate and Lumsden, 1996). In both cases, cell mixing is

asymmetric: in rhombomeres 2 to 6, there is a lack of spread along the longitudinal axis and an extensive mediolateral cell mingling; in the cerebellar lateral territory, there is a pronounced longitudinal clonal dispersion but a restricted mediolateral distribution (Fig. 4). Considering that the presumptive cerebellar territory undergoes a morphogenetic rotation (see below), these data imply that there is a conservation of cell mingling behaviours both along the two axis and between rhombomeres and the cerebellar lateral territory.

Because of this asymmetric cell mingling, clones participating to these territories have a distribution that is not directly dependent of the polyclonal border: even medium clones have a rostrocaudal organisation whatever their position. They are restricted in their mediolateral dimension on both sides. Therefore, it raises the possibility that, at least the lateral territory, is rapidly constituted of small parasagittal domains and/or that all cells have an asymmetric behaviour, dispersed in the rostrocaudal dimension and restricted in the lateromedian dimension.

In contrast, we tentatively propose that cells in the median territory have a different behaviour, since only large clones with no mediolateral restriction have been obtained. This second territory may, therefore, be specified in a different manner. The examination of more clones is necessary to confirm this interpretation and to deduce the rules at work in this territory.

In the absence of a correlation between these territories and the domains of developmental gene expression, it is not possible to definitively conclude whether these two territories are merely polyclonal growth units or developmental compartments. If, however, cerebellar development has a segmental basis, similar to that of rhombomeric organisation (Marin and Puelles, 1995, Wingate and Lumsden, 1996), such early organisation clearly persists in adults since our analysis focused on postmitotic neurons.

Morphogenetic rotation of the hemispheres primordia

In the quail/chick system, it has been established that the rostralmost part of the cerebellar presumptive territory in the neural tube later occupies an anteromedial position in the mature cerebellum (Martinez and Alvarado-Mallart, 1989, Hallonet et al., 1990, Alvarez-Otero et al., 1993). The caudal portion of the presumptive territory occupies a lateral and posteromedial position. The extent of variations between grafts in those studies makes it impossible to draw a precise fate map of the cerebellum in the neural tube (Alvarez-Otero et al., 1993).

In contrast, our analysis of individual cell behaviour indicates a **systematic** longitudinal arrangement of medial clones from the most rostral to the most caudal lobes (Figs 3, 4) and a similar **systematic** longitudinal orientation of lateral clones throughout the cerebellum (Figs 3, 4). Because the area occupied by the large clones probably reflects their extension into the ventricular epithelium, this suggests that cells of each hemisphere apparently undergo a significant rostrocaudal and caudolateral rotation between their relative positions in the early neural tube and the mature cerebellum. The final mediolateral organisation of cells in the cerebellum is therefore orthogonal to the organisation of their precursors in the neural tube.

This apparent rotation could be due to either a global deformation of the neural tube, passively displacing anterior territories towards the midline, or may result from differential modes and rates of cell growth in relation to their position along

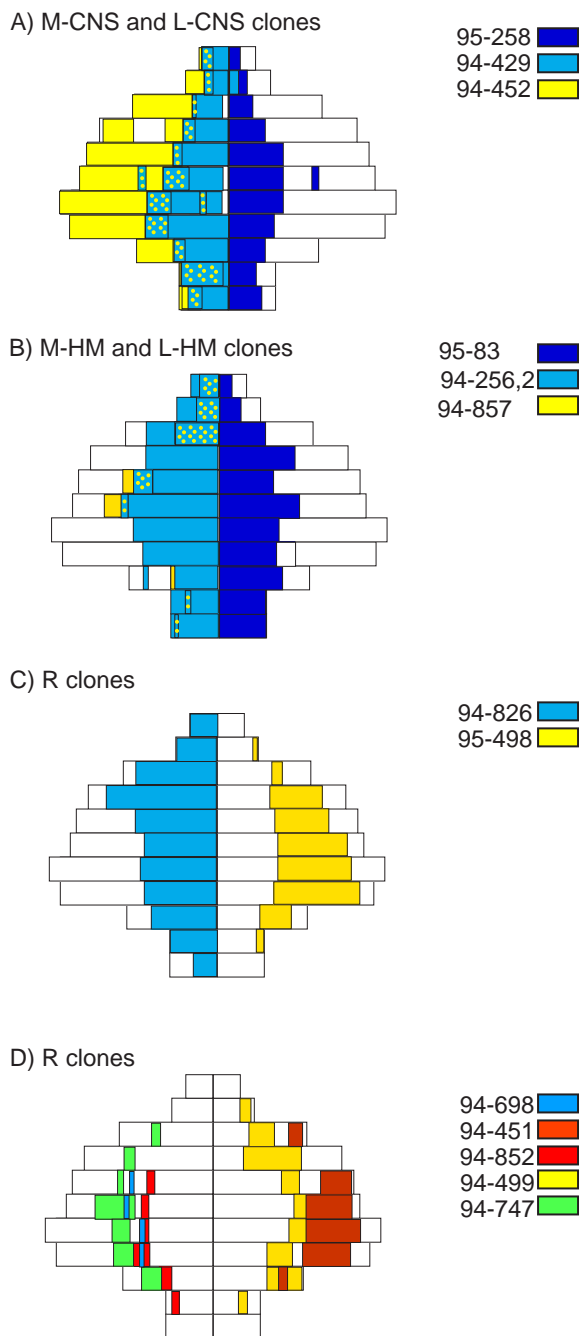


Fig. 5. Comparison of the clones classified by their contribution to structures outside the cerebellum. Large clones have been classified in three categories: those contributing also to neuronal structures outside the cerebellum, M-CNS and L-CNS; those contributing also to structures adjacent to the cerebellum, M-HM and L-HM; and those restricted to the cerebellum only, M-R and L-R. Lateral and median clones of the same category have been represented by different colours on the same cerebellum. (A) M-CNS and L-CNS clones (clone 95-258 has been represented on the left side for clarity). (B) M-HM and L-HM clones. (C,D) M-R and L-R clones.

the neural tube. According to this last hypothesis, distinct cell behaviours would create the different clonal domains observed in this study. An analysis of a larger sample of clones is necessary to define more precisely the differential cell growth between lateral and medial domains and to deduce a complete model of associated morphogenetic movements. Finally, such an analysis would have to be done at the cellular level in conjunction with an examination of the spatiotemporal expression of pivotal developmental growth factors, such as the *Wnt-1* and *Fgf-8* gene products.

Parasagittal clonal patterning of the lateral domain

The expression pattern of certain genes (Calbindin, cGMP-dependent protein kinase, Purkinje-cell-specific glycoprotein, PEP 19 et *L7/pcp-2*) has shown that the cerebellum is subdivided very early into parasagittal transcriptional domains (Wassef et al., 1992). In the case of the *L7pcp-2* gene, the expression in bands was shown to be cell-intrinsic Purkinje cells (Oberdick et al., 1993) and to be apparent as soon as E15. Later, between E15 and E17.5, the expression patterns of *En-2*, *En-1* and *Pax-2* delimit parasagittal domains in the cerebellum anlage (Millen et al., 1995). It has been hypothesised that these genes are instrumental in the subdivision of the cerebellum anlage into sagittal domains (Millen et al., 1995).

The retrospective analysis of the clones that were generated in this study suggests that the cerebellum anlage is clonally organised in prospective longitudinal domains during its formation (see clones 95-258, 94-256, 94-452 and 95-498, 94-852, 94-698 and 94-857 for examples). Patterning of cell growth is therefore already operating during the formation of the cerebellum anlage, that is, before E14. This clonal patterning shares similarities with the genetic patterning already apparent from E15 just described, in that the general orientation of the clonal restriction domains is reminiscent of the sagittal orientation of the expression pattern of *L7-pcp-2 lacZ*. However, in contrast to the genetic analysis, which dissociates a proliferative period (E9, E15) from a patterning period (E17.5-P10), the clonal analysis clearly indicates that clonal patterning occurs at a time of cell proliferation and growth. This raises the hypothesis that there might be many small parasagittal domains of clonal restriction in at least the lateral domain. Although it remains to be determined whether clonal patterning precedes genetic patterning and if there is a molecular link between the two processes, our results suggest that clonal and genetic patterning may, indeed, be coordinated.

80 cells at the origin of the cerebellar cortex

The problem related to the ancestral origin of cells of the cerebellum has already been approached using chimera and XX inactivation mosaics. Wetts and Herrup (1982a) have proposed that all Purkinje cells descend from 8 founders per hemiserebellum, each with an equal contribution. In a similar study based on the analysis of mice exhibiting mosaicism for a marker localised on the X chromosome, 65 founders were proposed (Baader et al., 1996).

We have addressed the issue of Purkinje cell lineage using retrospective clonal analysis. The mean cell number obtained from cohorts of Purkinje cells, in clones whose precursors are labelled before the allocation of cells to the cerebellum (clones M-HM and L-HM), suggests that a pool of founder cells comprises about 80 cells per hemiserebellum (Table 4). The

founder pool would be organized into two clonal domains (lateral and median) of equivalent size. The mean cell number obtained from cohorts of Purkinje cells, presumably labelled

even earlier in development (clones M-CNS), does not indicate a reduction in the size of the pool of founder cells (Table 4) and this suggests that the genealogical relationship of Purkinje cells cannot be traced beyond 80 independent cells. This number is close to the 65 founder cells calculated by Baader et al. (1996) who labelled cells early, at about E5. Therefore, it is likely that the value of 80 obtained here approaches the actual size of the biologically significant pool of founders for the Purkinje cell lineage.

In addition, based on our discovery that the **size** and **cell number** of Purkinje cell cohorts in some of the earliest labelled clones are smaller than those of older clones, we suggest that, at the time of the allocation of the founders to the cerebellum, each founder may have a distinct potentiality. Alternatively, it could be that the pool of allocated cells does not correspond to the 80 founder cells but to a subset of their descendants. In the later case, the pool of 80 cells that we traced retrospectively would correspond to an earlier stage in the formation of the cerebellum. The analysis of more large clones will help to distinguish between these two possibilities.

Nonetheless, it is clear that the developmental phase represented by the 80 founder cells corresponds to the initiation of a period of clonal growth – that is, a period during which cells remain contiguous. This period is necessarily preceded by a phase of non-coherent growth that distributes unrelated cells into several distinct territories: two for the primary germinative epithelium and a separate one(s) for the secondary germinative epithelium. It is also apparent that the 80 founders are not only at the origin of Purkinje cohorts but also give rise to cohorts of other neuronal classes produced by the primitive germinative epithelium. Following this rationale, the estimate of 80 cells as the number of Purkinje cohorts suggests that this period of clonal growth takes place in the neural tube and involves very small groups of cells. This period of clonal growth in the neural tube is presumably followed by a period of dispersion, which is achieved through the intercalation of

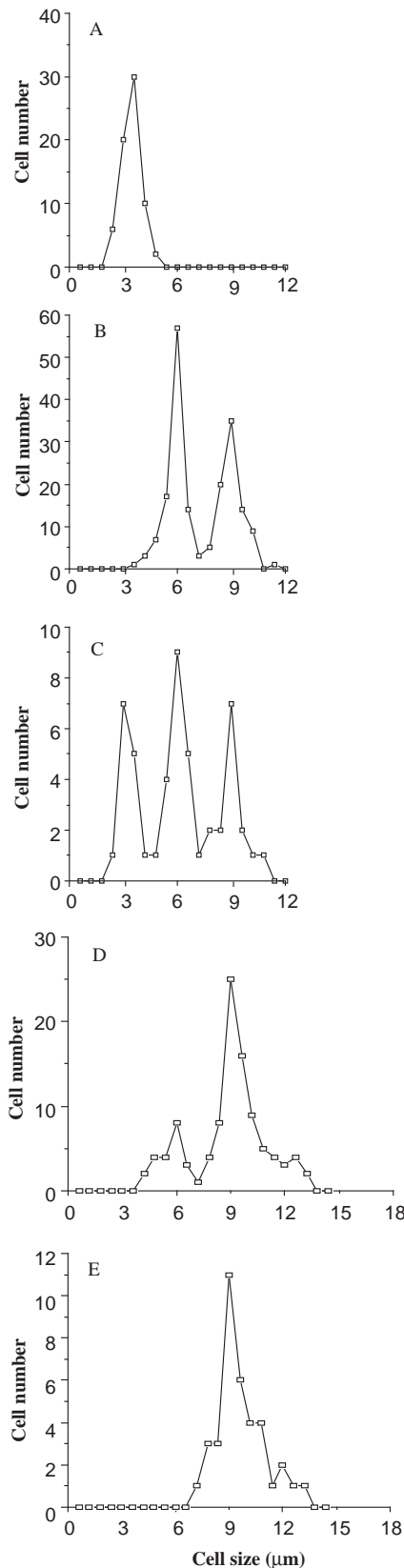


Fig. 6. Histograms for the size distributions of labelled nuclei in the different cerebellar layers in large clones. (A) Clones composed of inner granule neurons in the IGL. The size of the nuclei of the labelled granule neurons was recorded on histological sections of clones 95-34, 94-844, and 95-345. Mean size is $3.4 \mu\text{m}$ with a s.e.m.=0.1. (B) Neurons in the IGL. Size distribution of nuclei in large clones 94-452, 95-83, 94-451, 94-256, 94-429, 94-258, 94-498, 94-857 and 94-499 has been recorded. The mean sizes of the nuclei are $5.9 \mu\text{m}$ with a s.e.m.=0.1, and $9.0 \mu\text{m}$ with a s.e.m.=0.1, which correspond to medium and large classes of Golgi neurons, respectively, but not to inner granule neurons. (C) Large clones 94-826 and 94-747. The size of the labelled nuclei in the IGL was recorded for sections containing, in addition to medium- and large-sized neurons, a patch of smaller labelled cells. This analysis shows the presence of both small-sized neurons (inner granule neurons) and medium- and large-sized Golgi neurons. (D) Neurons in the PCL and ML. Two populations of neurons are present in clone 95-83: medium-sized neurons (nucleus size is $5.6 \mu\text{m}$ with s.e.m.=0.4) and large-sized neurons (nucleus size is $9.7 \mu\text{m}$ with s.e.m.=0.3). (E) Size of the nuclei in Purkinje cells stained positive for Calbindin. Mean nucleus size is $9.7 \mu\text{m}$ with s.e.m.=0.5. The main peak ($9 \mu\text{m}$) corresponds to round-shaped Purkinje neurons whereas the secondary peak ($12 \mu\text{m}$) corresponds to more elongated Purkinje neurons. Therefore in D, large-sized neurons are most likely Purkinje neurons and the medium-sized neurons are probably basket and stellate neurons.

cells during and/or after proliferation in the germinative epithelium but which respects the limits of the lateral and medial territories and is clonally organised. Such a model of clonal cell growth and subsequent dispersion would explain the observed distribution of cells in both the large and the small clones. Finally, it is intriguing to note that these successive cell behaviours (dispersive growth, followed by clonal growth and then again by further dispersive growth delimited by boundaries) are those expected for territories organised into polyclonal units and, as has been discussed above, these kinds of units have been described in the rhombencephalon (Simon et al., 1995).

Overview and final comments

In summary, the results presented here show the following. (1) There are common clonal precursors for all classes of neurons found in the adult cerebellum, except for granule cells of the IGL which have a distinct origin (Fig. 1; Table 3); therefore (2) there is a clonal independence of the PGE and SGE from the earliest stages of cerebellar formation. (3) Precursor cells of the PGE are organised into two polyclonal groups (Fig. 5) that are not clonally related, since clones labelled very early, but which are not restricted to the cerebellum, contribute to either of these territories but not to both (Table 2; Fig. 4). (4) Cerebellar precursor cells have the same histogenic potential independent of the polyclonal group to which they belong (Tables 3, 4). (5) Cell behaviour is probably particular for different precursor groups: the group whose descendants will occupy a lateral final position exhibit a sagittal patterning (Fig. 5A-D); whereas, the group whose descendants will occupy a medial final position exhibit an unrestricted distribution up to the midline (Fig. 5A-C). Differential patterning of cell growth is therefore probably operating during the formation of the cerebellum anlage and there might be many small parasagittal domains of clonal restriction in at least the lateral domain. (6) There are limited lateromedial but less limited rostrocaudal movements at the clonal level for both Purkinje and Golgi cells and their precursors deriving from the primary epithelium. (7) The final mediolateral organisation in the cerebellum is orthogonal to the organisation of the precursors in the neural tube and this clonal patterning therefore shares similarities with the patterning defined by developmental gene expression domains. A possible hypothesis is that distinct cell behaviours create the different clonal domains observed in this study and that the cellular and genetic organisation of the cerebellum are coordinated. (8) The genealogical relationship of precursors at the origin of these two territories cannot be traced to less than 34 and 45 independent cells, respectively (Table 4). Since these numbers do not diminish when the earliest clones are examined, they likely represent the ultimate limit of the genealogical origin of the primary germinal epithelium. We hypothesise that the developmental phase represented by

these 80 founder cells initiates a period of clonal growth and follows a phase of non-coherent growth, which distributes unrelated cells into several territories: a medial and a lateral territory for the primary germinative epithelium, and separate territories for the secondary germinative epithelium. The PGE and SGE would then subsequently develop following specific cellular patterns. Finally, the pluripotent neuroepithelial cells of the cerebellar plates would produce the various neuronal classes of the cerebellum by sequential asymmetric divisions.

What is the biological significance of the novel findings described in this study? The existence of several distinct polyclonal groups at the origin of the cerebellum indicates a shift from a dispersive mode of growth to a more coherent mode of growth during development. During this period, the cells at the origin of the two germinative epithelia would be segregated and the cells of the PGE would be isolated into two groups. This may define a period during development when the fate of small groups of cells is fixed according to their position, a phase which then would necessitate a change in cell behaviour for the descendants of the chosen cells. From a clonal view, therefore, the cerebellum develops from several independent units. The existence of two groups of cells in the PGE maybe

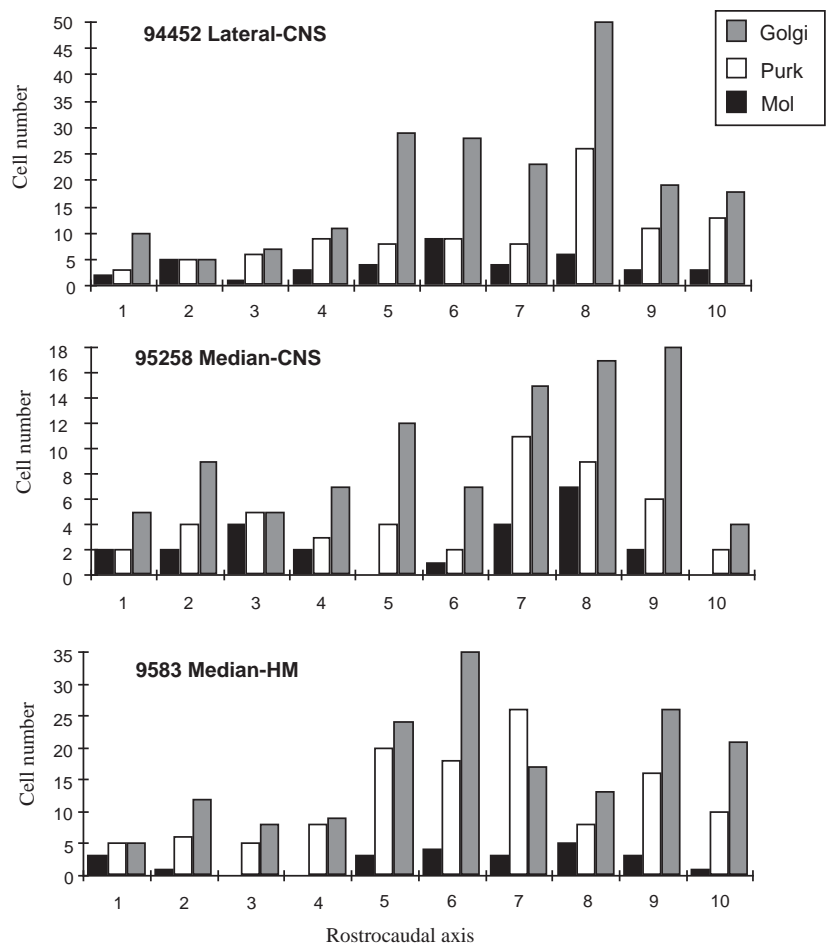


Fig. 7. Rostrocaudal dispersion of labelled molecular, Purkinje and Golgi cells in the large clones. The X-axis corresponds to ten representative transverse sections along the rostrocaudal dimension of the cerebellum. The Y-axis corresponds to the number of labelled cells for each neuronal class in each section. Dispersion of the three neuronal classes along the rostrocaudal axis is similar.

the consequence of two different modes of clonal patterning, which may be instrumental in mediating the morphogenetic movements of the neural tube that lead to the formation and the positioning of the cerebellar plates. It is also apparent that cells with the same histogenic potential may have different clonal patterning and this would suggest a certain independence of clonal patterning and cellular differentiation. The existence of a clonal border at the midline suggests that, at the time the bilateral territories join, lateral dispersion of neuroepithelial cells becomes minimal. The basis for this behaviour could be morphogenetic – for example, an arrest in lateral growth and expansion, or developmental, involving a decision that helps determine cell fate, or may be a combination of both.

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