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Dynamic Organization of Developing Purkinje Cells Revealed by Transgene Expression

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The cerebellum has many properties that make it a useful model for investigating neural development. Purkinje cells, the major output neurons of the cerebellar cortex, have drawn special attention because of the availability of biochemical markers and mutants that affect their development. The spatial expression of L7, a protein specific for Purkinje cells, and L7βGal, a gene expressed in transgenic mice that was constructed from the L7 promoter and the marker β-galactosidase, delineated bands of Purkinje cells that increased in number during early postnatal development. Expression of the transgene in adult *reeler* mutant mice, which show inverted cortical lamination, and in primary culture showed that the initial expression of L7 is intrinsic to Purkinje cells and does not depend on extracellular signals. This may reflect an underlying developmental map in cerebellum.

THE MURINE CEREBELLAR CORTEX has been widely used to investigate neural development because its structure is relatively simple and ordered, its developmental progression is well documented, and cell-specific markers and viable mutants that affect its maturation are available. In particular, considerable emphasis has been placed on determining the contributions of cell-intrinsic and -extrinsic mechanisms to the development of Purkinje cells, the major output neurons of the cerebellar cortex. Analysis of transplants (*1*) and chimeras (*2*) indicates that Purkinje cell development is largely autonomous of the environment. However, studies with biochemical markers have shown that Purkinje cell maturation is not synchronous (*3, 4*). This raises the questions of whether Purkinje cell development is ordered with respect to time and position and whether this process is cell-intrinsic. Using molecular markers for murine Purkinje cells, we have shown that these neurons follow a stereotypical developmental map that is largely dictated by cell-autonomous processes.

A strain of mice has been derived that carries a transgene (L7βGal) that directs expression of β-galactosidase almost exclusively to cerebellar Purkinje neurons (*5*). Therefore, we have used β-galactosidase activity to follow developmental events that occur in individual or entire populations of Purkinje cells. The developmental expression of the L7βGal transgene was determined in tissue sections and whole mounts of cerebella from normal and mutant mice heterozygous for L7βGal.

By embryonic day 14 (E14), the cerebellum already contains its complete complement of Purkinje cells (*6*). However, at E17, the earliest time at which the transgene product can be detected, only subpopulations of Purkinje cells express L7βGal in normal mouse cerebellum (Fig. 1A). Expression is localized to four parasagittal bands of Purkinje cells, two on each side of the midline. The most medial of these bands spans nearly the entire anterior-posterior extent of the cerebellum, whereas the lateral band is only present in the posterior area.

After E17, the number of Purkinje cells expressing the gene increased such that all cells were L7βGal-positive by postnatal day 9 (P9) (Fig. 1, D and F). The induction of L7βGal after E17 occurred in a stereotyped topographical and temporal manner (Fig. 1). At P0 (day of birth), three bilateral bands (two medial and one lateral) were evident (Fig. 1, B and E). The medial bands were thicker in the posterior cerebellum, and the most lateral band was discontinuous (Fig. 1E). From P0 to P3, the pattern of gene expression did not change. At P4, the number of major bilateral parasagittal bands in the transgenic cerebellum had increased from three to five (two medial and three lateral) (Fig. 1C).

At all developmental stages, L7βGal and L7 were expressed in the same population of cells (Fig. 2, C and D). From P4 to P7, all Purkinje cells in the vermis became L7βGal- and L7-positive, whereas most of those in the lateral parts of the hemisphere were still negative. By P9, all Purkinje cells expressed the L7 gene and the L7βGal transgene (Fig. 1, D and F) (*4*). This inductive pattern of L7βGal was stereotyped: the initial expression was posteromedial, and expression progressed anteriorly and then laterally. Two other Purkinje cell markers, PEP-19 and calbindin 28kD (*7*), did not show the same banding pattern as that revealed by L7 in normal cerebellum (Fig. 3, A to C). Nevertheless, antibodies to calbindin 28kD and PEP-19 stain clusters of Purkinje cells early in development (*3, 4*).

In the central nervous system of the homozygous *reeler* mouse, there is an inversion of lamination, most likely a consequence of an abnormality in neuronal migration (*8*). In the cerebellum this results in Purkinje cells that are incorrectly positioned and largely organized into clusters (*9*). Therefore, we could test the effect of cell position on L7 expression by examining L7βGal expression in *reeler* mice. In adult *reeler* mice, all Purkinje cells expressed L7βGal (Fig. 2B). However, two bilateral bands of Purkinje cells expressed large amounts of the transgene, whereas Purkinje cells located medial and lateral to these bands expressed smaller amounts of the transgene (Fig. 2B). This is in contrast to expression in wild-type adult cerebellum, which was uniform throughout (Fig. 2A).

Although it is difficult to make direct comparisons between *reeler* and wild-type cerebella because of the lack of foliation in *reeler*, several features of transgene expression are apparent. (i) The initial induction of L7βGal expression in Purkinje cells is the same in *reeler* and wild-type cerebellum (Fig. 2E). (ii) As in wild-type cerebellum, the expression of L7βGal is concentrated in medial Purkinje cells during early postnatal development (compare Fig. 1, A through C, to Fig. 2, E through H). (iii) In contrast to the situation in wild-type mice, in *reeler* mice the amount of L7βGal decreases with age in

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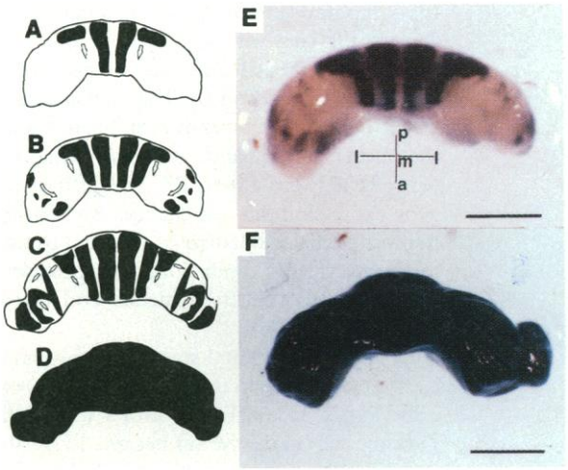

Fig. 1. Developmental expression of L7βGal in cerebellum. Graphic representation of expression of L7βGal at E17 (**A**), P0 (**B**), P4 (**C**), and P9 (**D**). Arrows show the dynamic pattern of developmental expression from P0 to P9. (**E**) Low-power photomicrograph of dorsal aspect of P0 cerebellum staining in toto for L7βGal, corresponding to (B). Posterior, p; medial, m; anterior, a; lateral, l. (**F**) Low-power micrograph of P9 dorsal cerebellum, corresponding to (D). Scale bars: (E) 1.0 mm; (F) 2.0 mm.

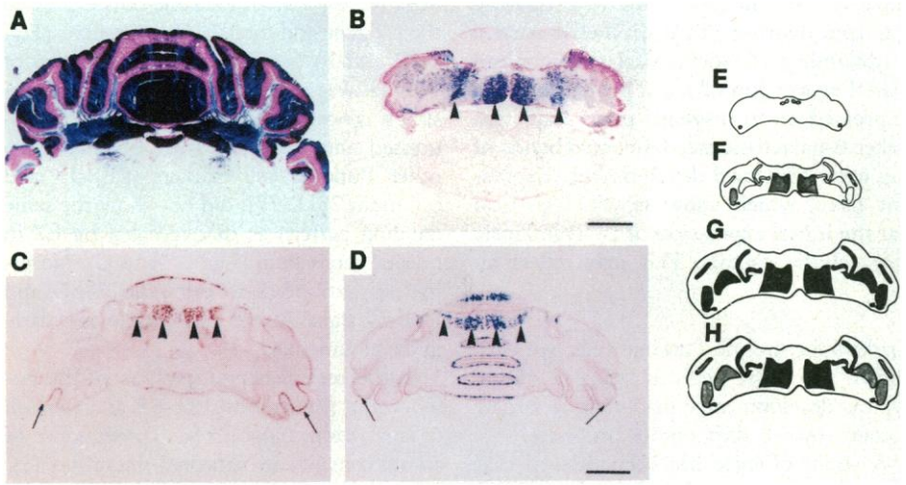

Fig. 2. Expression of L7βGal and L7 in wild-type and *reeler* cerebellum. (**A**) In coronal sections of adult wild-type cerebellum, all Purkinje cells express the L7βGal transgene. (**B**) In coronal sections of *reeler*, the transgene is expressed in two bilateral bands of Purkinje cells (arrowheads), whereas all other cerebellar Purkinje neurons located laterally and in between the bands express lower amounts of the transgene. (**C**) Horizontal section immunostained for L7 through the cerebellum of P4 nontransgenic animals reveals two bilateral medial bands, as well as staining of Purkinje cells located in the most lateral part of the cerebellum (arrows). The pattern of expression of the cognate gene (C) is identical to that of the transgene (**D**). (**E** through **H**) Graphic representation of L7βGal expression in developing *reeler* cerebellum. Scale bars: (A and B) 1.0 mm; (C and D) 0.5 mm.

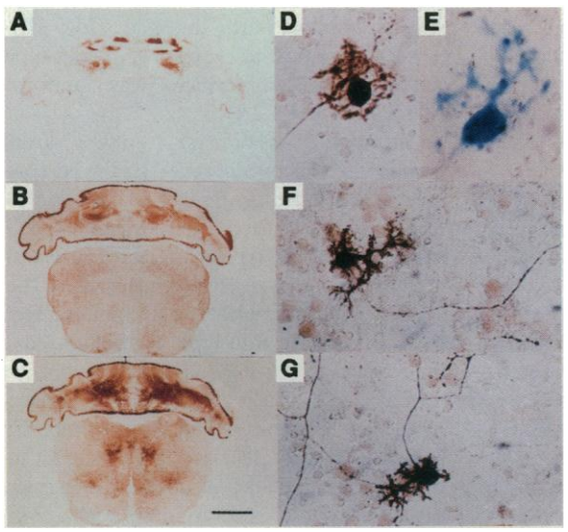

Fig. 3. Comparison of markers in developing cerebellum and Purkinje cells in vitro. (**A** through **C**) Serial horizontal sections of P2 cerebellum immunostained for L7 protein (A), PEP-19 (B), and calbindin 28kD (C). Bands of immunoreactive Purkinje neurons are evident in (A), whereas in (B) and (C) the entire row of Purkinje cells is immunopositive. (**D** through **G**) Purkinje neurons in dissociated cultures express immunoreactivity for L7 (D), PEP-19 (F), and calbindin 28kD (G), as well as β-galactosidase activity from the L7βGal transgene (E) (*17*). Scale bar: (A through C), 0.95 mm.

lateral cerebellum (Fig. 2, G and H). Therefore, the weakly L7βGal-positive Purkinje cells observed in adult *reeler* (laterally and between L7βGal-positive bands) (Fig. 2B) are neurons that either have failed to maintain L7βGal expression or have increased protein degradation.

Because all Purkinje cells expressed L7βGal, irrespective of their position, it is likely that the induction of the gene is cell-autonomous. However, the presence of regions of high and low expression in *reeler* suggested some contribution of positional or afferent cues to the amount of transgene expression. To further characterize the contribution of the cellular environment to L7 expression, we established primary dissociated cultures from both normal transgenic and nontransgenic mice.

Cerebellar anlagen were removed at E15, 2 days before the onset of L7 expression in vivo, and were established in culture (*10*). Purkinje cells were identified by expression of PEP-19 (Fig. 3F), calbindin 28kD (Fig. 3G), and cognate (Fig. 3D) and transgenic L7 genes (Fig. 3E). L7 was first detected after 3 to 5 days in culture, although Purkinje cells expressed PEP-19 and calbindin 28kD shortly after plating (*11*). After 2 weeks in culture, each marker was expressed in approximately the same number of Purkinje cells. The timing of this expression coincided with the onset of gene expression in vivo. The cultures lacked the two main afferent systems to the cerebellum, mossy and climbing fibers; thus, the induction of L7 must be autonomous of these inputs. Because unidentified cells in these cultures, most of which are cerebellar interneurons, establish functional synapses with Purkinje cells (*12*), we cannot completely exclude the possibility that other afferent systems may influence L7 induction. However, chronic blockade of electrical activity with tetrodotoxin or of synaptic transmission with elevated Mg^{2+} did not affect induction of L7 (*12*). Therefore, extrinsic signals do not appear to influence L7 induction significantly, suggesting the involvement of cell-intrinsic mechanisms.

The expression of the L7βGal transgene in the normal cerebellum defines a unique organization of Purkinje cells during development that is distinct from the parasagittal bands of Purkinje cells observed by others (*3, 13*). Initially, L7βGal appeared in the posterior and medial aspect of the fetal cerebellum in a position reminiscent of the distribution of the earliest born Purkinje cells. Subsequently, two gradients of L7 expression were observed. First, the number of positive cells in a particular band increased in the posterior-to-anterior axis of the cerebellum. Second, the number of

bands increased in the medial-to-lateral axis (Fig. 1). No changes in band number occurred between E17 to P0 and P1 to P4. This developmental pausing could indicate staggering of an underlying inductive signal or the presence of multiple regulatory processes that are separated in time.

When sharp, segmental boundaries in gene expression exist during early development, such as in rhombomeres, genes encoding nuclear proteins frequently delineate these segments or regions (*14*). Indeed, the presumptive T cell oncogene, rhombotin, not only has an early rhombomeric expression but also reveals a banding pattern in neonatal cerebellum that may be complementary to L7βGal (*15*). Afferent projections to cerebellum also are distributed in an anterior-to-posterior, bandlike organization (*16*). Thus, the cell-autonomous mechanisms that establish the Purkinje cell map delineated by L7 expression may be the same as those that guide the establishment of functional circuitry in cerebellum.

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Interaural Phase Coding in Auditory Midbrain: Influence of Dynamic Stimulus Features

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A laterally located sound source stimulates the two ears at slightly different times, generating interaural phase disparities (IPDs) that are used for sound localization. Under natural conditions, such interaural cues are likely to be constantly changing, or dynamic. In the inferior colliculus of gerbils and cats, the nonlinearities in the coding of dynamic interaural phase cues are demonstrated. Responses to ecologically realistic phase cues are more reflective of the change of IPD than of the absolute IPDs over which that change occurs. This observation is inconsistent with the established view that directional information is coded in terms of absolute IPD.

IPD IS AN IMPORTANT SOURCE OF SPATIAL information in audition. Because an observer's head is normally free to move and many sound sources are mobile, most naturally occurring IPDs are likely to be dynamic. Temporal variation of IPD can be viewed either as a source of information or confusion for the auditory system. The former view is suggested by psychophysical data demonstrating that human subjects can accurately discriminate velocity (*1*) and direction (*2*) of moving sounds; the latter view by demonstrations that spatial acuity of the human auditory system is best for static sound sources and decreases as a function of sound source velocity (*3, 4*). Physiological studies have emphasized the similarity of responses to static and dynamic IPD stimuli for the majority of IPD-sensitive neurons throughout the ascending auditory pathway (*5–8*). We now report consistent differences between responses of inferior colliculus (IC) neurons to static and dynamic IPDs in both gerbils (*Meriones unguiculatus*) and cats. By varying the depth of interaural phase modulation (IPM) to approximate the changes of IPD generated by sound-source motion more closely than was possible before, we demonstrate strong influences of dynamic IPD on the responses of the majority of phase-sensitive units (neurons). These neuronal response properties may underlie the sensitivity of the auditory system to dynamic IPD stimuli.

For sounds in the range of tonal frequencies to which neurons are phase sensitive, the largest possible change in IPD, corresponding to motion of a sound source from one side of the head to the other, is less than 360° in mammals. Furthermore, the IPD modulations associated with naturally occurring sound-source motion are usually smaller than the maximum that is possible. The current understanding of neural responses to dynamic interaural phase cues derives from use of the binaural beat stimulus, which produces a continuous, unidirectional change of interaural phase through 360°. For this study, we developed an alternative binaural stimulus that permits the generation of more ecologically realistic IPDs. A phase-modulated, tonal signal is presented at one ear, while the other ear receives an unmodulated reference tone, identical in frequency and sound-pressure level (SPL) to the carrier of the modulated signal. By adjusting parameters of the modulation waveform, including its depth (Fig. 1), we can generate IPM approximating various aspects of simulated auditory motion.

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